# 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 corneas. 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.[1] 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,[2] to ameliorate the tear film instability in a rabbit model,[3] and to treat mild to moderate dry eyes in patients.[4] 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.[5] 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 Labes 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. The rabbits were fed approximately 130 g food per day, the rats were allowed access to food, 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 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 (Somnopentyl; Kyoritsu Seiyaku Corp., Tokyo, Japan), and the eyes were enucleated. The corneas were stained with 50 μL of 1% MB solution again, and then rinsed with PSS to remove excess MB. The corneas were isolated, and the MB in the isolated corneas was extracted with acetone/sodium sulfate solution for 3 or 4 days. Finally, the absorbance at 660 nm of the MB in the acetone/sodium sulfate solution was determined with a spectrophotometer. Rabbit eyes that were similarly kept open but moistened with PSS were used as positive controls. Rabbits whose eyes were kept closed served as negative controls. The right eyes of a total of 12 rabbits were tested for each ocular lubricant group.

### 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.[8] 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 (Dai-ichi 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% $CO_2$ 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 $1 \times 10^5$ cells/well and cultivated for 5 days. The supernatant was removed and replaced with different lubricants of 0.1% SH, 0.3% 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 refilled 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

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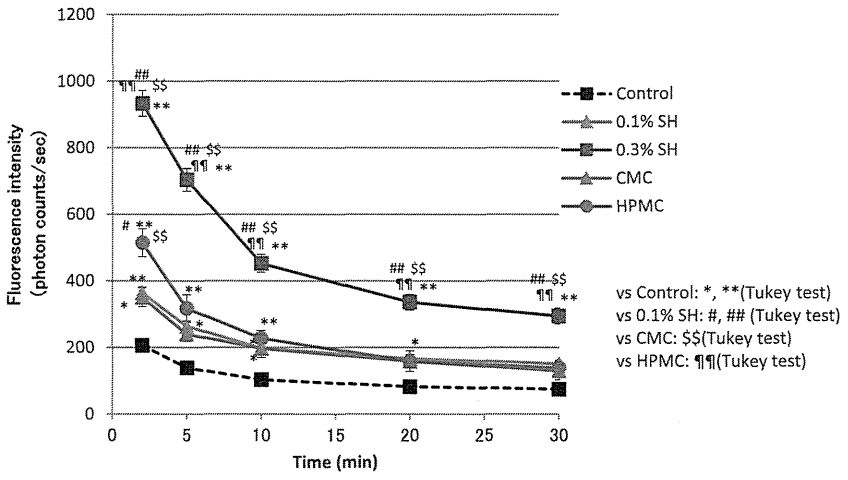


FIGURE 1. Ability of three types of ocular lubricants: 0.1% and 0.3% SH, CMC, and HPMC to be retained on the corneal surface. Fifty microliters of each ocular lubricant containing 0.002% fluorescein was dropped on the rabbit cornea. After 2, 5, 10, 20, and 30 minutes, the fluorescein intensity on the cornea was measured with a fluorometer. The data plotted are the mean ± SEM of six eyes of six rabbits. Physiological saline solution was used as the control. Data were analyzed by Tukey variant tests, and the statistical significances are depicted as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $\#P < 0.05$ and $\#\#P < 0.01$ versus 0.1% SH; $\$\$P < 0.01$ versus CMC; $\P\P P < 0.01$ versus HPMC.

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 (all $P < 0.01$; Tukey test). The fluorescein intensity of HPMC was significantly higher than that of 0.1% SH and CMC 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 (Fig. 1).

### 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 ± 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 ± 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).

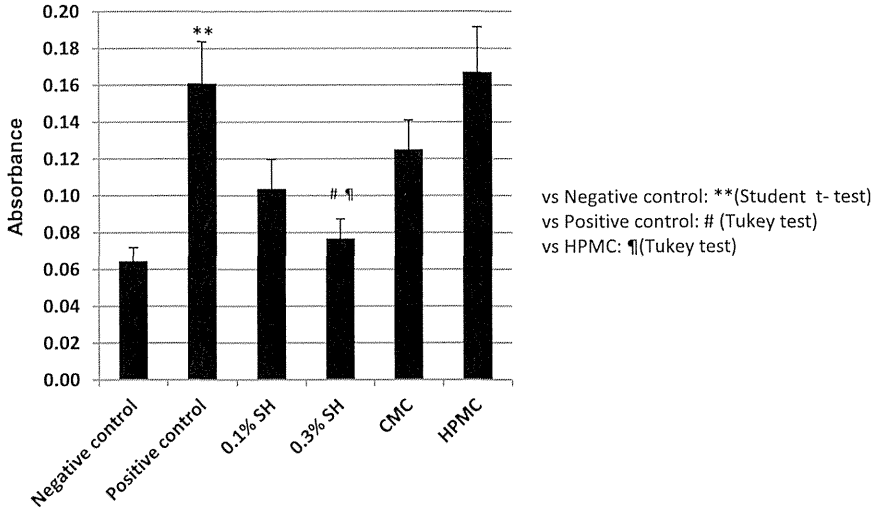


FIGURE 2. Protective effectiveness of three types of ocular lubricants. Rabbit eyes were held open with a speculum and 50 μL of each lubricant was dropped on the cornea. After 3 hours, the rabbits were killed, and the corneas were stained with MB. The absorbance of MB extracted from stained cornea was measured. Data represent the mean ± SEM of 12 eyes of 12 rabbits. Rabbit eyes that received PSS were used as positive controls, and eyes that were kept closed served as negative controls. The statistical significances are shown as $^{**}P < 0.01$ versus negative control (Student's *t*-test); $\#P < 0.05$ versus positive control (Tukey variant test); $\P P < 0.05$ versus HPMC (Tukey variant test).

## DISCUSSION

Dry eye syndrome can be classified into the evaporative types and the aqueous-deficient types. Application of ocular lubricants to keep the ocular surface moistened is the most common therapy for treating dry eyes. This treatment is convenient and simple, and many different formulations of ocular lubricants are commercially available, among which SH, CMC, and HPMC are the ingredients most often used.

Sodium hyaluronate is a glycosaminoglycan disaccharide biopolymer and consists of repeating alternating sequences of N-acetylglucosamine and glucuronate in linear chains. Sodium hyaluronate has a huge capacity to bind water, and the affinity is 1000-fold of its own weight. In addition, it resists evaporation. Carboxymethylcellulose is an anionic cellulose polymer with a carboxylic group. It is available in several viscosities which correspond to the different molecular weights. Carboxymethylcellulose has bioadhesive properties, and its anionic characteristic may be beneficial in increasing 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.[10] 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.[11] 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[5] or when placed atop an agar gel in a dose-dependent manner.[12] In addition, our findings support the data reported by Snibson et al.[13] 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 as a short-term model for the study of the evaporative type of dry eyes.[6,7] Application of 0.3% SH significantly reduced the MB absorbance in the cornea, indicating effective protection by this ocular lubricant. This finding is consistent with the results of the first part of our study. It is reasonable to extrapolate that the higher capacity of ocular surface retention by SH would allow it to protect the cornea, reduce the loss of epithelial integrity, and therefore inhibit the MB staining.

To test the therapeutic efficacy of the ocular lubricants, the lacrimal gland–extirpated rat dry eye model was used. This model has been used to study the aqueous-deficient type dry eye syndrome.[8,9] 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.[14] 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.[15,16]

