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Rabbit Fungal Keratitis Model of *Fusarium solani* Tested Against Three Commercially Available Antifungal Drugs

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Objectives: The purpose of this study was to develop a reproducible preclinical *Fusarium solani* keratitis model, which would allow comparative testing of currently available antifungals (NATACYN [Alcon, Fort Worth, TX], voriconazole 1%, and amphotericin B 0.1%) as well as efficacy testing of new antifungals for translation into clinical practice in the future.

Methods: The rabbit *F. solani* keratitis model was developed in New Zealand white rabbits using local and systemic immunosuppression. Infection was introduced by intrastromal injection of *F. solani* spores into one of the immunosuppressed rabbit eyes while the contralateral eye was a control. Progress of the infection was assessed by the clinical features, histopathology, and viable fungal counts. In this study, the efficacy of currently available antifungals (NATACYN [Alcon], voriconazole 1%, and amphotericin B 0.1%) was compared. Rabbits were randomly divided (n=4 in each group), and the respective antifungal was instilled topically 5 times/day for 7 days. Treatment effects were analyzed by evaluating the anterior segment with the help of slit-lamp, histopathological findings and viable fungal culture at the end of the experiment.

Results: We report the development of a reproducible and progressive rabbit *F. solani* keratitis model as shown by the substantial viable fungal counts (3 log CFU), the presence of large patchy lesions and substantial hypopyon in the 12-day model correlated with specific histopathological analysis for fungus (extended *F. solani* hyphae from midcorneal stroma into the anterior chamber and traverse Descemet membrane with anterior chamber suppurative plaque). Voriconazole 1% and NATACYN revealed significant reduction of the fungal wound area (P=0.02 and 0.021), respectively, while amphotericin B 0.1% exhibited P value of 0.083 compared with their infected nontreated controls. Voriconazole 1% and amphotericin B 0.1% showed significant viable fungal count differences (P=0.004 and 0.01), respectively, whereas P value of NATACYN was 0.337 compared with control infected corneas.

Conclusion: The reported rabbit fungal keratitis model can be used for screening new antifungals and evaluating currently available antifungals to facilitate better clinical outcomes. Voriconazole 1% showed the best efficacy among the three tested currently available antifungals by showing the significant differences in both wound size and viable fungal count comparisons in our *F. solani* rabbit keratitis model.

Key Words: Antifungals—Efficacy—Infection—Experimental animal models—Cornea.

(*Eye & Contact Lens* 2020;46: 274–280)

Corneal infections or diseases are the second major leading cause of blindness worldwide after cataract according to the WHO report.[1] Approximately 1.5 to 2 million new cases of corneal blindness have been reported annually due to ocular trauma and corneal ulceration.[1] Corneal keratitis, inflammation of the layers of the cornea, can be caused by bacterial, viral, protozoa, or fungal organisms, which invade the corneal layers, causing inflammation of corneal tissue, destruction of the layers, and, ultimately, blindness. Of these organisms, fungi remain as one of the most challenging pathogens for the ophthalmologists to diagnose and treat effectively.[2] One report suggests that fungal keratitis can be more virulent, damaging to corneal tissue, and leading to corneal perforation compared with bacterial keratitis.[3] Seventy species of fungal organisms are reported, and 2 major fungal organisms, filamentous fungi or yeast, are stated to cause fungal keratitis.[4] Yeasts commonly inhabit the digestive or genital tract, skin, and environment[5] while filamentous fungi are frequently found in the environment such as soil, water, and air in the form of spores.[6] Yeast-related keratitis (mainly *Candida* species) is commonly found as the pathogen in 30% to 52% of fungal keratitis occurring in temperate climate countries such as Australia, Northern United States, and Europe.[7,8] Filamentous fungi-related keratitis (mainly *Fusarium* and *Aspergillus*) commonly occur in tropical climates such as Southern United States, Mexico, Africa, China, South America, Central America, Middle East, India, and Southeast Asia.[2,9] The greater risk of corneal destruction and visual morbidity, poor prognosis,[10] and the past *Fusarium* outbreak[11,12] has stimulated the need for developing a standard, reproducible rabbit model to compare and to develop better treatments. There have been several reports published in the literature regarding the *Fusarium solani* keratitis rabbit model using diverse methodologies. Most of the reported studies use the method of direct intrastromal inoculation of fungal spores into the naive rabbits' cornea.[13–18] A few studies have used subconjunctival injection of steroids to suppress the local immunity before infection into

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The authors have no conflicts of interest to disclose.

