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Comparison of Clinical, Microbiological, and Histopathological Effects of Topical Caspofungin, Anidulafungin, and Voriconazole in *Candida albicans* Keratitis

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Abstract

Purpose: The aim of this study was to compare the clinical, microbiological, and histopathological effects of topical caspofungin, anidulafungin, and voriconazole in the treatment of *Candida albicans* keratitis.

Methods: *Candida albicans* (ATCC 10231) was inoculated on the mechanically de-epithelialized corneas of 28 male New Zealand White rabbits. Topical treatment was initiated after 72 h of inoculation. The rabbits were randomly assigned to treatment with 0.9% NaCl (control group), 0.5% caspofungin, 0.5% anidulafungin, or 1% voriconazole (n=6 rabbits per group). Eye drops were instilled every 30 minutes for the first 12 h, then hourly for 3 days. Clinical evaluations were done after 36 and 72 h of treatment. Clinical evaluation scores were calculated. Twelve hours after the final topical application, corneal tissue was removed under general anesthesia. In sterile conditions, the excised corneas were divided into two pieces for histopathological and microbiological examination. The efficacies of the treatments were measured by clinical assessment, fungal culture, and histopathology.

Results: After 72 h of treatment, mean clinical assessment scores of the control, caspofungin, anidulafungin, and voriconazole groups were 9.60 ± 0.54 (9-10), 4.66 ± 1.63 (3-7), 2.50 ± 0.83 (1-3), and 6.50 ± 1.64 (4-8), respectively. Viable cell counts of the groups were 5549.20 ± 5113.54 (857.14-13333.33), 809.52 ± 1120.00 (0-2857.14), 0, and 8678.57 ± 10078.62 (0-25000) CFU/g, respectively. Histopathological analysis yielded mean hyphal densities of 50 µm, 20 µm, 0 µm, and 85 µm, respectively.

Conclusions: According to clinical, microbiological, and pathological data, the most effective agent against *Candida albicans* keratitis was anidulafungin, followed by caspofungin.

Keywords: *Candida albicans*; Fungal keratitis; Anidulafungin; Caspofungin; Voriconazole

Introduction

Fungal keratitis is an important cause of unilateral blindness worldwide, and corneal scarring due to suppurative keratitis is the most common cause of preventable blindness [1]. Studies indicate that the incidence of fungal keratitis has increased over the last 40 years [2,3]. Its etiology varies depending on geographical location. In tropical and subtropical countries dealing with agriculture, molds are the preponderant causal organisms, mainly *Aspergillus* species. The most widespread predisposing factor is ocular trauma involving organic material. In contrast, *Candida albicans* is the most frequently isolated pathogen in temperate countries. Risk factors include weakened local defense mechanisms and epithelial erosion resulting from contact lens use, atopic diseases, and topical or systemic use of corticosteroids [4,5].

Fungal keratitis presents a serious threat due to delayed diagnosis and the insufficient efficacy of antifungal medications. Various antifungal agents such as topical 0.15% amphotericin B, 5% natamycin, and azole derivatives are used in the treatment of Candida keratitis [5,6]. Voriconazole is a new-generation triazole produced from fluconazole that inhibits sterol biosynthesis by inhibiting the enzyme 14-α sterol demethylase dependent on cytochrome P450 [7,8]. Caspofungin and anidulafungin are new antifungal agents of the echinocandin class that noncompetitively inhibit beta-(1,3)-D-glucan synthesis in fungal cell walls [9]. Caspofungin has potent activity against *C. albicans* biofilms [10]. A study demonstrated that intravenous administration of anidulafungin results in dose-dependent penetration into the vitreous humor [11]. However, there is not enough studies about topical anidulafungin in the treatment of fungal eye diseases.

The purpose of this study was to determine the efficacy of topical anidulafungin in an experimental *Candida* keratitis model and to compare it with the efficacies of caspofungin and voriconazole.

Materials and Methods

Animals

Twenty-four male New Zealand White rabbits weighing 1.5-2.5 kg were divided into four groups (6 animals per group). They were raised in an authorized breeding center and were housed in separate cages under standardized conditions (in a temperature- and humidity-controlled room with a 13:11 hour light:dark cycle). Rabbits were fed with standard dry food and water ad libitum. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The regional ethics committee approved the treatment protocol.