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

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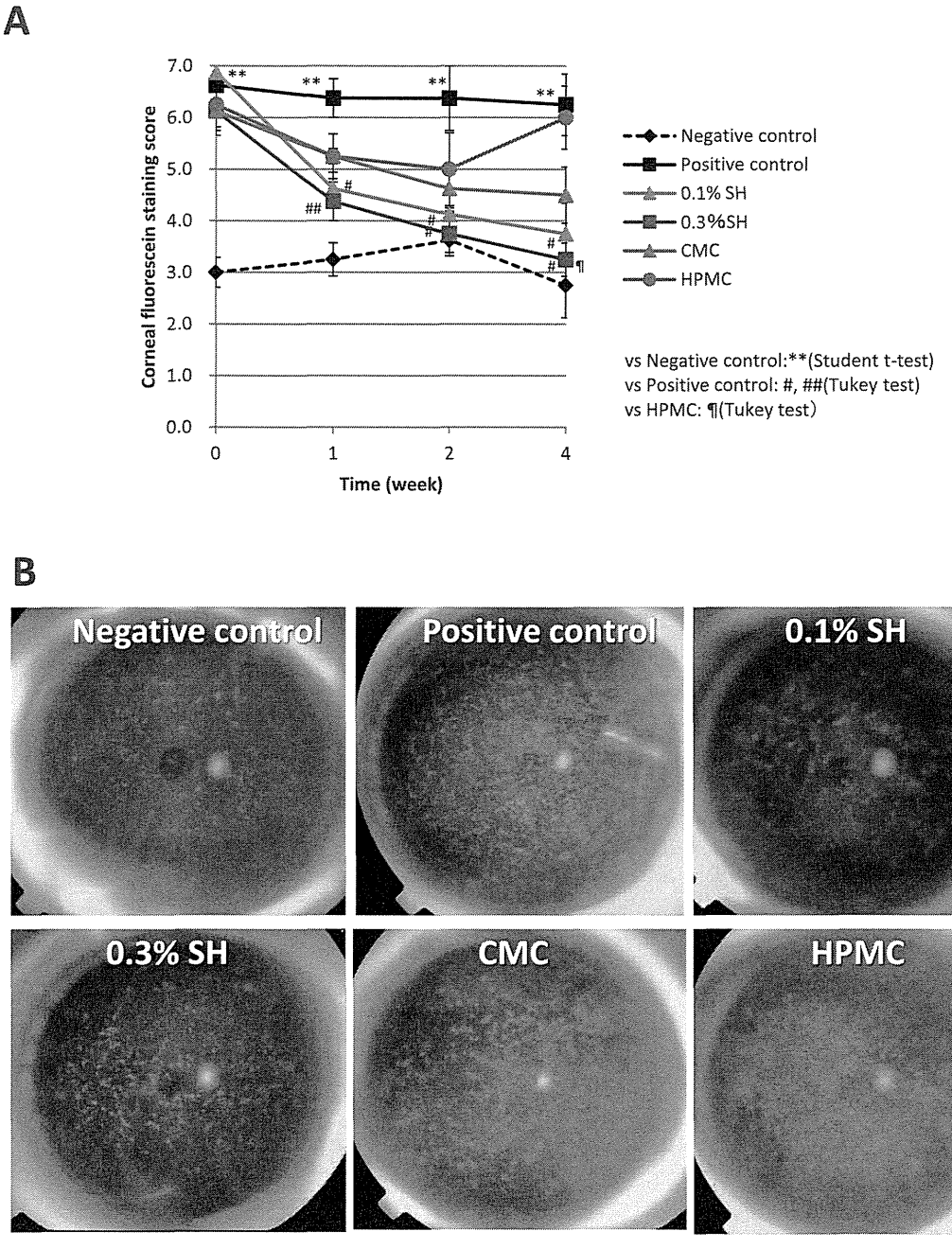


FIGURE 3. Therapeutic effectiveness of three types of ocular lubricants in an exorbital lacrimal gland-extirpated rat dry eye model. (**A**) Corneal fluorescein staining scores indicative of loss of the epithelial integrity are shown. Data represent the mean ± SEM of four eyes of four rats. Negative 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 $^{\P}P < 0.05$ versus HPMC (Tukey variant test). (**B**) Representative slit-lamp microscopic photographs showing corneal fluorescein staining at 4 weeks after ocular lubricant exposure.

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.[15] Sodium hyaluronate has also been shown to be beneficial for corneal epithelial cell migration and elongation in vitro.[17,18] 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.

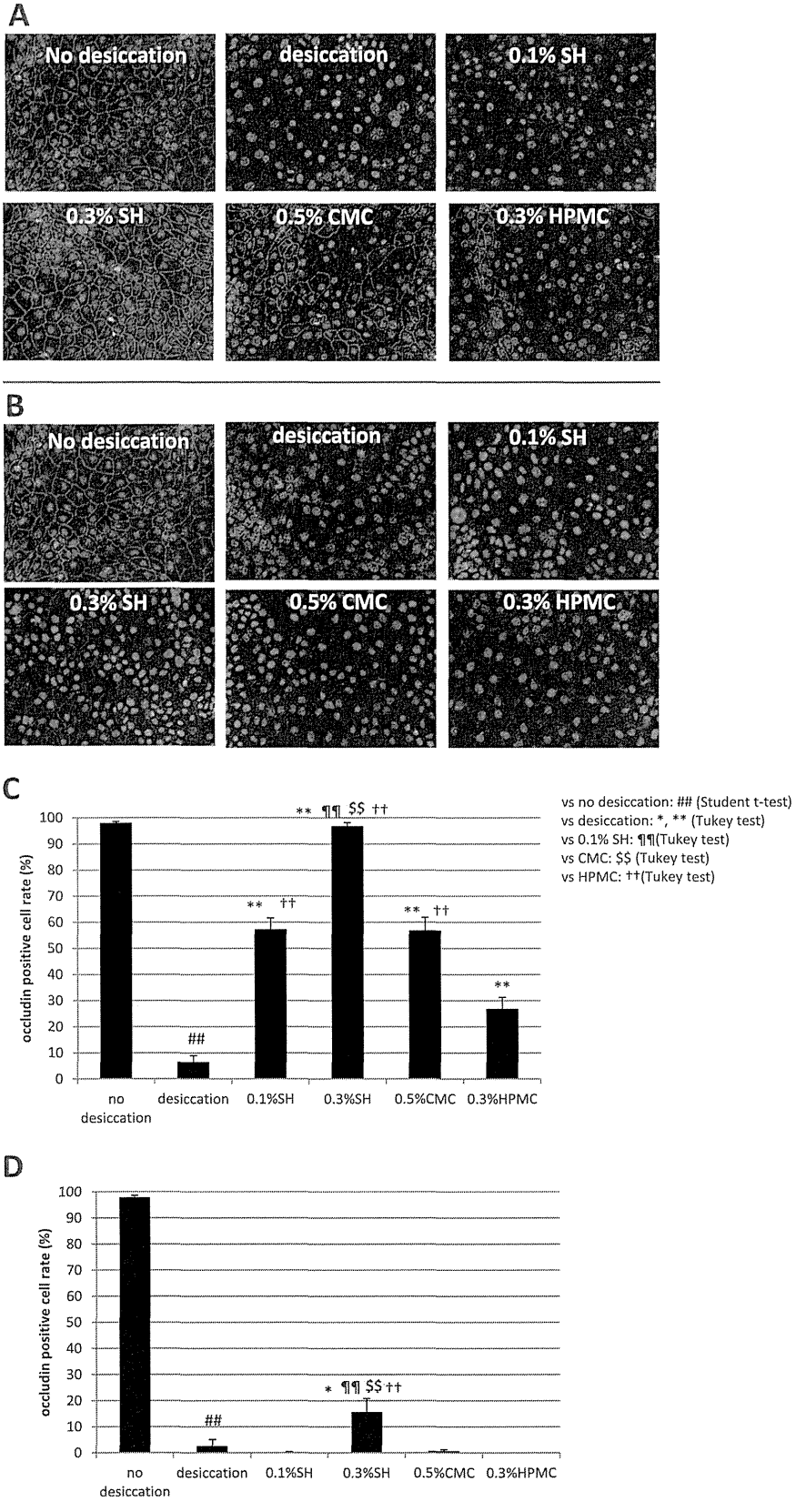


FIGURE 4. Epithelial cell barrier function protein assay against desiccation. Human corneal epithelial cells were pretreated with different lubricants and desiccated for 20 minutes (**A**, **C**) and 60 minutes (**B**, **D**). Cells were stained with anti-occludin antibody with propidium iodide (PI) 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 because of the higher ability of SH to be retained on the ocular surface, its effectiveness in protecting against desiccation, and its protection of corneal epithelial cells in a dry eye disease model.

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# Polymicrobial Sclerokeratitis Caused by *Scedosporium apiospermum* and *Aspergillus cibarius*

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**Purpose:** The aim of this study was to report the first case of polymicrobial fungal sclerokeratitis caused by infection with both *Scedosporium apiospermum* and *Aspergillus cibarius*, and notify the medical community of the possibility of infection caused by *A. cibarius* in humans.

**Methods:** A 78-year-old woman presented to a medical practitioner with epiphora and a purulent discharge in her left eye. After concurrent administration of topical antibiotics and systemic steroids, her symptoms worsened, and she was referred to the Tokushima University Hospital. Because of suspected fungal infection, microscopic examination and cultivation of both corneal and scleral scrapings were performed.

**Results:** Fungi were observed on microscopic examination, and *S. apiospermum* was isolated only from the sclera in the early stage of the clinical course. Although administration of an adequate medication regimen comprising topical and systemic antifungal drugs resulted in an improvement in the sclera, keratitis persisted, and the infected sclera was melted. After scleral transplantation, administration of systemic caspofungin and high concentrations of voriconazole solution eye drops resulted in a gradual improvement in keratitis. A strain of filamentous fungus was isolated from the cornea 6 weeks after the cultivation on a Sabouraud agar plate, and it was identified as *A. cibarius*.

**Conclusions:** *A. cibarius* may infect human tissue. Coinfection of the cornea and the sclera with 2 different species of fungi is likely to follow a complex clinical course.

**Key Words:** *Aspergillus cibarius*, *Scedosporium apiospermum*, sclerokeratitis, coinfection

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Fungal sclerokeratitis is a rare infectious disease of the anterior segment of the eye, and coinfection of sclerokeratitis caused by multiple microbials is a rare condition. To the best of our knowledge, their rarity is reflected in the fact that only 7 articles regarding fungal sclerokeratitis and no articles regarding coinfection of sclerokeratitis caused by 2 different species of fungi have been published to date.[1–7] Here, we describe our experience in the identification and treatment of the first case of coinfection of sclerokeratitis caused by 2 different species of filamentous fungi—*Scedosporium apiospermum* and *Aspergillus cibarius*. We also report the detection of *A. cibarius* as a pathogen capable of causing infectious disease in human tissue, for the first time in the literature.