Supported by TCR R1018.

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Accepted December 13, 2019.

DOI: 10.1097/ICL.0000000000000689

the corneal stroma[19–21] while others used systemic immunosuppression of dexamethasone or cortisone to suppress the systemic immunity.[22,23] Hence, we have used an integrated approach to achieve a successful *F. solani* rabbit keratitis model and reported a useful, reproducible rabbit model of *F. solani* keratitis which we used to compare three commonly used commercially available antifungals.

MATERIALS AND METHODS

Ethical Statement

New Zealand white rabbits (combination of male and female) were purchased from the National University of Singapore. All animal research conducted in this study followed the SingHealth Institutional Animal Care and Use Committee guidelines, and all the animal experiments were in accordance with the recommendations of the Association for Research in Vision and Ophthalmology for the conduct of animal research. All rabbits were initially examined by slit-lamp with fluorescein application to ensure that the anterior segment was clinically normal.

F. solani Keratitis Model

Rabbits were immunosuppressed by subconjunctival injection of dexamethasone 0.8 mg once/day for 5 days only before infection. In addition, dexamethasone 0.1% was applied topically 5 times/day for 5 days before infection and for 5 days after infection. A standard systemic immunosuppression protocol used for rodents was applied with minor changes.[24] In brief, cortisone acetate 100 mg/kg and cyclophosphamide 100 mg/kg were given subcutaneously at 2 days before infection and 3 days after infection. Rabbits received ceftazidime (50 mg/kg) subcutaneously every day from the beginning of the systemic immunosuppressive protocol. Sedation of rabbits was performed by injecting the combination of ketamine 35 mg/kg and xylazine 5 mg/kg intramuscularly, and local anesthesia was achieved by the topical application of lignocaine (5 mg/mL) before the infection procedure. *F. solani* ATCC 46492 was subcultured onto potato dextrose agar (PDA) at 30°C for 3 to 5 days. 0.1% Tween 80 in sterile phosphate-buffered saline (PBS) was used to wash the fungal growth, and the suspension was filtered through sterile gauze to remove hyphal elements. An initial inoculum $\approx 7\times10^5$ colony-forming units (CFU), 15 μL was administered by midstromal injection (estimated depth—200 μm) of a fungal spore suspension using BD insulin syringe with 31-G needle to the right eye while the left eye remained normal. Rabbits were followed for up to 12 days. Euthanasia was achieved by intracardiac injection of sodium pentobarbital 100 mg/kg in the sedated rabbits.

Disease Progression Follow-up by Slit-Lamp Microscopy

Infected rabbit corneas were monitored using a new-generation Zoom clinical Slit Lamp, NS-2D, Righton, Japan, at 1, 5, 8, and 12 days after infection (DPI). Minims fluorescein sodium eye drops (Bausch and Lomb, 2% wt/vol) was used to check for the presence of an ulcer with the aid of cobalt-blue filter equipped slit-lamp biomicroscopy at DPI-5 and 12. Corneal ulcer wound area was calculated by ImageJ 1.52k.