Induction of keratitis

The *C. albicans* keratitis model was based on contact lens-induced infection [12]. Before all interventions, the rabbits were anesthetized with intramuscular xylazine (5 mg/kg; Xylapan; Chassot, Bern, Switzerland) and ketamine hydrochloride (35 mg/kg; Ketalar; Parke-Davis, Ann Arbor, MI, USA). Corneal anesthesia was achieved using topical proparacaine HCl 0.5% (Alcaine, Alcon, Puurs, Belgium). The epithelium of each animal's right eye was completely removed by gently scraping with a 15 Bard-Parker blade and the eye was rinsed with balanced salt solution. We chose *C. albicans* (ATCC 10231) to inoculate the de-epithelialized cornea because it has been suggested that this strain may be an appropriate indicator organism to determine loss of antimicrobial activity in multipurpose contact lens solutions due to partial evaporation [13]. We applied 100 µl of fungal inoculum containing approximately 10^9 blastoconidia on each de-epithelialized cornea, and then covered it with a soft contact lens made from etafilcon A (14.5 mm diameter; Acuvue; Johnson & Johnson Vision Products, Jacksonville, FL, USA). The eyelids were closed with 5/0 silk suture to prevent the contact lenses from falling out. After 72 h, the blepharorraphy sutures were opened and the contact lens was removed. The same ophthalmologist clinically evaluated all the eyes for keratitis by hand-held slit-lamp examination. Swab cultures were immediately taken from the infected corneas and used to inoculate Sabouraud dextrose agar plates. The culture plates were incubated at 37°C for 24 hours and *C. albicans* were observed in all of the plates. The existence of *C. albicans* keratitis was verified in all infected eyes.

Antifungal agents

Anidulafungin (Eraxis; Pfizer, New York City, NY, USA) and caspofungin (Cancidas; Merck & Co., Whitehouse Station, NJ, USA) were diluted to 0.5% and voriconazole (Vfend; Pfizer, New York City, NY, USA) was diluted to 1% using sterile water. Concentrations of drugs used in the experiments were determined by the results of *in vitro* toxicology studies [14]. Drug solutions were prepared daily and protected from light and stored at 4°C between each use.

Treatment protocol

Treatment was repeated every 30 minutes for the first 12 h and at hourly intervals thereafter for a total of 3 days. This treatment protocol was based on a study of amphotericin B usage in a rabbit model [15]. Topical balanced salt solution was administered to the control group at the same intervals.

Tissue sample collection

Twelve hours after the final topical application, corneal tissue was removed under general anesthesia. Following tissue collection, the animals were killed using high-dose anesthesia. In a sterile environment, the corneas were separated from the limbus and divided into two pieces. One half of the cornea was used for fungal culture and the other half was fixed with 10% paraformaldehyde for histopathological examination.

Clinical grading and scoring

Clinical evaluation was performed before treatment and after 36 and 72 hours of treatment. The grading system previously defined by Ishibashi and Kaufman [16] was used with little alteration (Table 1). Lesion size and hypopyon were evaluated by hand-held slit-lamp. The total clinical scores for all eyes, and the severity of the ocular infections were determined based on the Table 1.

Infection	Intensity	Score
Corneal ulcer	Diameter<2 mm	1
	2 mm<diameter<4 mm	2
	4 mm<diameter	3
Haze of peripheral cornea	Mild	1
	Middle	2
	Severe	3
Protrusion of cornea		1
Exudate in anterior chamber and plaque of posterior surface of cornea		1
Hypopyon	Height<1 mm	1
	1 mm<height<3 mm	2
	3 mm<height	3

Hyphema		1
Infection of iris		1
Stromal infiltration	Light stromal infiltration	1
	Dense stromal infiltration	2
	Corneal perforation	3

Table 1. Grading of corneal infection in *Candida albicans* keratitis.

Quantitative fungal recovery

Under sterile conditions, the corneal halves were weighed on sensitive scales and shredded to small pieces of approximately uniform size with 15 Bard-Parker blades, transferred to a test tube containing 2 ml sterile saline, and ground on ice with a tissue grinder 3 times for 10 seconds each at 20 second intervals. A serial dilution with 1 µl, 10 µl, and 100 µl of the tissue suspension was plated in triplicate on Sabouraud Dextrose Agar plates, and the plates were incubated at 35 ± 2°C for 48 h. Colony-forming units (CFU) were enumerated and divided by corneal weight to yield a value of CFU/g of cornea tissue.

Histopathology

The cornea halves were embedded in paraffin and 3-µm sections of the central cornea were cut and stained with hematoxylin-eosin and Periodic Acid-Schiff reagents by standard protocols. We measured the maximal depth of *C. albicans* penetration in micrometers.