## CASE REPORT

### Clinical Course

A 78-year-old woman presented to a medical practitioner with epiphora and a purulent discharge in her left eye that lasted for 3 days. Although she had undergone uneventful cataract surgeries in both eyes several years ago, her history of systemic disease was unremarkable. Upon presumptive diagnosis of bacterial keratitis without the cultivation of corneal scrapings, topical antibiotics with systemic steroids (30 mg/d of oral prednisolone) were administered. Details pertaining to the duration of corticosteroid dosage remained unclear. Because the condition of the anterior segments seemed to worsen after treatment initiation, she was referred to the Tokushima University Hospital.

At the first visit, the patient's best-corrected visual acuity in the left eye was limited to hand motion. Slit-lamp microscopy revealed conjunctival hyperemia, peripheral corneal infiltration, corneal abscess, whitish scleral opacity at the nasal side, and severe iritis with hypopyon (Fig. 1A). Two days after the patient's initial visit, the size of the corneal abscess increased rapidly (Fig. 1B). Fungiflora Y staining of scraping samples collected from both the corneal and scleral abscesses for microscopic examination and pathogen detection revealed a large quantity of fungi in the corneal samples (Fig. 2), and the absence of any microorganisms in the scleral sample. The scraping specimens were inoculated onto 2 Sabouraud dextrose agar plates and incubated at 37 and 25°C for the identification of the pathogen.

In accordance with these findings, the following treatment regimen was initiated: intravenous micafungin sodium, oral itraconazole, frequent administration of 1% voriconazole eye drops, and 1% pimaricin ophthalmic ointment 3 times a day. The patient had responded poorly to treatment 5 days later, so intravenous micafungin sodium was discontinued, and intravenous voriconazole and 3 intrastromal injections of 1% voriconazole were included in the treatment plan. The oral itraconazole was continued for 2 weeks. The voriconazole solution

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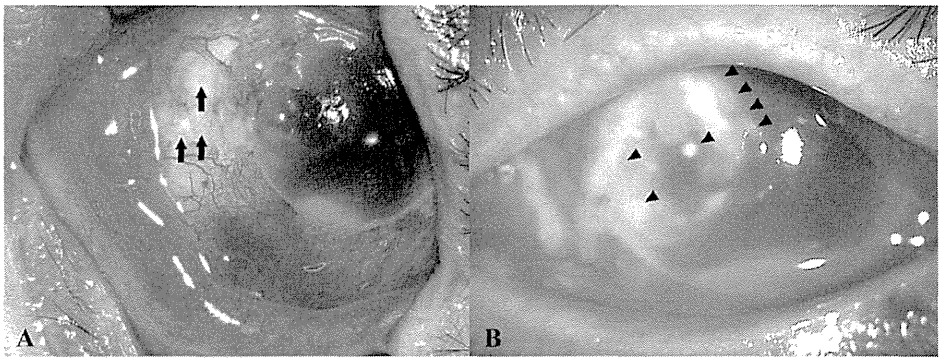


**FIGURE 1.** Images of the anterior segment on the first visit (A) and 3 days after the first visit (B). A, A white avascular area suggestive of calcification is marked on the nasal side of the sclera (black arrows). B, Corneal opacity because of increased keratitis (black arrowheads) persisted for 2 days.

administered topically was increased in strength from 1% to 3% after scleral transplantation for nasal scleral thinning, which was performed 35 days after the initiation with antifungal medication. Intravenous caspofungin acetate was also administered for 5 days after the transplantation.

## Isolation and Species Identification of Pathogen

One week after the initial inoculation of scraping samples onto the peripheral area of a Sabouraud dextrose agar plate, a whitish hyphate colony was observed at the site at which the scleral specimen had been inoculated and cultivated at 25°C (Figs. 3A–C). In contrast, no colonies were observed at the center of the Sabouraud dextrose agar plate on which the corneal specimen had been inoculated and cultivated 25°C, nor on the plate on which the samples had been incubated at 37°C.

Seven weeks after the initial culture of scraping samples, a yellow to reddish-brown colony was observed at the center of the agar plate where the corneal specimen had been inoculated (Figs. 3B, D). Analysis of the partial sequence of the β-tubulin gene of the fungi isolated from the cornea for detection of species level showed the presence of a novel species, *A. cibarius* (Eurotium state). Based on sequence of the internal transcribed spaces of the ribosomal DNA gene regions, the strain isolated from the sclera was identified as *S. apiospermum*. The minimum inhibitory concentrations (MICs) of several antifungal drugs and minimal effective concentration of micafungin to the 2 strains were determined by the use of the broth dilution method according to M38-A2 of the Clinical and Laboratory Standards Institute (Table 1). Seven months after the initiation of antifungal therapy, the patient's best-corrected visual acuity was limited to light perception because the sclerokeratitis had caused bullous keratopathy.

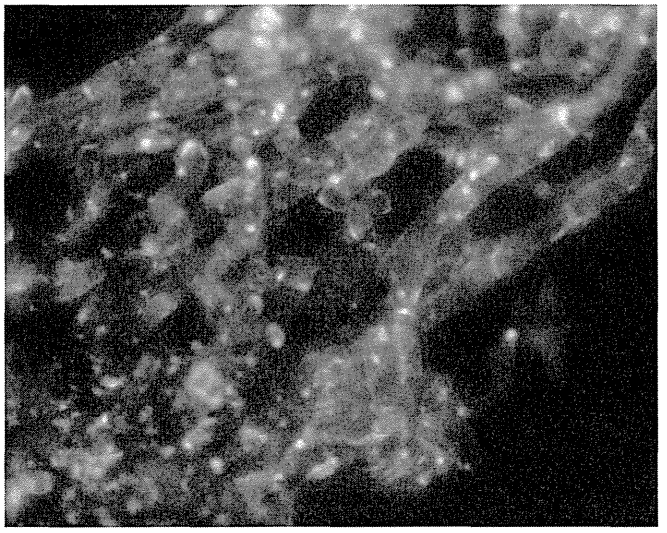

**FIGURE 2.** Fungiflora Y staining of *A. cibarius* isolated from the corneal scrapings (original magnification, ×1000). A large quantity of fungi is seen.

## DISCUSSION

*A. cibarius* was first reported in 2012 by Hong et al,[8] who had isolated it in meju, a brick of dried fermented soybeans in South Korea. It was subsequently isolated in black beans, bread, and salami in the Netherlands. To the best of our knowledge, this case is the first to describe infection of human tissue with *A. cibarius*. In accordance with the observation, this novel species should be regarded as a possible pathogen in infectious keratitis.

When considering the risk of developing infection with *A. cibarius*, 2 crucial considerations are the timing and temperature of the environment in which the species is grown. However, rigorous isolation and species identification may not be possible in clinical settings. Nevertheless, ophthalmologists should attempt to isolate pathogenic fungi to the greatest extent possible, because this will assist in identifying novel species involved in keratitis, and accumulation of new pathogens with species identification by the use of a molecular genetic method is crucial.

Previous reports have described fungal sclerokeratitis occurring after trauma and pterygium surgery.[1–4] Although the exact means by which the *Scedosporium* species is involved in sclerokeratitis remains unclear, most of the cases reported were caused by infection with *Scedosporium* species in immunocompromised patients, or the patients had experienced disturbance in local ocular morphology. Regarding these cases, Moriarty et al[1] found that calcification at the base of an ulcer bed was frequently a nidus of infection, whereas Singh and McCluskey[2] reported that the pathogenesis of infectious scleritis after pterygium surgery was related to an avascular change in the sclera. Considering that the normal structure of the sclera had been disrupted on the side of calcification in the current case, this area might have been the focus of fungal infection.