Efficacy Testing of Three Commercially Available Antifungals

NATACYN (natamycin ophthalmic suspension 5%, Alcon, Fort Worth, TX), voriconazole 1%, and amphotericin B 0.1% prepared in PBS (pH~7) by Singapore General Hospital laboratory were used to treat the rabbit *Fusarium* keratitis model. Three independent experiments were performed: (1) natamycin 5% and control, (2) voriconazole 1% and control, and (3) amphotericin B 0.1% and control, whereas the control group was the *F. solani*–infected group with PBS treatment. Four animals (n=4) were used for each group. All the treatments started at DPI-5, and topical application of the respective antifungal was performed 5 times/day for 7 days. At the end of the experiment, rabbits were sacrificed humanely, the corneas (n=3) were dissected and

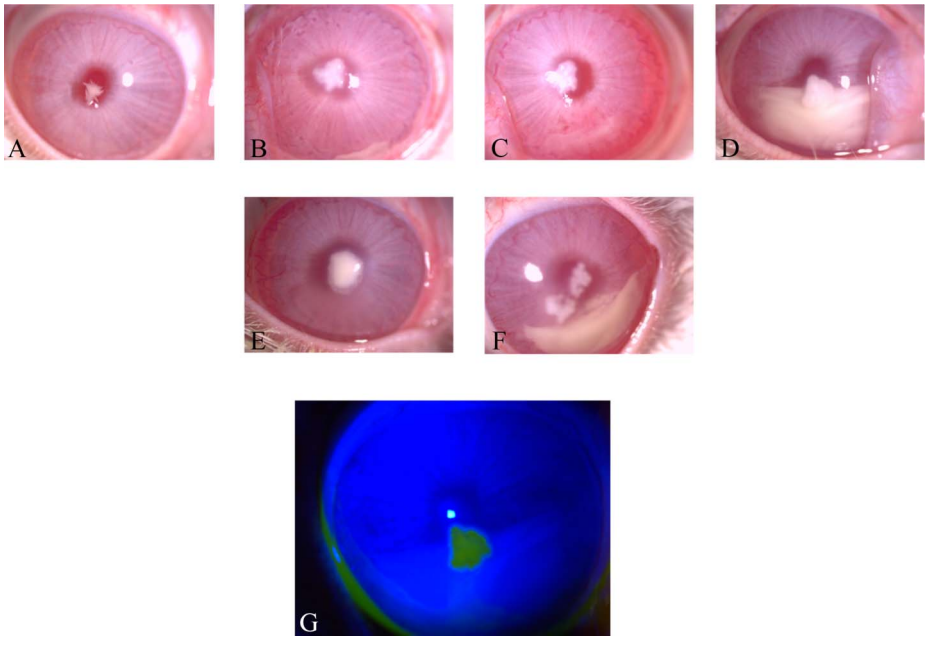

FIG. 1. Slit-lamp microscopy images of rabbit *F. solani* keratitis model (A) DPI-1, (B) DPI-5, (C) DPI-8, and (D) DPI-12. Corneal neovascularization was occurred at 2 o'clock and 9 o'clock positions at DPI-12 (E and F). The corneal wound was visualized in the DPI-12 fluorescein-stained slit-lamp microscopy image (G). DPI, days postinfection. *$P \leq 0.05$ was set to be statistically significant. full color online