Antifungal susceptibility test for *Candida albicans* ATCC 10231 strain

C. albicans ATCC 10231 strains were tested using Sensititre Yeast OneTest Panel Y010 (Trek Diagnostic Systems, USA) colorimetric microdilution method following the CLSI M27-A3 guidelines [17]. Active yeast suspension of nearly 1.5×10^3 cells/ml was prepared in Yeast One inoculum broth (Trek). The Yeast One Panels were inoculated with the working yeast suspension by placing 100 µl into each well using an appropriate multichannel pipetting device. The panel was closed with the adhesive seal and incubated at 35°C for 24 h. Yeast growth was evident as a color change from blue (negative, no growth) to pink (positive, growth). The colorimetric minimum inhibitory concentration (MIC) result for each test agent was defined as the lowest concentration of antifungal agent that inhibited pink color development (first blue well).

Statistical analysis

Clinical scores are shown as mean ± standard deviation and range (minimum-maximum). SPSS package software version 21.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The differences between groups in clinical grading, quantitative isolate recovery, and histological data at hour 0, hour 36, and hour 72 of treatment were assessed by Kruskal-Wallis one-way analysis of variance. Repeated measures within groups were compared using the Friedman test. When statistical significance was determined, Wilcoxon signed-rank sum test was used. A P value less than 0.05 was considered statistically significant.

Results

Clinical evaluation

The clinical scores obtained at hour 0, hour 36, and hour 72 of treatment is given in Figure 1 and Table 2. There was no statistically significant difference between the groups before treatment (p=0.536; Kruskal Wallis test).

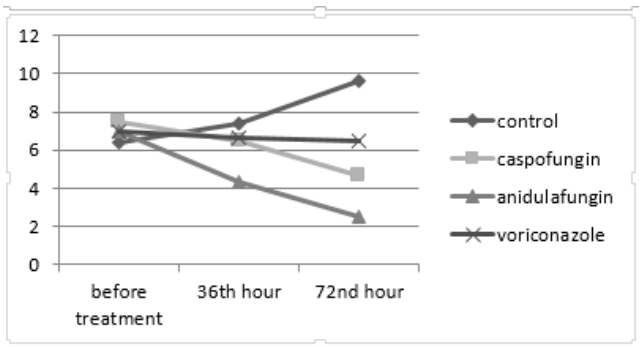

Figure 1: Clinical scores among groups during the treatment. Before treatment there is no difference between the groups. Clinical scores decreased in the anidulafungin and caspofungin groups; but increased in the control group; and have been nearly the same in the voriconazole group.

Group	Before treatment	36th hour	72nd hour	p (Before treatment - 72nd hour)
Control	5-7 (6.40 ± 0.89) (a)	6-8 (7.40 ± 0.89) (a)	9-10 (9.60 ± 0.54) (b)	p=0.007
Caspofungin	6-10 (7.50 ± 1.37) (a)	5-8 (6.50 ± 1.04) (ab)	3-7 (4.66 ± 1.63) (b)	p=0.004
Anidulafungin	6-8 (7.00 ± 0.89) (a)	2-6 (4.33 ± 1.36) (b)	1-3 (2.50 ± 0.83) (b)	p=0.002
Voriconazole	4-9 (7.00 ± 1.67) (a)	4-8 (6.66 ± 1.36) (a)	4-8 (6.50 ± 1.64) (a)	p=0.472

a, letters indicate differences between groups.

b, letters are discussed separately for each group.

Table 2: Clinical scores in groups.

Clinical scores at 72 h differed significantly between the groups (p<0.001; Kruskal Wallis test). There were significant differences between the clinical scores obtained before treatment and at 36 and 72 h of treatment in all groups except the voriconazole group. Clinical scores at 72 h decreased drastically in the anidulafungin group (p=0.002; Friedman test) and caspofungin group (p=0.004; Friedman test) but increased significantly in the control group (p=0.007; Friedman test); the clinical scores of the voriconazole group were nearly unchanged after 72 h of treatment (p=0.472; Friedman test) (Figures 1-4).

Figure 2: Representative photographs of the control group. A, An eye in the control group before treatment (clinical score= 7). Large corneal lesion of 7 × 8 mm, middle corneal haze, silier injection and middle stromal infiltration. B, The same eye in 72nd hour (cilinical score=10) continuing large corneal lesion of 8 × 9 mm, diffuse and severe corneal haze, conjunctival chemosis, silier injection and dense stromal infiltration. C, Representative examples of the histopathology of cornea in the control group with light microscopic examination maximal depth of penetration Candida albicans, Periodic acid-Schiff stain. Magnification, X400.