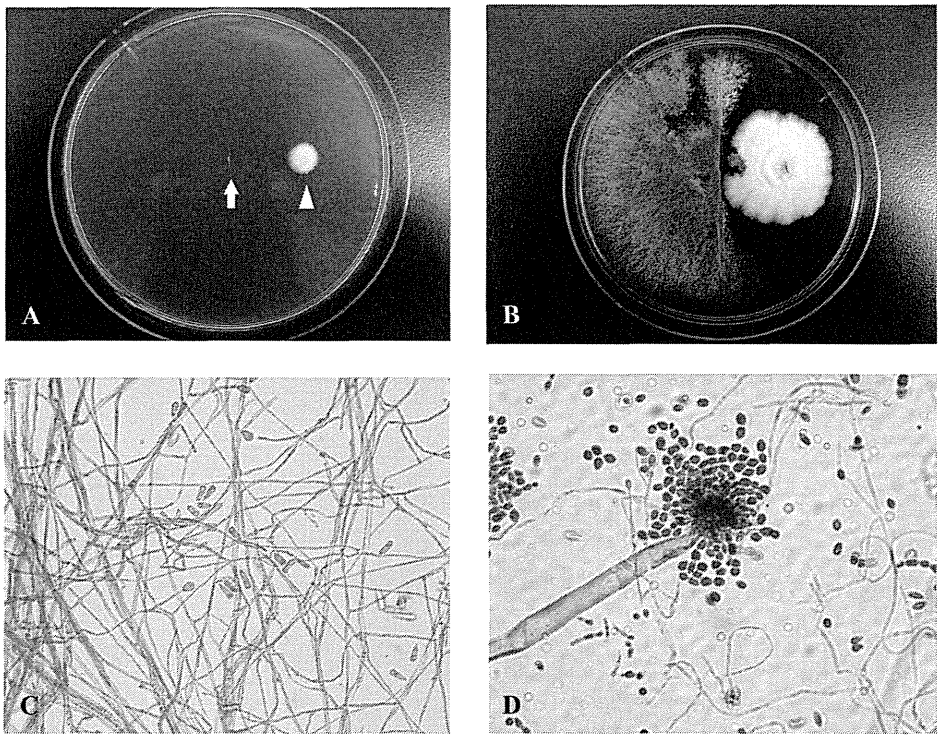


**FIGURE 3.** Inoculated Sabouraud agar plate 7 days after the inoculation (A) and 49 days after inoculation (the diameter of the Petri dish is 9 mm) (B), and images of lactophenol cotton blue mounts of the strain isolated from the sclera (original magnification, ×400) (C), and the cornea (original magnification, ×400) (D). A, Only scraped corneal tissue, but no colony, is observed in the center of the agar plate where the corneal scraping had been inoculated (white arrow). A white hyphate colony of *S. apiospermum* is seen where the scleral scraping had been inoculated (white arrow head). B, A yellow to reddish-brown hyphate colony of *A. cibarius* is observed at the center of the agar plate. C, Oval conidia on the tip of the conidiophores of *S. apiospermum* are observed. D, Conidia with a thin wart-shaped surface on the tip of the conidiophore of *A. cibarius* are observed.

Based on observation that the area of keratitis had progressed for 3 days after the first visit, we had originally hypothesized that 1 causative strain of fungus had spread from the infected sclera to the nasal cornea. However, we subsequently isolated 2 different species of fungi from the cornea and the sclera, and the keratitis persisted after the scleritis had been resolved by surgical intervention. We therefore believed that the infection in both the cornea and the sclera occurring simultaneously, or with a modest time lag, with 2 different species of fungi might result in a poor outcome. Although we found that the MICs of *S. apiospermum* for several of the antifungal drugs that we had administered were higher than those for *A. cibarius*, the avascular changes in the sclera caused by calcification may impair the permeability of antifungal drugs to the infected region. The differences in the MICs of 2 species and the differences in the tissue permeability of antifungal drugs between the sclera and the cornea may be related to the difference between healing rates of the scleritis and the keratitis.

In conclusion, polymicrobial sclerokeratitis may result from infection with 2 different species of fungi. In all such cases, rigorous isolation of the causative pathogens from the sclera and the cornea, along with the consideration of *A. cibarius* as a possible causative pathogen, is crucial.

**TABLE 1.** MICs of the 2 Strains of the Fungus

| | MIC, μg/mL | |
|---|---|---|
| **Drugs** | ***A. cibarius*** | ***S. apiospermum*** |
| MCFG | <0.015 | 0.25 |
| AMPH | 0.25 | 2 |
| 5-FC | 16 | >64 |
| FLCZ | 16 | 16 |
| ITCZ | 0.5 | 1 |
| VRCZ | 0.25 | 0.25 |
| MCZ | 1 | 0.5 |

AMPH, amphotericin B; 5-FC, flucytosine; FLCZ, fluconazole; ITCZ, itraconazole; MCFG, micafungin; MCZ, miconazole; VRCZ, voriconazole.

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# In Vitro Evaluations of Topical Agents to Treat *Acanthamoeba* Keratitis

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***Purpose:*** To evaluate the effectiveness of topical agents for the treatment of *Acanthamoeba* keratitis (AK).
***Design:*** Laboratory research.
***Participants:*** Fifty-six *Acanthamoeba* isolates from 56 patients with clinically proven AK were studied.
***Methods:*** The effectiveness of 7 agents against *Acanthamoeba* cysts was determined in vitro. The agents were 1.0% povidone-iodine, 0.05% benzalkonium chloride (BZC), 0.02% chlorhexidine gluconate (CHG), 0.1% propamidine isethionate, 0.02% polyhexamethylene biguanide (PHMB), 5.0% natamycin, and 1.0% voriconazole (VRCZ). These concentrations are those recommended for patients. In addition, 10-fold dilutions of each of the agents were tested. After exposing the cysts to each agent at 35°C for 1 hour or 24 hours, the agents were removed by centrifugal washing. The exposed cysts were observed by optical microscopy for 7 days. In addition, the fine structures of the exposed isolates were examined by transmission electron microscopy (TEM). The genotype of the isolates was determined by 18S rDNA fragment sequencing.
***Main Outcome Measures:*** The in vitro susceptibility was determined by complete growth inhibition, and the morphologic appearance was determined by TEM. The genotypes of the 56 isolates were determined by 18S rDNA fragment sequencing.
***Results:*** The *Acanthamoeba* cysts were most susceptible to natamycin, followed by povidone-iodine, BZC, PHMB, propamidine, and CHG. None of the strains was susceptible to VRCZ. The susceptibilities to PHMB and CHG may be time dependent and to propamidine may be concentration dependent. Transmission electron microscopy showed changes in the inner structure of the cysts exposed to natamycin and povidone-iodine. The *Acanthamoeba* genotype was T4 in 52 isolates, and cysts with the same genotype had different agent susceptibilities.
***Conclusions:*** Natamycin and povidone-iodine had excellent cysti-static (or cystcidal) effects, and PHMB and propamidine did not. There was no correlation between agent effectiveness and *Acanthamoeba* genotype. Therefore, susceptibility tests of isolates are needed to choose the most appropriate agent, and our results can be a guideline for choosing the most appropriate agent for immediate empirical treatment of AK. *Ophthalmology 2014;121:2059-2065 © 2014 by the American Academy of Ophthalmology.*

*Supplemental material is available at www.aaojournal.org.*

The initial step in treating eyes with bacterial infections is identifying the bacterium and performing agent effectiveness tests to determine the most effective antibacterial agent. In cases of *Acanthamoeba* keratitis (AK), effectiveness tests are rarely performed, and empirical treatments are generally used. Susceptibility tests of amoeba to different agents are not generally performed because the procedures for the tests are cumbersome and require a longer time because amoebae grow more slowly than bacteria. In addition, a standardized method of performing effectiveness tests of different agents against amoebae has not been established, although various methods have been reported.[1–5] In addition, the concentration of the topical agents in the conjunctival sac is rapidly reduced because of the continuous dilution by the lacrimal fluid. This must be considered when susceptibility tests are performed in vitro. Thus, an appropriate treatment strategy for AK has not been determined in relation to not only the most effective agent but also the optimal frequency and duration of the treatments.

*Acanthamoebae* were initially classified by their morphology, but they have recently been classified on the basis of their 18S rDNA sequence because the morphology can vary with the culture media and incubation temperatures.[6] On the basis of this classification, the *Acanthamoebae* have been classified into genotypes T1 to T16. Earlier studies have shown that the majority of the AK-causing amoebae had the T4 genotype.[7–9] At present, it has not been established whether the susceptibility of *Acanthamoebae* to different agents is dependent on the genotype.

Thus, the purpose of this study was to perform effectiveness tests of 7 agents that are clinically used topically against *Acanthamoeba* isolates obtained from 56 patients with AK. We also analyzed the relationship between the



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pattern of effectiveness of these agents and the genotype of the 18S rDNA sequences, and the morphologic classifications of cysts, including the size.

## Methods

### *Acanthamoeba* Isolates

A total of 56 nonaxenic *Acanthamoeba* isolates were obtained from 56 patients with clinically proven AK from the Osaka University Hospital, Fujita Health University Banbuntane Houtokukai Hospital, and Ehime University Hospital. The isolates were collected between 1994 and 2011 and stored at 4°C. We also studied the *Acanthamoeba castellanii* strain 300100, which was obtained from the American Type Culture Collection (ATCC). For encystment, amoebae were inoculated onto non-nutrient agar plates that were seeded with the heat-killed (70°C for 30 minutes) ATCC *Escherichia coli* strain 25922. The plates were incubated at 25°C in an airtight container that included a saturated pad in a petri dish. After encystment, the cysts were harvested in 2 ml of Page's amoeba saline solution and stored at 4°C until the assays.

The study protocol was approved by the Osaka University Ethics Committee, which decided that it was not necessary to obtain an informed consent form from the original patients because this study did not include any clinical involvement of the patient.