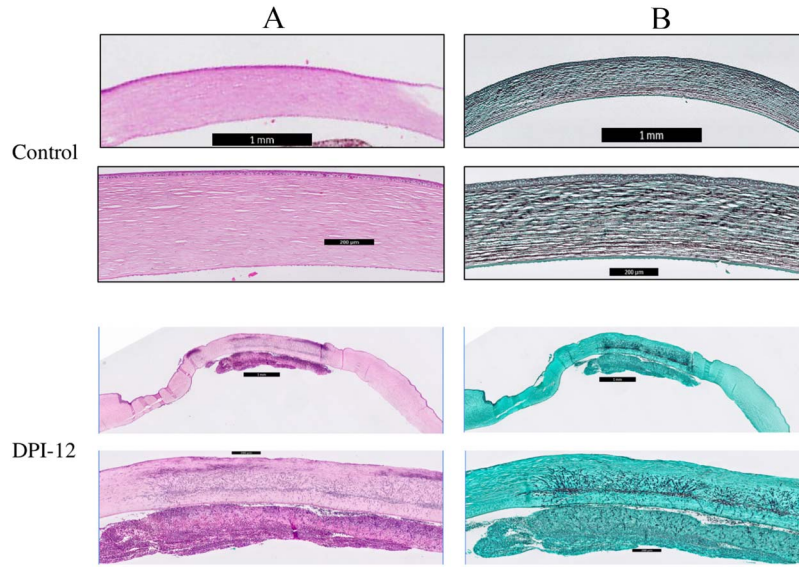

FIG. 2. (A) Hematoxylin and eosin staining images of normal uninfected cornea after immunosuppressive regimes and DPI-12. Presence of fungal hyphae and spores was observed in the midcorneal stroma accompanying with the presence of suppurated anterior chamber endothelial plague. (B) Grocott–Gömöri methenamine silver staining images showing the confirmation of fungal hyphae presence in the midstroma of DPI-12 sample. DPI, days post-infection. full color online

homogenized in sterile PBS for fungal counts,[25] and one cornea (n=1) was fixed in a mixture of 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) and 2.5% neutral-buffered formalin solution (Leica Surgipath; Leica Biosystems Richmond, Inc., Richmond, IL) for histopathological analysis. The homogenates were serially diluted in sterile PBS, duplicated, plated in PDA, and incubated at 30°C for 3 to 5 days. Results were recorded as the log10 number of CFU/cornea,[26] and the difference in log CFU/cornea between the experimental and control groups was reported. Viable fungal counts and corneal wound sizes at 7 days post-treatment (DPT) were compared for their statistical difference between the treatment group and its respective control group. Data were analyzed by the Mann–Whitney *U* test (PASW statistic 18), and *P* value was set to be significant at 0.05.

Histopathological Assessments of *Fusarium* Keratitis and Antifungal Treatments

Fixed corneal specimens were sent to the Ophthalmic Pathology service at the Singapore National Eye Center/Singapore General Hospital in a mixture of 4% paraformaldehyde (PFA) (Sigma-Aldrich) and 2.5% neutral-buffered formalin solution (Leica Surgipath; Leica Biosystems Richmond, Inc.). They were processed and embedded in paraffin (Leica-Surgipath; Leica Biosystems Richmond, Inc.) according to standard clinical protocols. Four-micrometer sections were then cut, and sections dried in an oven at 37°C for at least 24 hr. To prepare the sections for histochemical stains, the sections were heated on a 60°C plate warmer, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Based on previously published protocols, hematoxylin and eosin stain and Grocott–Gömöri methenamine silver (GMS) stain were performed.[27,28] Sections were then read using standard light microscopy (Olympus BX-40) and imaged on a Philips Digital Pathology Solution platform.

RESULTS

Rabbit *F. solani* Keratitis Model

To define the model after preliminary work, the corneas of four animals (n=4) were inoculated. At DPI-1, small satellite lesions developed in the central cornea where the fungal spores were injected (Fig. 1A). The lesion extent increased in size as the presence of patchy lesions was observed and corneal haze was observed by slit-lamp microscopy at DPI-5 (Fig. 1B). In half of the experimental animals (n=2), hypopyon was present starting from DPI-5 (Fig. 1B). Progression of the infection continued to DPI-12 as noted by the presence of increased large patchy lesions and substantial hypopyon (Fig. 1D). Corneal ulcers were detected at the patchy lesions (wound size=4.72 mm^2) starting at DPI-5 and ulcer increased to 9.14 mm^2 at DPI-12 as shown by Minims fluorescein sodium eye drops (Fig. 1G). Central corneal ulcers were observed in all

TABLE 1. *Summary Table of Different Treatment Effects in Experimental* F. solani *Keratitis Rabbit Model*