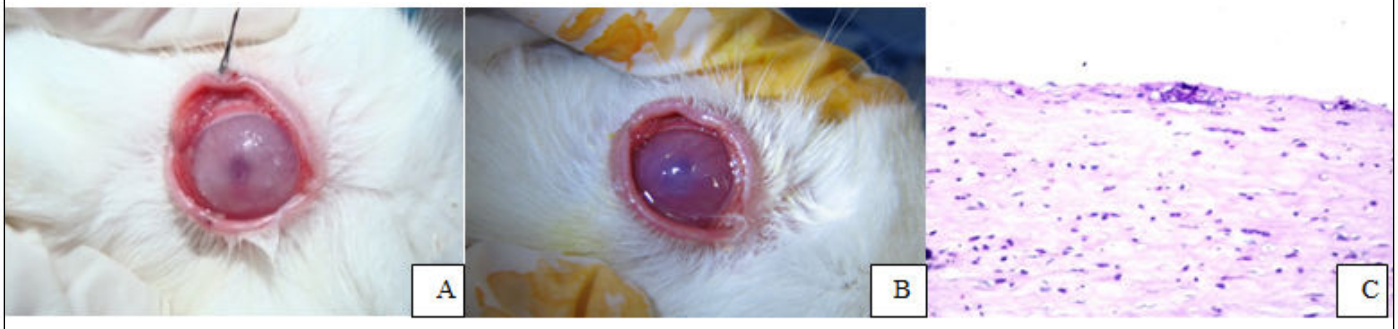

Figure 3: Representative photographs of the caspofungin group. A, An eye in the caspofungin group before treatment (clinical score= 8). Large corneal lesion of 6 × 4 mm, silier injection, middle haze of peripheral cornea and dense stromal infiltration. B, The same eye in 72nd hour (Clinical score=5) continuing corneal lesion of 2 × 3 mm, mild central corneal haze, silier injection and light stromal infiltration. C, Representative examples of the histopathology of cornea in caspofungin group with light microscopic examination maximal depth of penetration *Candida albicans*, Periodic acid-Schiff stain. Magnification, X400.

Microbiological and histo-pathological examination

Fungal load in the cornea tissue (CFU/g) determined by microbiological analysis is shown in Table 3. Mean fungal tissue load was 5549.20 ± 5113.54 (857.14-13333.33) CFU/g in the control group, 809.52 ± 1120.00 (0-2857.14) CFU/g in the caspofungin group, 0 CFU/g in the anidulafungin group, and 8678.57 ± 10078.62 (0-25000) CFU/g in the voriconazole group. CFU/g values were significantly lower in the caspofungin and anidulafungin groups compared to the control group (p=0.008; Kruskal Wallis test), but there was no significant difference in CFU/g between the voriconazole and control groups.

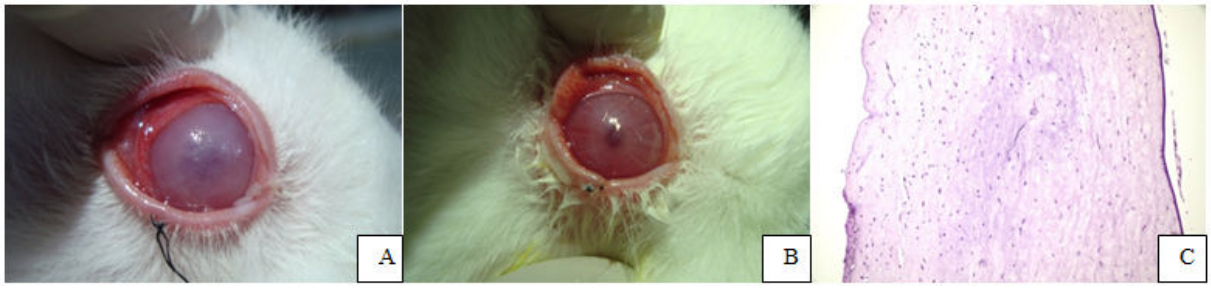

Figure 4: Representative photographs of the anidulafungin group. A, An eye in the anidulafungin group before treatment (clinical score= 8). Large corneal lesion of 8 × 4 mm, diffuse corneal haze, silier injection, severe haze of peripheral cornea and dense stromal infiltration. B, The same eye in 72nd hour (clinical score=2) continuing minimal corneal lesion of 1 × 1.5 mm, light central corneal haze, any stromal infiltration. C, Representative examples of the histopathology of cornea in anidulafungin group with light microscopic examination maximal depth of penetration Candida albicans, Periodic acid-Schiff stain. Magnification, X200.

MIC values for anidulafungin, caspofungin and voriconazole were determined as 0.12, 0.12, and 0.03 µg/ml, respectively. All of these MIC values were classified as sensitive according to CLSI standards [17].

Comparison of the 72 h clinical scores and the results of microbiological analysis yielded a Spearman's rho test correlation coefficient of 0.731, indicating a significant positive correlation ($p<0.001$; Spearman's rho test) (Figure 5).