### Agent Effectiveness Assays

We selected 2 ophthalmic solutions, 1 injection drug, and 4 disinfectants for the AK treatment. The concentrations of these agents were 5.0% natamycin (Pimaricin; Senju, Osaka, Japan), 0.1% propamidine isethionate (Golden Eye; Typharm Limited, Norwich, UK), and pre-prepared solutions of 1.0% voriconazole (VRCZ) (Vfend; Pfizer Japan Inc, Tokyo, Japan), which were used as stock solutions. We also used 0.02% polyhexamethylene biguanide (PHMB) (Spalux; Daikin Co, Chiba, Japan), which is the concentration usually used for topical AK treatment. In addition, 1.0% povidone-iodine (ISODINE; Meiji Co, Ltd, Tokyo, Japan), 0.05% benzalkonium chloride (BZC) (Wako, Osaka, Japan), and 0.02% chlorhexidine gluconate (CHG) (Wako, Osaka, Japan), which are antiseptic agents for ophthalmic surgery, were tested. Ten-fold dilutions of these 7 agents also were tested. Distilled water was used as a control and referred to as nonexposure.

To prepare *Acanthamoeba* suspensions of the cysts that were stored at 4°C, they were washed three times in quarter-strength Ringer lactate (RL) solution (SOLULACT; Terumo, Tokyo, Japan) by centrifuging (1000 rpm for 5 minutes). The pellet after the third wash was resuspended in 3.5 ml of the RL solution. The number of cysts in the suspension was counted using a hemacytometer (Burker–Turk cell counter) and adjusted to a final concentration of $10^4$ cysts/ml in RL solution. Then, 100 μl of the cyst suspension was mixed with 100 μl of each agent in sterile microtubes and incubated in a stationary position at 35°C for 1 hour or 24 hours. Then, the exposed cysts were washed 3 times (1000 rpm for 5 minutes) with the RL solution, and the sediment was applied to each non-nutrient agar *E. coli* plate. These plates were incubated at 25°C in an airtight container that had a saturated pad in a petri dish. The appearance of the amoebae was observed by optical microscopy for 7 days.

To determine the effectiveness of an agent, the cysts of the isolate were exposed to the agent for 1 hour or 24 hours and then examined by optical microscopy. If even 1 excystment was present, the isolate was classified as being resistant to the agent. However, if no excystment was present, the isolate was considered to be susceptible. This method was described in detail by Narasimhan et al[1] in 2002. We verified its good repeatability by performing triplicate tests of the ATCC strain, and then we used the same techniques for the measurement of agent effectiveness on all clinical isolates.

### Genotyping

The cysts, which had been stored at 4°C, were cultured in peptone yeast-extract glucose medium for 2 to 4 weeks, and the excysted trophozoites were collected by centrifugation (1000 rpm for 5 minutes). DNA was extracted from the trophozoites with a commercially available kit (MORA-EXTRACT; Kyokuto Pharmaceutical, Tokyo, Japan). Polymerase chain reaction (PCR) was performed as described by Corsaro and Venditti for 18S rRNA gene sequences of *Acanthamoeba* with the following genus-specific primers: JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCA-CAAGCTGCTAGGGAGTCA-3′).[10]

The PCR conditions were initial incubation for 7 minutes at 95°C, followed by 45 cycles of 1 minute at 95°C, 1 minute at 60°C, and 2 minutes at 72°C. The PCR products were purified with the QIAamp PCR purification kit (Qiagen K.K., Tokyo, Japan). Direct sequencing of the PCR products was performed with the ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA) using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). The obtained sequence data were analyzed with the Sequencher 5.1 DNA sequence assembly and the analysis software (available at http://genecodes.com/, accessed January 17, 2012).

### Observations of Morphologic Characteristics

Morphologic classification of the *Acanthamoeba* isolates was performed according to the Pussard–Pons classification. Twenty microliters of the cyst suspension that had been stored at 4°C were put on a glass slide and covered with a cover slip. The slide was observed by optical microscopy, and all the cysts were classified into *Acanthamoeba* groups I, II, and III. In addition, the cyst size was measured at a magnification of 400× with an optical micrometer microscope.

### Transmission Electron Microscopic Observations

The ATCC *Acanthamoeba* strain was used for testing. After an adequate number of trophozoites had been inoculated onto the peptone yeast-extract glucose medium, the trophozoites were collected by centrifugation (1000 rpm, 5 minutes) and allowed to encyst in Page's amoeba saline solution supplemented with magnesium chloride (50 mmol/l) for 2 weeks. Agents used included 5.0% natamycin, 1.0% povidone-iodine, 0.05% BZC, 0.02% PHMB, 0.02% CHG, 0.1% propamidine, and 1.0% VRCZ. Distilled water was used as a control for nontreatment. After exposing the cysts to these agents for 1 hour, the agents were removed as described for the susceptibility tests. The cysts were then washed in phosphate-buffered solution by centrifuging (1000 rpm for 5 minutes), and the pellets were fixed with 2% (weight/volume) glutaraldehyde in phosphate-buffered solution. They were post-fixed in osmic tetroxide. The fixed cysts were dehydrated and embedded in an epoxy resin, and ultrathin sections were cut. The sections were placed on carbon-coated copper grids and stained with aqueous uranyl acetate and lead citrate, and observed by transmission electron microscopy (TEM) (Hitachi H-7650, Hitachi, Japan).

Table 1. Quality of Susceptibility Test of ATCC Strain

| Agents | Concentration | Exposure time | Measurement time | | | Quality |
|---|---|---|---|---|---|---|
| | | | *1st-try* | *2nd-try* | *3rd-try* | |
| Pimaricin (natamycin) | 5.0% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| | 0.5% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| Povidone-iodine | 1.0% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| | 0.1% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| Benzalkonium chloride (BZC) | 0.05% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| | 0.005% | 24 h | − | − | − | 100% |
| | | 1 h | + | + | + | 100% |
| Polyhexametylene biganaide (PHMB) | 0.02% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | + | 66.7% |
| | 0.002% | 24 h | − | − | + | 66.7% |
| | | 1 h | − | + | + | 66.7% |
| Propamidine isetionate | 0.1% | 24 h | − | − | − | 100% |
| | | 1 h | − | − | − | 100% |
| | 0.01% | 24 h | − | − | − | 100% |
| | | 1 h | + | + | + | 100% |
| Chlorhexidine gluconate (CHG) | 0.02% | 24 h | − | − | − | 100% |
| | | 1 h | + | + | + | 100% |
| | 0.002% | 24 h | + | + | + | 100% |
| | | 1 h | + | + | + | 100% |
| Voriconazole (VRCZ) | 1.0% | 24 h | + | + | + | 100% |
| | | 1 h | + | + | + | 100% |
| | 0.1% | 24 h | + | + | + | 100% |
| | | 1 h | + | + | + | 100% |

+ = growth; − = no growth; ATCC = The American Type Culture Collection; h = hour.

# Results

## Effectiveness of Anti-*Acanthamoebic* Agents

The effectiveness tests of the ATCC strain to 7 topical agents were performed in triplicate (Table 1), and the results for the 3 tests were not significantly different except for that of PHMB. The degree of susceptibility of the cysts of the *Acanthamoeba* isolates to the different agents is shown in Table 2. All of the data on the 56 isolates are shown in Table 3 (available at www.aaojournal.org). *Acanthamoeba* cysts of all of the isolates were susceptible to natamycin under all 4 measurement conditions, that is, at 1-hour and 24-hour exposures and at base concentration and 10-fold dilution. This was followed by decreasing susceptibility to povidone-iodine, BZC, PHMB, propamidine, and CHG. One of the 56 isolates was susceptible to povidone-iodine at a concentration as low as 0.1%, but was unexpectedly resistant at a concentration of 1.0% that was confirmed by retesting twice. This isolate was morphologically classified into group I. All isolates were resistant to VRCZ under the 4 measurement conditions. Polyhexamethylene biguanide and CHG were more effective after 24 hours of exposure than after 1 hour of exposure, and propamidine was more effective in 0.1% solutions than in 0.01% solutions.

## *Acanthamoeba* Genotyping

Sequencing data were obtained for 52 of the 56 clinical isolates, and all were genotyped as T4 (Table 3, available at www.aaojournal.org). Two of the 4 strains that could not be genotyped accurately were morphologically classified into groups II and III, suggesting that these 2 isolates probably had different genotypes. The other 2 isolates were difficult to genotype, but we believe that they had different genotypes.

## Morphologic Classification and Size of Cysts

The morphologic classification by optical microscopy was group II in 47 isolates (83.9%), group III in 6 isolates (10.7%), 2 isolates in combined groups II and III (3.8%), and 1 isolate in group I (1.8%) (Table 3, available at www.aaojournal.org). The mean cyst size was 16 μm, with a range of 7 to 22 μm.

