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ORIGINAL ARTICLE

Antifungal Efficacy of Luliconazole in an Experimental Rabbit Model of Fungal Keratitis Caused by *Fusarium solani*

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Abstract Fungal keratitis is a corneal fungal infection that potentially leads to blindness and is mainly caused by filamentous fungi, such as *Fusarium*, with limited drug options available, such as natamycin and voriconazole. Therefore, this study aimed to evaluate the therapeutic effects of the imidazole antifungal drug—luliconazole—using a rabbit experimental model of fungal keratitis caused by *Fusarium solani*, which is the dominant causative agent of fungal keratitis. *F. solani* was inoculated into rabbit corneas. luliconazole 1% suspension or natamycin 5% eye drops were administered four times a day (N = 6 for each group) 3 days after inoculation. Signs were scored up to 14 days after inoculation to evaluate the efficacy of the drugs. Compared with the peak mean sign scores of the placebo control group, there was a significant decrease in the mean sign scores of both the treatment groups ($P < 0.05$). Sign score trends were similar between the two treatment groups. In conclusion, luliconazole demonstrated therapeutic efficacy comparable to that of natamycin in treating experimental fungal keratitis. This suggests that luliconazole can be a novel therapeutic agent for human fungal keratitis.

Keywords Fungal keratitis · *Fusarium solani* · Luliconazole · Natamycin

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Introduction

Infectious keratitis is a corneal infection that is caused by bacteria, fungi, viruses, and parasites. Infectious keratitis is known to cause severe visual impairment and ultimately lead to functional blindness [1]. A prospective observational study conducted in Asia revealed trauma and wearing of contact lenses as the major risk factors for infectious keratitis and reported a 32.7% rate (the percentage of cases diagnosed with the cause) of fungal keratitis, second only to 38.0% for bacterial keratitis [2]. Additionally, the most

frequently detected organisms in the study were *Fusarium* spp. (18.3%), followed by *Pseudomonas aeruginosa* (10.7%) and *Aspergillus flavus* (8.3%). A prospective observational study conducted in Japan revealed that 52 of 94 (55.3%) isolated fungi were filamentous fungi, and *Fusarium* spp. were the most frequently detected species (23 isolates) [3]. These findings indicate that *Fusarium* spp. are the major causative agents of fungal keratitis in Asia. In addition, healthy individuals engaged in agriculture and outdoor activities may develop fungal keratitis due to vegetative injury [4]. Infectious keratitis treatment includes drug therapy and surgical methods; however, the probability of complete cure remains low even with these treatment methods. A combination of drug administration and lesion scraping is recommended to enhance therapeutic efficacy [5]. Scraping the lesion physically reduces the number of fungi in it and enhances tissue penetration of the drug. Regarding pharmacotherapy, natamycin (NAT) 5% eye drops are the only Food and Drug Administration (FDA)-approved drug that is listed in the World Health Organization's essential medicine list [6], but its therapeutic effect is limited because of poor corneal stromal layer penetration [7]. Additionally, amphotericin B is an alternative drug of NAT, but it has toxicity concerns and accessibility issues as its use requires access to compounding pharmacy [8]. Recently, voriconazole has been used alternatively for the topical treatment of fungal keratitis, and clinical trials have been conducted to compare voriconazole with NAT. However, in cases with *Fusarium* spp. as the causative organism, voriconazole treatment was reported to be less effective than NAT treatment [9]. Currently, limited therapeutic options are available for fungal keratitis compared with bacterial keratitis, for which ocular antibiotics are widely available; thus, the development of new agents is much anticipated.

Luliconazole (LLCZ) is a new imidazole antifungal agent that inhibits the synthesis of ergosterol, which is a major fungal cell wall component. Topical formulations of 1% LLCZ are used for treating superficial fungal infections, such as tinea pedis, whereas 5% LLCZ is used for treating onychomycosis. However, LLCZ has not been used for fungal keratitis. Reportedly, LLCZ not only exhibits strong antifungal activity against *Trichophyton* species, which are the causative agents of superficial fungal infections [10, 11], but also against nondermatophytes, such as *Aspergillus* and *Fusarium* [12, 13]. Additionally, LLCZ exerts strong antifungal activity against *Fusarium* complex isolated from patients with fungal keratitis and *Aspergillus* and *Fusarium* spp. isolated from equine fungal keratitis samples [14, 15].