	Clinical Response	Corneal Fungal Wound Size Difference at DPT-7	Histopathological Analysis	Log Reduction of Viable Fungal Counts at DPT-7
Efficacy testing of NATACYN (natamycin ophthalmic suspension 5%, Alcon)	Decrease in conjunctival inflammation (Fig. 3B)	4.03 mm^2 (Fig. 3B)	Slight decrease in fungal burden (Fig. 3B)	0.77 (Fig. 4A)
Efficacy testing of voriconazole 1%	Marked inhibition of conjunctival inflammation (Fig. 3C)	5.6 mm^2 (Fig. 3C)	Significant reduction of fungal loads (Fig. 3C)	0.99 (Fig. 4B)
Efficacy testing of amphotericin B 0.1%	Distinct decrease in conjunctival inflammation (Fig. 3D)	3.43 mm^2 (Fig. 3D)	Noticeable inhibition of fungal loads (Fig. 3D)	0.75 (Fig. 4C)

TABLE 2. *Different Experimental* F. solani *Rabbit Keratitis Models*

Animal Infection Procedure	Clinical Features	Experimental Evaluation	Fungal Counts	Tested Antifungals and Its Effects	Reference
Injection of *F. solani* spores into the corneal stroma.	*F. solani* keratitis started 3 days after infection.	Slit-lamp microscopy, histopathology, and viable fungal counts	2.5 log CFU	Corneal cross-linking was found to be effective.	13
Inoculation of *F. solani* spores into the corneal stromal incision	*F. solani* keratitis started 2 days after inoculation.	Slit-lamp microscopy	No CFU data	Natamycin was found to be effective in controlling *F. solani* keratitis.	14
Injection of *F. solani* spores into the corneal stroma.	Clinical features were evident at 3 days postinfection.	Slit-lamp microscopy, histopathology, and viable fungal counts	Approximately 3.3 log CFU	Combination of corneal collagen cross-linking (PACK-CL) and voriconazole was useful to manage the early stage of *F. solani* keratitis.	15
				Amphotericin B 0.15%, itraconazole 1% and voriconazole 1% were found to be effective.	16
				Topical caspofungin was effective in controlling *F. solani* keratitis.	17
Intrastromal injection of *F. solani* spores	*F. solani* corneal ulcer developed 7 days after inoculation.	Slit-lamp microscopy, histopathology, and viable fungal counts	1.6 log CFU	Intrastromal voriconazole injection was more effective than topical natamycin and topical voriconazole.	18
Local immunosuppressant for 5 days and inoculation of *F. solani* spores into the corneal stromal incision	*F. solani* keratitis started 5 days after inoculation.	Slit-lamp microscopy and viable fungal counts	Fungal count was zero in Sabouraud agar after 1 day incubation in Brain Heart Infusion (BHI) agar	Topical 0.5% povidone-iodine demonstrated no advantages in the management of *F. solani* keratitis when compared with 5% natamycin.	19
Intrastromal injection of *F. solani* spores with local immunosuppression	Small infiltrated lesion was evident as early as 2 days postinfection.	Slit-lamp microscopy, histopathology, and protease analysis	No CFU data	Mechanism of matrix turnover in *F. solani* keratitis was investigated.	20
Local immunosuppressant for 5 days and intrastromal injection of *F. solani* spores	Fungal keratitis developed 3 days after inoculation and severe inflammation was evident at 8 days after inoculation.	Slit-lamp microscopy, histopathology, and viable fungal counts	2 log CFU	Combination of ultraviolet A and voriconazole was more effective than voriconazole alone.	21
Topical application of *F. solani* spores into the scratched cornea after systemic immunosuppression for 3 days	*F. solani* keratitis started 5 days after inoculation	Slit-lamp microscopy, histopathology, and viable fungal counts	1.2 log CFU	Combination of voriconazole and epigallocatechin gallate was effective in treating *F. solani* keratitis.	22
Intrastromal injection of *F. solani* spores into the scratched cornea after systemic immunosuppression for 3 days	*F. solani* keratitis started 3 days after infection.	Slit-lamp microscopy, histopathology, and confocal microscopy	No CFU data	Combination of cryotherapy and antifungal agents was effective in treating *F. solani* keratitis.	23

CFU, colony-forming unit.

the experimental rabbits. The above findings suggested that a full-blown *F. solani* keratitis infection was in place at DPI-5, and DPI-5 was set as a starting time point for the treatment part of the experiment. Initially, an ulcer was localized at the fungal spore injection site. Corneal neovascularization was evident at DPI-12 at 2 and 9 o'clock positions in half of the experimental rabbits (Fig. 1E, F). There was no lid edema or secretion in all the experimental animals during the course of infection. Fungal retrieval count was 3.4 log CFU.