## Relationships among Agent Effectiveness, Genotypes, Morphologic Groups, and Size of Cysts

The agent effectiveness, genotypes, morphologic groups, and size of the cysts are shown in Table 3 (available at www.aaojournal.org). Although 52 of the 56 strains had the same T4 genotype, the agent effectiveness differed among these isolates. In addition, the agent effectiveness differed in the 46 strains with a morphologic classification of group II; however, all strains were susceptible to natamycin and povidone-iodine regardless of morphologic classification. There was no significant correlation between the agent effectiveness and the size and morphology of the *Acanthamoeba* cysts.

## Transmission Electron Microscopic Observations

The changes in the morphology of the cysts of the ATCC strain after exposure to each agent are shown in Figure 1. In comparison

Table 2. Susceptibility Rate of Acanthamoeba Cysts

| Rank order | Agents | Concentration | Exposure time | Number of susceptibility strains | Susceptibility rate of 56 strains (%) |
|---|---|---|---|---|---|
| 1 | Pimaricin (natamycin) | 5.0% | 24 h | 56 | 100.0% |
| | | | 1 h | 56 | 100.0% |
| | | 0.5% | 24 h | 56 | 100.0% |
| | | | 1 h | 56 | 100.0% |
| 2 | Povidone-iodine | 1.0% | 24 h | 56 | 100.0% |
| | | 0.1% | | 56 | 100.0% |
| | | 1.0% | 1 h | 49 | 87.5% |
| | | 0.1% | | 50 | 89.3% |
| 3 | Benzalkonium chloride (BZC) | 0.05% | 24 h | 56 | 100.0% |
| | | | 1 h | 56 | 100.0% |
| | | 0.005% | 24 h | 55 | 98.2% |
| | | | 1 h | 26 | 46.4% |
| 4 | Polyhexametylene biganaide (PHMB) | 0.02% | 24 h | 56 | 100.0% |
| | | 0.002% | | 46 | 82.1% |
| | | 0.02% | 1 h | 26 | 46.4% |
| | | 0.002% | | 8 | 14.3% |
| 5 | Propamidine isetionate | 0.1% | 24 h | 56 | 100.0% |
| | | | 1 h | 35 | 62.5% |
| | | 0.01% | 24 h | 17 | 30.4% |
| | | | 1 h | 1 | 1.8% |
| 6 | Chlorhexidine gluconate (CHG) | 0.02% | 24 h | 56 | 100.0% |
| | | 0.002% | | 25 | 44.6% |
| | | 0.02% | 1 h | 25 | 44.6% |
| | | 0.002% | | 2 | 3.6% |
| 7 | Voriconazole (VRCZ) | 1.0% | 24 h | 0 | 0.0% |
| | | | 1 h | 0 | 0.0% |
| | | 0.1% | 24 h | 0 | 0.0% |
| | | | 1 h | 0 | 0.0% |

h = hour.

with the exposure to distilled water (Fig 1A), the cysts after exposure to natamycin for 1 hour showed a thickening of the wall and a withdrawal of the cytoplasm from the wall of the cyst (Fig 1B). After exposure to povidone-iodine, the ridges of the outer wall became smooth, the inner wall separated from the outer wall, and some of the cytoplasmic microstructures were lost and others formed clusters (Fig 1C). After exposure to BZC, the inner and outer walls of the cysts separated and the cytoplasmic elements were lost (Fig 1D). With PHMB, there was a loss of the electron-dense material in the cytoplasm (Fig 1E). With CHG, cytoplasmic clusters were observed (Fig 1F), and with propamidine, a separation of the cyst inner walls from the outer walls and the loss of cytoplasmic elements were observed (Fig 1G). With VRCZ, no major changes in intracellular organization were observed, although there was a reduction in the protrusion of the outer wall of the cysts (Fig 1H). These results indicated that the action sites of the agents against the *Acanthamoeba* structures differed according to the action of each topical agent.

## Discussion

Our results showed that the *Acanthamoeba* isolates tested had high in vitro susceptibility to natamycin, an antifungal agent, and povidone-iodine, an iodine-based disinfectant, but not to PHMB and propamidine. The genotype of most of these strains was T4, and this genotype is known to be the most common cause of AK.

Many susceptibility assays for *Acanthamoeba* have been reported, and they can be broadly divided into those testing anti-amoeba agents and those assessing contact lens cleaning and soaking solutions. However, methods of encystment, exposure to the agents, and exposure times differed, and the results differed.[1–5]

In 2012, Shoff and Eydelman[4] evaluated the optimal conditions for testing the multipurpose contact lens solution against *Acanthamoeba.* They reported that the encystment method had the greatest impact on the results, and that the starvation method was the most suitable encystment method. They also presented a quantitative method that was important for the standardization. These proposals may become evaluation tests for agents against amoebae in the future. When this method is applied for the agent susceptibility tests for the best therapeutic agent, the exposure time of *Acanthamoeba* cysts to the agent should be brief because the topical agents are continuously diluted by the lacrimal fluid and the effective concentration of the eye drops in the conjunctival sac can be extremely short.

In 2013, Kowalski et al[5] reported on a new method to test anti-*Acanthamoeba* drugs. Their method was relatively simple to perform but required 1 to 3 weeks to obtain results after exposure to the drugs. Therefore, it would be more practical to shorten the assay time to use it clinically.

The method used in our study was based on the method of Narasimhan et al.[1] The advantages of this method are that it can be used on mature cysts obtained by the starvation method and the exposure time of the cysts and agents can be made as short

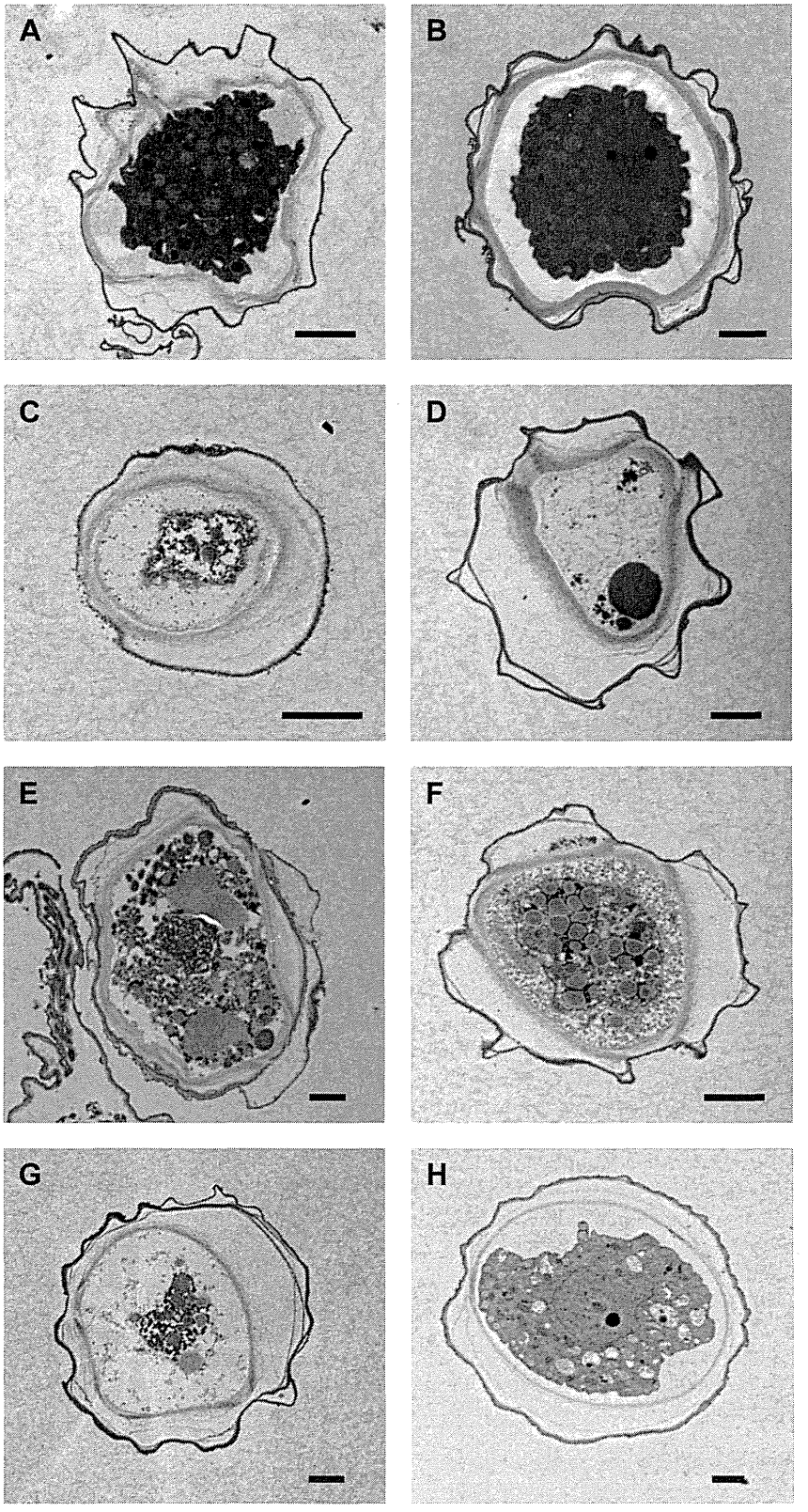


**Figure 1.** Transmission electron microscopy images of *Acanthamoeba* cysts after a 1-hour exposure to the following 7 agents. Bar is 2 μm. Untreated **(A)**; treated with 5% natamycin **(B)**; treated with 1.0% povidone–iodine **(C)**; treated with 0.05% benzalkonium chloride (BZC) **(D)**; treated with 0.02% polyhexamethylene biguanide (PHMB) **(E)**; treated with 0.02% chlorhexidine gluconate (CHG) **(F)**; treated with 0.1% propamidine isethionate **(G)**; treated with 1.0% voriconazole (VRCZ) **(H)**.

as possible. However, there are some problems with this method; the agents may not be completely removed after exposure and the 7-day observation period may be too short. In fact, white crystalline sediment was detected after natamycin exposure and centrifugal washing; thus, it was unclear whether the effect of the agent was completely removed. Thus, *Acanthamoebae* may not grow unless the agent is completely removed, because they can remain encysted for extended periods under adverse conditions. Therefore, to confirm the viability of *Acanthamoeba* cysts, we examined them by TEM and confirmed that natamycin severely damaged their morphology. This method also takes 2 to 4 weeks to obtain results after isolation, which is too long for the initial treatment.