However, no studies have evaluated the efficacy of LLCZ in experimental infection models. With the hypothesis that LLCZ exhibits therapeutic efficacy against *Fusarium* infection, this study aimed to evaluate the antifungal activity and therapeutic efficacy of LLCZ against *Fusarium* spp., which are major causative agents of fungal keratitis, in an experimental rabbit model of *Fusarium solani*-induced fungal keratitis [16].

Materials and Methods

Fungal Isolates

F. solani (NBRC 5890) strains were purchased from the National Institute of Technology and Evaluation. The cryopreserved strains were inoculated in Sabouraud's agar medium (Eiken Chemical, Tokyo, Japan) and incubated at 27 °C for 5 days for recovering culture. Saline was added to the agar medium to retrieve the conidia. Subsequently, the saline was collected and filtered through a sterile mesh (Falcon®, 100-μm cell strainer, NY, USA), to which more saline was added to achieve a final inoculum concentration of approximately 1×10^7 conidia/mL. The minimum inhibitory concentration of LLCZ against this strain is reported to be 0.06 μg/mL and that of NAT is reported to be 4 μg/mL[14].

Animal

A total of 19 Slc: JW/CSK 9-week-old male specific pathogen-free rabbits (SLC, Shizuoka, Japan) were used for animal studies. A prehousing period of 5 days was provided. The animals were housed in a holding room that is maintained at controlled temperature (18–28 °C), controlled humidity (30–80% RH), and light/dark cycle of 12 h each (illumination: 6:00 am to 6:00 pm). The animals were individually housed in aluminum cages (350 × 500 × 400 mm, width × depth × height) and were fed solid feed (LRC4,

Oriental Yeast Industry Co., Ltd., Tokyo, Japan) in a feeder and tap water in a water bottle.

Experimental Model

Inoculation procedure was carried out based on the methods previously reported [16]. Animals with no abnormalities in body weight or general condition, after a preliminary rearing period, were divided into the following three groups of six animals each: (1) placebo control group; (2) LLCZ 1% group who were administered LLCZ 1% suspension; and (3) NAT 5% group who were administered NAT 5% eye drops. Grouping was done by random sampling method so that the mean and variance of each group's weight were approximately equal. Medetomidine hydrochloride at 0.5 mg/kg (Fujita Pharmaceutical Co., Ltd., Tokyo, Japan), midazolam at 2 mg/kg (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan), and butorphanol tartrate at 0.5 mg/kg (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) were intramuscularly injected into the thigh (0.5 mL/kg) once a day for 5 days before and on the inoculation day to anesthetize the animals. To suppress the animal's immunity and allow for the establishment of the fungus infection, 16 µL of dexamethasone (50 mg/mL, FUJIFILM Wako Pure Chemicals Co., Ltd., Osaka, Japan) was injected into the conjunctiva of the right lower eyelid where the inoculum was to be injected once a day for 5 days before and on the inoculation day. Before dexamethasone injection, 0.4% oxybuprocaine hydrochloride (Benoxil® ophthalmic solution 0.4%, Santen Pharmaceutical Co. Ltd., Osaka, Japan) was injected for superficial anesthesia.

On the inoculation day, the right external eye was washed with saline solution under anesthesia after dexamethasone administration. The eye was dislocated by applying pressure using a medicine spoon, and a 27G needle was used to create a wound in the central cornea, reaching 1/2 the depth of the corneal stroma. The wound was then widened using a 27G needle with a rounded tip to create a pocket-like wound. Subsequently, 10 µL of the inoculum solution (1×10^5 conidia) was injected into the pocketed corneal wound. The date of inoculation was set as day postinfection (DPI) 0. After inoculation, atipamezole hydrochloride at 2.5 mg/kg (Mepacia Injection, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) was intramuscularly injected into the thigh to awaken the rabbits.