Histopathological Assessment

Inspecting slides after H&E staining revealed fungal elements composed of spores and hyphae (Fig. 2A). The hyphae were seen extending from the nidus of the infection in the midcorneal stroma into the anterior chamber and traverse Descemet membrane (Fig. 2A). The inflammatory response was seen within the corneal stroma with a marked suppurative anterior chamber endothelial plaque (Fig. 2A). GMS stain also showed fungal hyphae extending from the midstroma to the peripheral parts (Fig. 2B).

Efficacy of Three Commercially Available Antifungals

After establishing the fungal infection model, the next step was to determine its usefulness in evaluating current antifungals. Natamycin, commercially available as NATACYN, along with voriconazole 1% and amphotericin B underwent testing in the rabbit *Fusarium* keratitis model to determine their comparative efficacy for this infection.

Treatment was started at DPI-5 when the infected cornea was cloudy with a central corneal ulcer associated with intense

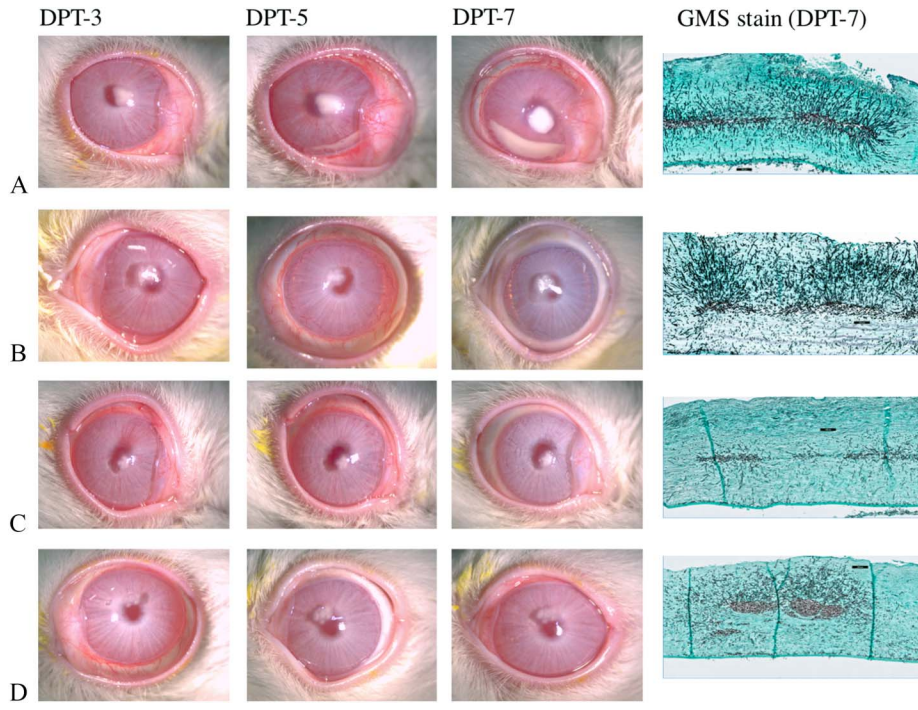

FIG. 3. Slit-lamp microscopy and GMS images of (A) control, (B) NATACYN, (C) voriconazole 1%, and (D) amphotericin B 0.1% along the course of rabbit *F. solani* keratitis treatment efficacy. Rabbit corneal fungal wound was reduced in all the treatments (amphotericin B 0.1% vs. control, P=0.083) along with the significant amount of reduction of fungal wound area in NATACYN (P=0.021) and voriconazole 1% (P=0.02) treatment groups. The best control slit-lamp microscopy and GMS images were chosen as representatives from three independent efficacy experiments. GMS, Grocott-Gömöri Methenamine Silver. full color online

conjunctival inflammatory responses. Effects of different treatment are summarized in Table 1.