Our results showed that natamycin had the highest efficacy among the agents against *Acanthamoeba* cysts. This agent has mainly antifungal activity by impairing membrane permeability. Against *Acanthamoeba,* similar effects such as intimal thickening and contraction of the intracellular elements were confirmed by TEM imaging. Although it is uncertain whether the influence of the agents was completely eliminated by centrifugal washing, it was clear from the TEM imaging that natamycin had a biocidal action against the *Acanthamoeba* cysts. Although Ma et al[11] and Kitagawa et al[12] reported that some patients with AK responded positively to a natamycin-based combination regimen, Inoue et al[13] reported a case that was unresponsive to natamycin. It is generally believed that the differences in the responsiveness are due to not only differences in the strain susceptibility but also the difference in the penetration of natamycin into the corneal tissue. Therefore, corneal debridements, which increase the tissue penetration of natamycin, may be useful for natamycin treatment of AK.

Povidone-iodine had the next highest efficacy in the agent susceptibility test of *Acanthamoeba* cysts after natamycin. We found that povidone-iodine clearly damaged the cysts as observed in the TEM images. In contrast, Lim et al[3] reported that povidone-iodine showed no cysticidal effects, probably because the povidone-iodine concentrations they used (0.125–256 μg/ml) were below the effective concentration.

Of note, we found that only 1 isolate was resistant to the higher (1.0%) concentration despite being susceptible at a lower (0.1%) concentration. Gottardi[14] reported that the concentration of free iodine had the maximal bactericidal activity in 0.1% solutions compared with higher concentrations. Therefore, we believe that this phenomenon was related to the concentration of free iodine. Moreover, free iodine is consumed by organic matter, such as the components of body fluids. Thus, when povidone-iodine is used to treat AK, attention should be paid to the concentration and quantity of the eye drops. However, further investigations are needed to assess the strains that exhibit resistance to povidone-iodine.

Benzalkonium chloride had the third highest effectiveness among the agents, and TEM images confirmed the separation of the cyst inner and outer walls and loss of cytoplasmic microstructures. Niszl and Markus[15] demonstrated the effectiveness of BZC in contact lens disinfecting solutions, and BZC is considered to be an effective treatment for AK. However, its toxicity to the human cornea needs to be considered.[16] Therefore, for treating AK, attention should be paid to the exposure time and concentration of BZC.

We found that the susceptibilities to propamidine, CHG, and PHMB, which are frequently used to treat AK, were low. These results support previous reports of cases that were unsuccessfully treated by these agents.[17–19] Although the TEM images of ATCC strain cysts exposed to these agents showed some damage, the clinical isolates might not be damaged by these agents. Moreover, it seemed that the susceptibilities to PHMB and CHG were time dependent and that the susceptibility to propamidine was concentration dependent. When using these agents, the exposure time of the amoebae to PHMB and CHG should be longer, and propamidine should be administered while maintaining the optimal concentration.

Voriconazole had no cysticidal effects against any isolates in the agent effectiveness tests. However, Bang et al[20] used VRCZ to treat patients with AK resistant to chlorhexidine and reported its effectiveness. The mechanism of the agent might be different in vitro than that in vivo.

### Study Limitations

Because the agents are diluted by the lacrimal fluid in vivo, the amoebae will be exposed to a continuously decreasing concentration to the agents. In addition, because the amoebae are located within the corneal tissue, the amoebae will be exposed to a lower concentration of the agent. Therefore, it would be preferable to choose an agent that has both an anti-amoebic effect and a high penetration into corneal tissue. To enhance the penetration, corneal abrasion should be performed.

In conclusion, natamycin and povidone-iodine have excellent cysti-static (or cystcidal) effects, but PHMB and propamidine do not. The current classification of *Acanthamoeba* based on the 18SrDNA sequencing is not a good indicator for the choice of an effective agent. Therefore, the agent effectiveness tests for individual isolates are important for choosing the appropriate treatment for AK, especially for refractory AK cases. A simple and rapid method for testing the agent susceptibility of *Acanthamoeba* needs to be developed in the future, because the present susceptibility tests are complicated and time-consuming. The results of this study may be useful for choosing the agents for the empirical treatment of AK.

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Abbreviations and Acronyms:
**AK** = *Acanthamoeba* keratitis; **ATCC** = American Type Culture Collection; **BZC** = benzalkonium chloride; **CHG** = chlorhexidine gluconate; **PCR** = polymerase chain reaction; **PHMB** = polyhexamethylene biguanide; **RL** = Ringer lactate; **TEM** = transmission electron microscopy; **VRCZ** = voriconazole.

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CASE SERIES

# Ineffectiveness of intrastromal voriconazole for filamentous fungal keratitis

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**Purpose:** The purpose of this study is to describe the ineffectiveness of intrastromal voriconazole injection for filamentous fungal keratitis by contrasting the effectiveness for yeast keratitis.

**Methods:** We examined seven fungal keratitis patients prospectively. All yeast was identified by molecular phylogenetic analyses of the chromosomal regions coding for the D1/D2 domain of the large-subunit 26S ribosomal RNA gene. All filamentous fungi were identified by the sequencing of internal transcribed spacers of the ribosomal DNA gene regions. Approximately 0.1 mL of voriconazole diluted with saline to 1.0% was injected with a 30-gauge needle inserted obliquely into the three to five clear cornea sites around the abscess. All subjects were administered natamycin ointment and oral itraconazole. When needed, intravenous micafungin, voriconazole, and/or intracameral voriconazole were added. Clinical courses were observed by the slit lamp microscope. Histopathology was examined when the corneas were removed.

**Results:** All cases that were caused by yeast healed quickly after injections. Two cases of keratitis caused by *Fusarium*, and one case caused by *Aspergillus*, did not heal completely. In the *Fusarium* cases, additional antifungal medications (3.0% topical voriconazole and intravenous injection of micafungin) were needed. After optical penetrating keratoplasty in one of the cases, fungi were found in the deep stroma of the removed cornea. In the case of *Aspergillus* keratitis, pathological findings also showed fungi deep in the stroma of the removed cornea and the keratitis recurred after therapeutic penetrating keratoplasty.

**Conclusion:** Intrastromal voriconazole injection is successful in treating yeast keratitis. However this is not the case for filamentous fungal keratitis.

**Keywords:** voriconazole, intrastromal injection, filamentous fungal keratitis

## Introduction

Fungal keratitis is a vision-threatening infectious disease. Currently the number of effective antifungal drugs is less than those of antibacterial drugs and they are less tissue-permeable.[1] Therefore fungal keratitis tends to have a worse prognosis than bacterial keratitis.[2] Since filamentous fungal keratitis is more intractable than yeast-related keratitis,[3] early diagnosis and targeted treatment for filamentous fungal keratitis is the key to managing the disease. Although natamycin, the only commercially available antifungal agent for ophthalmic use, is effective in eliminating both yeast and filamentous fungi, there are some cases where its effectiveness is limited.

Voriconazole has been reported to have a broad-spectrum of antifungal properties and has been shown to be effective on ophthalmic clinical isolates, including the *Fusarium* and *Aspergillus* species.[4] In recent years, some reports have shown a curative effect of intrastromal injection of antifungal drugs.[5–9] Although in most of these reports, a solution of 0.05% voriconazole was effective, at our center a number of cases of filamentous keratitis recurred, despite the use of multiple intrastromal injections

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of voriconazole at concentrations of 0.05% to 1%. In those cases, some causative strain showed low minimum inhibitory concentration (MIC) of voriconazole. It is important to recognize that voriconazole intrastromal injection success rate may vary depending on the underlying pathogen. Hence, we evaluate the effects of voriconazole intrastromal injection therapy for fungal keratitis and describe the ineffectiveness of the treatment for filamentous fungal keratitis by contrasting the effectiveness for yeast keratitis.