Drug Administration

LLCZ (purity: ≥ 98.0%) was obtained from Nihon Nohyaku Co., Ltd. (Tokyo, Japan). LLCZ was grinded in a mortar and pestle, and then suspended at 1.0% (w/v) into phosphate-buffered saline containing polysorbate 80 (FUJIFILM Wako Pure Chemicals Co., Ltd., Osaka, Japan) added to a final concentration of 0.1% (v/v). Meanwhile, NAT 5% eye drops (Pimaricin ophthalmic suspension 5%) was purchased from Senju Pharmaceutical Co., Ltd. (Osaka, Japan) and used as obtained. Each drug was dropped into the conjunctival sac of the right eye. The dose of each drug was 50 µL and administered four times a day (3-h intervals). Drugs were administered for 11 days from DPIs 3–13 (Fig. 1). Placebo control group was received only same phosphate-buffered saline containing polysorbate 80 during the same period.

Scoring

The infected area was observed once a day before drug administration under a slit lamp (Kowa Co., Ltd., Aichi, Japan) from DPIs 0–14 according to the reported sign scoring table (Table 1) of Ishibashi et al. [16], and the total score was calculated for each animal.

Histopathological Examination

After the observation of infected areas was completed at DPI 14, the rabbits were euthanized via the removal of blood from their abdominal aorta under anesthesia by administering intra-auricular venous thiamylal sodium (ISOZOL for INJECTION, Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) at 2 mg/kg. The right eye was removed, and the removed eyeballs were fixed in 3.125% (v/v) glutaraldehyde and 2.5% (v/v) formalin for approximately 2–3 h and then refixed in 20% (v/v) neutral-buffered formalin. The specimens were then paraffin-embedded following the standard procedure, and thin sections (about 3 µm) were prepared. Each specimen was stained with periodic acid–Schiff stain (PAS) following the standard histological observation method.

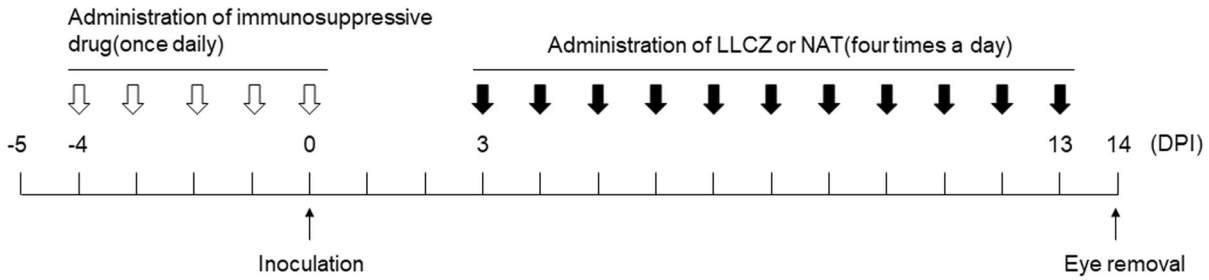

Fig. 1 Experimental scheme. Four days prior to the inoculation date of the fungi, each animal was received daily conjunctival doses of immunosuppressant (dexamethasone). The animals were given the drug (LLCZ or NAT) three days after inoculation with the fungus (DPI 3) and the drugs were administered four times a day until DPI 13. The cornea of each animal was collected for histopathology at DPI 14

Table 1 Ocular infection finding score

Site/findings	Intensity	Score
1. Corneal ulcer	Diameter of < 2 mm	1
	Diameter of 2–4 mm	2
	Diameter of ≥ 4 mm	3
2. Haze of peripheral cornea	Mild	1
	Moderate	2
	Gross	3
3. Protrusion of the cornea		
4. Perforation of the cornea		
5. Exudate in anterior chamber and plaque of the posterior surface of the cornea	Mild	1
	Gross	2
6. Hypopyon	Height of < 1 mm	1
	Height of 1–3 mm	2
	Height of ≥ 3 mm	3
7. Hyphema		1
8. Infection of the iris (increasing plica, congestion, bloating, and circumcorneal hyperemia)		1

Statistical Analysis

Significant differences in right eye sign scores between placebo control and NAT 5% groups and between placebo control and LLCZ 1% groups were evaluated using Steel's many-to-one rank test, which is similar to Dunnett's test, allowing comparisons between nonparametric Wilcoxon-type ranked data. Statistical analysis was performed using a commercially available statistical program (SAS system: SAS Institute Japan, Tokyo, Japan). The significance level was set at $P \leq 0.05$.

Results

Change in Sign Scores

In this study, *F. solani* was inoculated into the right corneas of rabbits. LLCZ 1% suspension or NAT 5% eye drops was administered to the cornea four times a day (each N = 6), 3 days after inoculation. Signs of the eyes were scored up to 14 days after inoculation.