Mean corneal hyphal density determined by histopathological analysis was 50 µm in the control group, 20 µm in the caspofungin group, 0 µm in the anidulafungin group, and 85 µm in the voriconazole group (Figures 2C,3C,4C and 5C).

Figure 5: Representative photographs of the voriconazole group. A, An eye in the voriconazole group before treatment (clinical score=7). Large corneal lesion of 8 × 7 mm, middle corneal haze, silier injection, haze of peripheral cornea and middle stromal infiltration. B, The same eye in 72nd hour (clinical score=10) continuing large corneal lesion of 9x8 mm, diffuse and severe corneal haze, conjunctival chemosis, silier injection and dense stromal infiltration. C, Representative examples of the histopathology of cornea in the anidulafungin group with light microscopic examination maximal depth of penetration *Candida albicans*, Periodic acid-Schiff stain. Magnification, X400.

Discussion

The treatment of fungal keratitis is challenging due to the limited tissue penetration, restricted antimicrobial spectrum, and toxicity of the antifungal agents which are currently available [18].

The usage of topical steroids after ocular surgery and contact lenses are increasing day by day. As a result, Candida keratitis is increasing in frequency. The prognosis of *Candida albicans* keratitis is bad and the need of penetrating keratoplasty in most cases is an important problem. In particular, ocular surface disease and long-term use of steroids after penetrating keratoplasty are thought to facilitate the development of fungal keratitis [19,20].

Voriconazole has a wide antifungal spectrum in the treatment of systemic and ocular diseases due to *Candida* species, *Aspergillus* species, *Cryptococcus neoformans*, *Paecilomyces lilacinus*, *Scedosporium* species, and *Curvularia* species [21-24] in a study that assessed corneal endothelial cell density after intrastromal injection of 0.1% voriconazole, intrastromal voriconazole was determined to have statistically significant toxicity to corneal endothelial cells [25]. Additionally, our clinical, microbiological, and histopathological investigations revealed no improvements in the group receiving topical voriconazole. We believe the lack of improvement with voriconazole may be attributed to low sensitivity of *C. albicans* to voriconazole, or to the toxic effect of voriconazole on the cornea. However, the corneal endothelial cells must be analyzed to determine whether the treatment is toxic.

Caspofungin is a key member of the echinocandin class, which exert their effects on the fungal cell wall by inhibiting the synthesis of (1,3)-D-glucan, and has strong activity against Candida and Aspergillus [26]. Caspofungin's inhibitory activity against Candida includes azole-resistant strains [8]. In a study using a rabbit model, it was suggested

that topical 0.5% caspofungin is as safe and effective as topical 0.15% amphotericin B. Amphotericin B is the most frequently used agent in the treatment of Candida keratitis [14]. Therefore, we used caspofungin in our study, as it is comparable to amphotericin B. Our results from the rabbit model demonstrated that topical 0.5% caspofungin is safe and more effective than voriconazole in the treatment of Candida keratitis. In addition, further animal studies are needed to compare and confirm the topical caspofungin and amphotericin B treatment approach in candida keratitis.

Anidulafungin, a new echinocandin, has been shown to have wide antifungal spectrum activity *in vitro* as well as clinical efficacy [27,28] and is approved for use in the treatment of esophageal candidiasis, systemic candidemia, and other Candida infections. Among the three echinocandins, anidulafungin reportedly has the lowest MIC's against Candida strains [29]; however, in our study, the MIC value of anidulafungin was equivalent to that of caspofungin, and voriconazole had the lowest MIC in Candida strains. Our study indicates that anidulafungin provides the best clinical improvement and histopathological and fungal recovery. Topical 0.5% anidulafungin was more effective than topical 0.5% caspofungin in the treatment of Candida keratitis. Anidulafungin is a new antifungal drug especially suitable for use in Candida keratitis. No adverse drug effects were observed in the infected eyes of the anidulafungin, caspofungin, or control groups. However, the voriconazole group exhibited clinical findings such as conjunctival injection, chemosis, and corneal clouding.

To conclude, anidulafungin and caspofungin are topical antifungal agents that can be used safely in the *C. albicans* keratitis. Further *in vivo* and *in vitro* studies on caspofungin and anidulafungin are necessary to determine their potential impact on human keratomycosis and their efficacy, corneal toxicity, and optimal dosage for topical usage. Corneal endothelial density can be investigated for improvement this article. Corneal toxicity may be caused the defect of cure. In addition to this work, the penetration of antifungal agents through the cornea into the aqueous humor after topical administration can be investigated.

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