## Participants and methods

### Participants

Seven patients (two males, five females), who were clinically diagnosed with culture-positive fungal keratitis, were enrolled in this study. All patients were consecutive fungal keratitis cases, who visited Tokushima University Hospital between January 2012 and June 2013. No patient had any antifungal medication prior to visiting our hospital, and no history of an immunocompromised state had been reported in medical interview. Their mean age was 72.3±10.8 years (range: 60–86 years). Informed consents for the voriconazole intrastromal injection were obtained from all subjects under the approval of the Ethics Committee of Tokushima University Hospital. We excluded patients whose intraocular pressure could not be measured due to corneal perforation, cases of an involuntary eye movement, and cases where we judged that the injection under local anesthesia was impossible.

Voriconazole (1.0%) eye drops were applied four to five times a day for at least 14 days in all cases except for Case 5. All subjects were treated with 1.0% voriconazole intrastromal injection after the diagnosis of fungal keratitis was confirmed. In the sixth case, 0.1% voriconazole was injected first and the concentration was gradually increased with each subsequent injection. In patients who had been prescribed other therapies for keratitis prior to visiting our hospital, topical antibacterial antibiotics were decreased to three or four times a day only for sterilization of the ocular surface before intrastromal injection. Additionally, topical steroids, lubricant eye drops (hyaluronic acid sodium), and the antiviral ointments were discontinued.

### Methods

All isolates of fungi were obtained from the corneal scrapings. Several kinds of agar plates were inoculated with scrapings, including two Sabouraud dextrose agar (Eiken Chemical Co., LTD., Tokyo, Japan), and they were incubated at both room temperature and 37°C. The species of yeast fungi were identified by molecular phylogenetic analyses of the chromosomal regions coding for the D1/D2 domain of the large-subunit 26S ribosomal RNA gene.[10] The species of filamentous fungi were identified by the sequencing of internal transcribed spacers of the ribosomal DNA gene regions.[10]

Voriconazole (VFEND® for intravenous use, 200 mg, Pfizer Japan Inc., Tokyo, Japan) was diluted with saline to 1.0%, 0.3%, or 0.1% and loaded in a 1.0 mL tuberculin syringe with a 30-gauge needle. Under topical anesthesia with 0.4% oxybuprocaine hydrochloride (Benoxil ophthalmic solution 0.4%, Santen Pharmaceutical Co., Ltd., Osaka, Japan), the needle was inserted obliquely, with the bevel down, into the clear cornea surrounding the abscess. Approximately 0.1 mL voriconazole was injected at each of three injection sites in most cases. In cases where the keratitis has progressed in the whole cornea, the same dose was injected at each of five injection sites. All injections were performed in the treatment room of the outpatient clinic, unless the eye was judged to be at risk for corneal perforation due to corneal thinning. In these cases, injections were performed in the operating theater. Just after injections, all eyes were examined with a slit lamp microscope to confirm the presence/absence of complications associated with treatment. Anterior segment optical coherence tomography (OCT) (Spectralis® OCT, Heidelberg Engineering, Heidelberg, Germany) was also performed just after the injection to confirm placement of the voriconazole solutions, as represented by stromal swelling in the mid and/or deep layers of the cornea.

## Results

Four yeasts were cultured on Sabouraud plate agar. One was identified as *Candida albicans* and three as *Candida parapsilosis*. Three filamentous fungi were cultured on Sabouraud plate agar and those species were identified as an *Aspergillus flavus* and a *Fusarium proliferatum*. An additional *Fusarium* species was identified only by morphological character in a lacto-phenol cotton blue mount. MICs of voriconazole to all strains were determined by use of a broth dilution method according to M38-A2 of the Clinical and Laboratory Standards Institute.

Four patients diagnosed with yeast-related keratitis had complete corneal healing after administration of intrastromal injection of 1.0% voriconazole (Table 1). MICs of voriconazole to four *Candida* species were low, eg ranged less than 0.015–0.06 μg/mL. Case 4 and 5, where the causative fungi were *A. flavus* and *Fusarium* species respectively, were refractory to multiple 1.0% voriconazole intrastromal injections and their keratitis recurred. The MICs of voriconazole to those strains were 0.5 μg/mL and 4 μg/mL, respectively.

**Table 1** Epidemiological data and outcomes of all subjects

| Case | Age (years) | Sex | 1st BCVA | Pathogen | MIC of voriconazole (μg/mL) | Size of opacity* (mm) | Number of injections | Additional medication | Outcome | Post BCVA |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 60 | F | HM | *Candida parapsilosis* | 0.015 | 4×3 | 2 | PMR-o, ITCZ MFLX-d | Healed | 20/40 |
| 2 | 61 | F | HM | *Candida parapsilosis* | 0.015 | 3×3 | 5 | PMR-o, ITCZ MFLX-d | Healed | 20/200 |
| 3 | 84 | F | LP | *Candida albicans* | 0.06 | 2×3 | 1 | PMR-o, MFLX-d | Healed | HM |
| 4 | 86 | F | LP | *Aspergillus flavus* | 0.5 | 11×11 | 6 | PMR-o, ITCZ, MCFG-iv, VRCZ-ici, MFLX-d | Recurred | LP |
| 5 | 73 | M | 20/2000 | *Fusarium* species | 4 | 4×3 | 6 | PMR-o, ITCZ, MCFG-iv, VRCZ-iv, MFLX-d | Recurred | 20/800 |
| 6 | 78 | F | HM | *Fusarium proliferatum* | 4 | 11×10 | 7 | PMR-o, ITCZ MCFG-iv, LVFX-d | Recurred | LP |
| 7 | 64 | M | 20/2000 | *Candida parapsilosis* | ≤0.015 | 3×4 | 1 | PMR-o, ITCZ | Healed | 20/200 |

**Note:** *Longitudinal diameter and transverse diameter.

**Abbreviations:** 1st BCVA, best corrected visual acuity at first visit; -d, eye drop; F, female; HM, hand motion; ici, intracameral injection; ITCZ, itraconazole 50 mg oral administration; iv, intravenous injection; LP, light perception; LVFX, levofloxacin; M, male; MCFG, micafungin; VRCZ, voriconazole; MFLX, moxifloxacin; MIC, minimum inhibitory concentration; PMR-o, natamycin ointment; Post BCVA, posttreatment best corrected visual acuity.

In Case 6 – the keratitis induced by *F. proliferatum* – we increased the concentration of voriconazole from 0.1%–0.3% or 1.0% because it progressed rapidly despite the additional systemic and local antifungal therapies. The MIC of voriconazole was 4 μg/mL.

With regard to other antifungal medication, we administered supplemental antifungal medications to all cases. Topical natamycin ointment (Pimaricin ophthalmic ointment 1% Senju®, Senju Pharmaceutical Co. Ltd., Osaka, Japan) was instilled into the conjunctival sacs to all cases, 100 mg per day of oral itraconazole capsule (Itrizole® Capsules 50, Janssen Pharmaceutical K.K., Tokyo, Japan) was given to all cases except for a mild case – Case 3 – for 2 weeks, and micafungin sodium (50 mg, Funguard®, Astellas Pharma Inc., Tokyo, Japan) was injected intravenously to three persistent cases. Additionally, one subject also received an intravenous injection and an intracameral injection of voriconazole because of aphakic eye. Intrastromal injections were free from complications such as other microbial infection, corneal perforation and corneal scaring.

## Discussion

In Case 4, the causative fungus was *A. flavus* (Figure 1), the patient did not completely recover from the keratitis after a second keratoplasty with a frozen cornea. Following the surgery, the subject was intensively treated with systemic and topical antifungal agents. Pathological examination of the first removed cornea revealed large amounts of fungi in the deep stroma adjacent to the Descemet's membrane (Figure 2) even though MIC of the causative strain of *A. flavus* was low. We think that either the concentration of voriconazole might have decreased faster than we expected or the solution did not reach the infectious focus. High-density OCT images

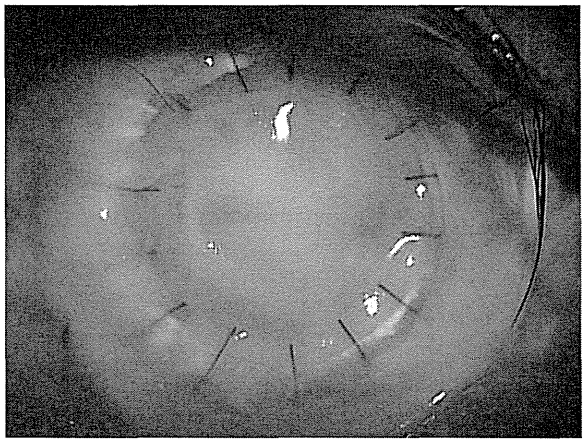

**Figure 1** Case 4 (pre-injection). The corneal abscess caused by *Aspergillus flavus* has spread to most of the grafted cornea.