DISCUSSION

The management of fungal keratitis is challenging because of the time required for diagnosis which increases the risk of a bad prognosis.[10] Corneal trauma,[29] contact lens wear,[7] topical steroid use,[29] corneal surface disorders, dry eye,[30] and laser-assisted in situ keratomileusis[31] have been identified as risk factors for fungal keratitis. A recent article from the Asia Cornea Society stated that filamentous fungi, *Fusarium* species, and *Aspergillus flavus,* are the first and third most common microorganism causing keratitis respectively in an Asian multicenter study.[32] Owing to the clinical difficulties with fungal keratitis and *F. solani* in particular, there have been a number of research reports describing experimental animal

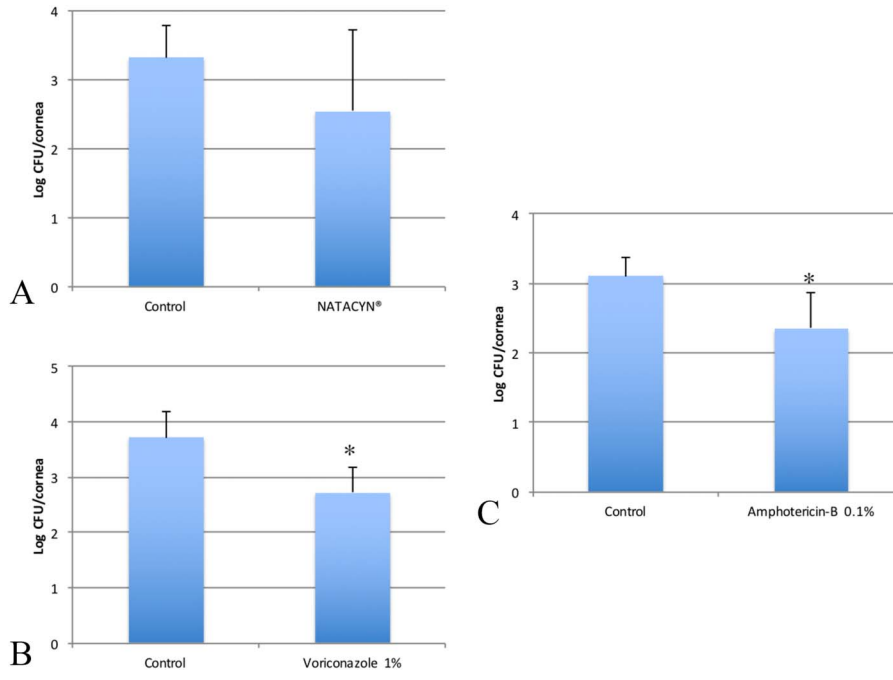

FIG. 4. Viable fungal loads in log colony-forming units (CFU) of (A) NATACYN, (B) voriconazole 1%, and (C) amphotericin B 0.1% in the rabbit *F. solani* keratitis model at DPT-7. Histograms show the comparison of viable fungal counts in log CFU after different treatments. Mean and SDs are shown. (A, NATACYN vs. control, P=0.337) (B, voriconazole 1% vs. control, P=0.004) (C, amphotericin B 0.1% vs. control, P=0.01). full color online