The mean sign scores after inoculation for each group are shown in Fig. 2. In placebo control group, corneal opacity was observed from DPI 1 and was present in five cases on the eye drop initiation day (DPI 3) (Fig. 3A). The mean sign score was 0.8 at DPI 3.

Subsequently, corneal opacity worsened, and ulceration; protrusion; anterior chamber abscess; and iritis were observed (Fig. 3B–D); the sign score gradually increased, peaking (4.3) at DPI 11, and remained at 4.0 until the last observation day (DPI 14).

The LLCZ 1% group showed the same signs as the placebo control group after the start of drug administration (DPI 3). Corneal opacity was observed in all the LLCZ 1% group at DPI 7. The score was 2 in only one case (Fig. 3E), while all others had a low score of 1 (Fig. 3F). Almost all animals had only a mild degree (score was 1) of corneal opacity at PDI 14, though one animal developed the symptom as same as the placebo control group. The mean sign scores for DPI 7–12 of the LLCZ 1% group were significantly lower than those of placebo control group ($P < 0.05$).

In the NAT 5% group, all animals were observed corneal opacity at DPI 7, and the score was 3 in only one case (Fig. 3G), while all others had a low score of 1 (Fig. 3H). Almost all animals had only a mild degree (score was 1) of corneal opacity at PDI 14 except for one case which had severe corneal opacity with ulcer. The mean sign scores for DPI 10–14 of the NAT 5% group were significantly lower than those of placebo control group ($P < 0.05$).

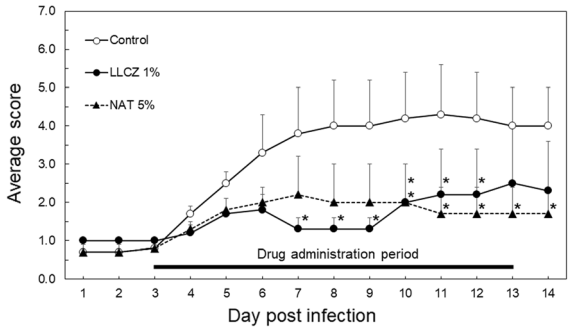

Fig. 2 Average infection scores during the observation period. The vertical axis shows the mean sign scores of animals in each group (N = 6). The group administered 1% LLCZ showed a significantly higher reduction in sign scores from DPI 7 compared with the placebo control group. Error bars represent standard error (SE). *$P < 0.05$, significant difference compared with the placebo control group (Steel's test). LLCZ, luliconazole; NAT, natamycin

No adverse ocular or systemic condition effects due to drug administration were observed in all animals in placebo control, NAT 5%, and LLCZ 1% groups.

Histopathological Examination

Histopathological examination (PAS staining) of the removed eyes revealed fungal infection of the stromal layer of the cornea. In these eyes, keratitis, corneal epithelium ulceration and erosion, corneal stromal layer melt necrosis, scar formation and corneal endothelial defects were observed (Fig. 4A). In placebo control group, fungi were detected in three animals (Fig. 4B), while fungi were detected in one animal both among LLCZ 1% group and NAT 5% group. In all groups, sign scores were more severe in animals in whom the fungi were found than in those in whom fungi were not. The eye of each one case in which fungi were detected in the LLCZ 1% group and NAT 5% group also showed lesions similar to those in the placebo control group, while only histopathological scars were observed in the other fungus-free eyes (Fig. 4C).

Discussion

In the fungal keratitis rabbit model established by infection with *F. solani* in this study, treatment with

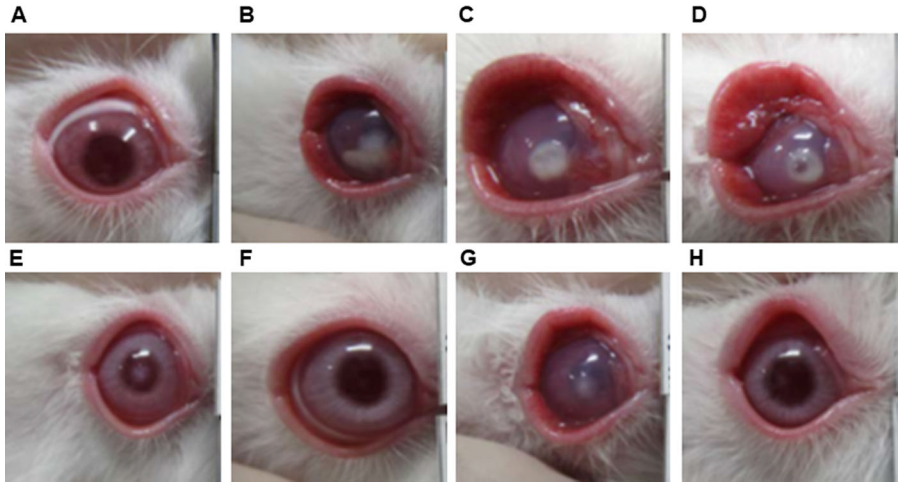

Fig. 3 Photographic images of sign history. Macro-photographs of rabbit eyes were taken during the observation period. **A** Placebo control group (DPI 3), **B** placebo control group (DPI 7), **C** placebo control group (DPI 10), **D** placebo control group (DPI 14), **E** LLCZ 1% group (DPI 7): score of corneal opacity was 2. **F** LLCZ 1% group (DPI 7): score of corneal opacity was 1. **G** NAT 5% group (DPI 7): score of corneal opacity was 3. **H** NAT 5% group (DPI 7): score of corneal opacity was 1

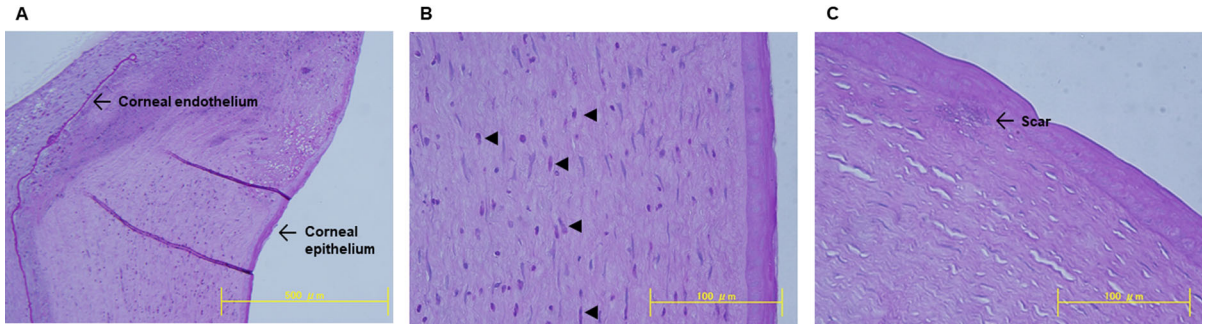

Fig. 4 Histopathological examination of the cornea by PAS staining. The cornea of each animal was collected and stained with periodic acid–Schiff stain at DPI 14. **A** Placebo control group (× 100), **B** Placebo control group (× 400), **C** LLCZ 1% group (× 400). Triangular arrows indicate fungi

LLCZ 1% suspension improved sign scores compared to placebo control group, suggesting that LLCZ 1% is effective.

Various models for fungal keratitis have been developed and reported for the evaluation of antifungal drug efficacy [17]. The model that was used in this study was based on the report by Ishibashi et al. [16]. For clinical features of fungal keratitis, patients typically present with a red, painful eye, together with reduced vision. Clinical examination will demonstrate conjunctival hyperaemia, making the eye appear red, in conjunction with a corneal infiltrate-an area of corneal opacity [18]. In addition to corneal infiltration, patients may have signs of inflammation of the anterior chamber with hypopyon. Gross granular infiltration of the corneal epithelium and the anterior stroma is the main finding in fungal keratitis with collagen destruction, coagulative necrosis, and stromal fungal infiltration seen on microscopy [19]. Fungal keratitis may eventually lead to corneal perforation and endophthalmitis in severe cases. These features have been observed to appear in this model and the course of symptoms is similar. Through histopathological examinations, Ishibashi et al. [16] found infection of the stromal layer of the cornea and the anterior chamber of the eye and inflammatory cell infiltration of the superficial stroma of the cornea 5 days after inoculation. We also could detect the infection in the stromal layer of the cornea at DPI14.

Therefore, this model also closely resembles the clinical presentation of humans.