models for the study of the pathogenesis and efficacies of antifungals using rabbit model with different experimental methodologies (Table 2).[13–23] The studies have shown mixed results for establishing a *F. solani* rabbit keratitis model (in terms of clinical features, viable CFU, and total experimental time reflective of sustained experimental infection duration [Table 2][13–23]). *F. solani* keratitis is difficult to maintain for a long period without losing its pathologic features and viable fungal count retrieval. It was reported that local pretreatment with steroid was essential for achieving sustained *F. solani* keratitis infection in rabbits,[20] and we have integrated both local and systemic immunosuppressive techniques while maintaining its pathologic features to develop the sustained and progressive *F. solani* rabbit keratitis for us to study the antifungals treatment effects over extended times Figure 1A–G. It was also observed that some of the animal models developed from previous reports yielded relatively few viable fungal counts which could lead to the false-negative reports (Table 2).[13–23] In this report, we have developed a highly reproducible rabbit *Fusarium solani* keratitis model evidenced by the substantial fungal retrieval amount and similar histopathogenic figures as found for human fungal keratitis (Figs. 1A–G and 2). The fungal retrieval count was stable with approximately 3 log CFU in all 4 different independent experiments, and all animals in each independent experiment successfully developed an active, progressive infection *F. solani* keratitis (Figs. 1F and 4). Dendritic ulcers appeared at DPI-1 in our model which is also noted as a common occurrence in patients (Fig. 1A).[33] Feathery borders or hyphae edges and the presence of hypopyon in our rabbit model were seen as well and these are also among the most common clinical findings in patients diagnosed with *F. solani* keratitis (Fig. 1B–D).[33] Moreover, our model also showed minimum inflammation and the absence of lid edema, which are similar to reports from infected patients (Fig. 1).[33] Most of the studies conducted 8 days of experimental infection model (3 days' waiting period for full-blown infection and 5 days for the treatment).[16,17,19,21] However, the delayed diagnosis and the requirement of prolonged treatment duration of *F. solani* keratitis in real scenario prompted us to study the late treatment effects with prolonged duration in our sustained *F. solani* rabbit keratitis model.[33] The late treatment effects (5 days' waiting period for full-blown infection) of commercially available antifungals for a prolonged duration (7 days) have been studied in our *F. solani* rabbit keratitis model (Figs. 3B–D and 4).

The reports have been published in the literature showing the effectiveness of voriconazole in the animal model,[15,16,18,21,22] and most of the animal models used 3 days of a waiting period to initiate the treatment which is indicated for treating superficial infections.[15,16,21] A report suggested that filamentous fungi tended to grow toward the anterior chamber as the infection progresses.[34] Five days of a waiting period was used in this study to start the treatment for the *F. solani* to settle in the deep stroma. Histopathology findings also revealed deep stromal or anterior chamber invasion of *F. solani* (Fig. 2A). To the best of our knowledge, our rabbit fungal keratitis model is the first experimental model showing the anterior chamber invasion of *F. solani* from corneal stroma. It could be considered as a severe or deep infection model, representing many actual clinical cases which reveal deep-lying infection or anterior chamber involvement, which is complicated to treat due to poor penetration of antifungals and natamycin in particular.[35,36]

We have compared 3 often used antifungals. Amphotericin B 0.1%–treated rabbit group showed that there was a significant decrease in fungal load evidenced by CFU count and histopathology (Figs. 3D and 4C) as shown by these studies.[16,17] However, its clinical use is limited by its toxicity.[37] Natamycin, the only US Food and Drug Administration–approved topical antifungal, showed minimal effectiveness in decreasing the fungal burden in histopathology experiments and fungal load in CFU in our study (Figs. 3B and 4A). Similar findings were found in the report that natamycin has poor penetration into the corneal stroma and does not provide good clinical outcomes.[35,36] In our study, voriconazole 1% showed the best antifungal activity in terms of CFU, wound area, and histopathological findings (Figs. 3C and 4B).

In conclusion, a new model of clinically relevant *F. solani* rabbit keratitis model is developed, and we have reported the first experimental antifungal drug efficacy study showing that voriconazole 1% is superior to NATACYN and amphotericin B 0.1%, suggesting the use of voriconazole 1% as a first-line treatment in the clinical management of *F. solani*. Efficacy testing of these three antifungals against other pathogenic filamentous fungi in this rabbit model is urgently warranted for assistance to ophthalmologists in managing affected patients.

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