NAT, a polyene antibiotic, is the only drug approved by FDA for external treatment of fungal keratitis and is used in many countries for fungal keratitis treatment. Its efficacy has been demonstrated in rabbit Fusarium keratitis models. It has been reported in a previous in vivo infection study to improve keratitis after 8–10 day daily eye drop administration of a 5% solution [20]. In the present study, we applied NAT four times per day (3-h interval) to avoid excessive stress of animals and succeeded in a detection of NAT efficacy. NAT delayed the development of infection and showed significant reduction in sign scores compared with placebo control group, thereby confirming the therapeutic effect of NAT. Therefore, this model is appropriate for evaluating the efficacy of NAT versus other drugs in treating fungal keratitis caused by *F. solani* corneal infection.

NAT is effective against *Fusarium* but has poor corneal stroma translocation and limited therapeutic efficacy [7, 21]. Voriconazole, a triazole antifungal agent used clinically, is effective against corneal fungal infections caused by *Aspergillus*, but its therapeutic efficacy against *Fusarium* is poor owing to its weak antifungal activity against *Fusarium* spp. [22].

LLCZ is a novel imidazole antifungal agent developed in Japan and is characterized by its strong antifungal activity against various filamentous fungi, including *Fusarium* and *Aspergillus*, which are the causative agents of fungal keratitis [10–13]. The in vitro antifungal activity of LLCZ is stronger than that of NAT and voriconazole [14] and is expected to have greater therapeutic efficacy. LLCZ showed therapeutic efficacy in this study. LLCZ delayed the development of infection. Severity of the symptoms were significantly improved by LLCZ and mean sign score of LLCZ 1% group was significantly lower than that of the placebo control group (DPI 7–12). In LLCZ

1% group, fungi were detected in than in only one animal, while fungi were detected in three animals in the placebo control group. This is likely to be an effect of the administration of the antifungal drug LLCZ. Animals in whom fungi were detected had more severe symptom scores than others, suggesting that there may be a correlation between the amount (or presence) of fungi and the severity of symptoms. Future research is needed to investigate the relationship between drug administration and symptom suppression, including quantification of fungi.

However, the therapeutic efficacy of LLCZ 1% suspension was not found to be superior to that of NAT 5% in this study. This may be attributed to a drug transfer problem, as the water solubility of LLCZ is 0.62 μg/L (unpublished data) and has been found to be extremely insoluble in water. The solubility in water of NAT is also sufficiently low (52 μg/L) [23], but LLCZ is more than 100 times less soluble in water than NAT. Particularly, the drug might not have sufficiently migrated to the cornea (the infection site) because LLCZ was suspended in an aqueous solvent and administered via eye drops, and its antifungal activity may not have been fully expressed.

Only suspension formulation of NAT was developed due to this insolubility in water of NAT. In recent years, novel water soluble formulation of NAT has been studied and revealed that the soluble formulation of NAT maintained its intraocular concentration 5 and 2.5 times higher at 4th and 6th hour compared to 5% NAT suspension [23]. Therefore, verifying the therapeutic efficacy and transferability of LLCZ by preparing a formulation suitable for eye drop application and devising a new administration method will be necessary to confirm the clinical superiority of LLCZ over existing drugs, including NAT. For example, nanoemulsions of LLCZ have been reported as a novel method of administration [24].

In conclusion, this is the first report demonstrating usefulness of LLCZ for the treatment of fungal keratitis caused by *F. solani*. Although the solvent condition was not adequate for LLCZ, LLCZ showed the significant therapeutic efficacy against fungal keratitis in this model. With further formulation development, LLCZ may become a promising antifungal drug for the treatment of fungal keratitis. Novel formulation that can dissolve higher concentration of LLCZ for treatment of fungal keratitis is waited.

Author Contributions All author contributed to the study conception and design. Material preparation, data collection and analysis were performed by SA and KI. The first draft of the manuscript was written by SA and all authors commented previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest Financial interests: Arimoto and Inagaki are employees of Nihon Nohyaku Co., Ltd. The other authors have no relevant financial or non-financial interests to disclose.

Ethics Approval The animal experiments were approved by the Animal Experimental Committee of the testing facilities (Nihon Bioresearch Inc.) and were conducted according to the Regulations Concerning the Management and Welfare of Laboratory Animals at the Nihon Bioresearch Inc. (Approval study No.39020).

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