Combined Topical Fluconazole and Corticosteroid Treatment for Experimental Candida albicans Keratomycosis

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PURPOSE. To determine the most efficient time point and concentration of topical corticosteroids in Candida albicans keratitis treated with fluconazole.

METHODS. Corneas of 105 rabbits were infected with viable yeast cells of C. albicans (2.5 × 10⁵). After a 48-hour incubation period, seven groups of animals were treated for 21 days with fluconazole, with group I acting as a control, and groups II to VII receiving adjunct therapy with the corticosteroid prednisolone (5 or 10 times daily; 3, 9, or 15 days after infection). The degree of corneal infiltration, ulceration, corneal clouding, hypopyon, conjunctivitis, neovascularization, and corneal perforation was monitored over a 24-day period, as well as recultivation and resistance to fluconazole of the C. albicans pathogen.

RESULTS. The control group showed the highest level of corneal clouding and neovascularization. In comparison, by day 24, the majority of groups also treated with prednisolone displayed significantly less corneal clouding and neovascularization. An immediate decrease in corneal clouding was observed in groups treated with additional low- or high-dose prednisolone from day 9 after inoculation. After additional prednisolone treatment from day 9 or 15 after inoculation, no significant difference was detected in the recultivation rate of C. albicans compared with the control. Early administration of prednisolone (day 3, low and high dose) resulted in the recultivation of significantly more C. albicans pathogens.

CONCLUSIONS. Fluconazole plus adjunct high-dose prednisolone treatment was most effective when administered 9 days after infection. The delayed application of corticosteroids after treatment with antifungal drugs in cases of fungal keratitis is therefore not contraindicated and may be beneficial in patients. (Invest Ophthalmol Vis Sci. 2005;44:2634–2643) DOI:10.1167/iovs.02-1135

Keratomycosis is an increasing problem in ocular infectious diseases.1,2 Corneal trauma and contact lens wear, especially extended wear and therapeutic bandage contact lens wear, are the most commonly associated risk factors. Studies have shown fungal keratitis to be associated with trauma in 35% to 100% of patients.3–7 Further risk factors for fungal keratitis are chronically applied topical medications, including corticosteroids, corneal anesthetic abuse with self-inflicted injury, and diabetes mellitus.1,8

The first sign of a fungal infection is a central, paracentral, or peripheral infiltrate with a marked conjunctival and intraocular irritation. Centrally located infections are usually more severe than infections near the limbus,9 and in most cases, the development of an ulceration follows. If untreated, complications frequently occur, such as hypopyon, neovascularization, corneal clouding, development of a descemetocele, and corneal perforation. The development of serious complications—total corneal clouding, staphyloma, and endophthalmitis—may ultimately cause blindness.10 The effectiveness of a penetrating keratoplasty as a treatment for these complications is limited.11 With the ineffectiveness of surgical intervention, a pharmacological approach may be beneficial in the treatment of fungal keratitis.

Fluconazole is a bis-triazodifluorophenyl-2-propanol antifungal compound with both in vitro and in vivo activity against C. albicans. It has a long plasma half-life of approximately 25 to 30 hours in humans, with a predominantly renal excretion mode, and the drug is effectively distributed throughout the tissues.12–14 Previous studies,15,16 including our own,17 have demonstrated that fluconazole is a safe and effective antifungal agent for the topical treatment of Candida keratomycosis.

The benefit of corticosteroids administered in combination with antifungal substances in the treatment of keratomycosis is somewhat controversial. If administered in addition to antifungal agents, nonspecific inflammatory processes, including corneal edema, intraocular irritation,18,19 and neovascularization,20 are reduced. However, many studies have demonstrated that treatment with corticosteroids in combination with antifungal agents has a negative effect on fungal infections.21–25 For example, recovery rates decline steadily in normal control corneas but remain stable over 15 days in corticosteroid-treated corneas. In addition, O’Day et al.21 observed that inflammation was equivalent or significantly less until day 10. At day 15, however, inflammation in corticosteroid-treated corneas was significantly worse than in animals that received no corticosteroid treatment.21

In those studies, corticosteroids were given before or immediately after inoculation of the pathogen. Initiating corticosteroid therapy a few days after inoculation and the subsequent use of antifungal therapy may be a beneficial approach to dual therapy.25 The dose of corticosteroids may also be important, and it has been shown that, in contrast to higher concentrations of corticosteroids, low-dose corticosteroids do not influence the recultivation of fungal pathogens20 and the therapeutic efficacy of amphotericin B.26

The present study was conducted to develop a systematic approach to examining the influence of the time factor and the

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concentration of corticosteroids in combination with antifungal treatment in keratomycosis.

**Materials and Methods**

**Animals**

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The project was approved by the local animal research review committee of the authors’ institution. A total of 105 (7 groups; n = 15) female, pigmented, inbred ChinChilla Bastard rabbits (Harlan, Borchen, Germany) were used, each weighing approximately 1.5 to 2.5 kg. The animals did not receive any treatment before ocular infection, and the slit lamp examination and indirect funduscopy showed no disease. All infections were induced in one eye only.

**Inoculation Technique**

The procedure is based on a model of experimental keratomycosis, as described elsewhere. Rabbits were anesthetized with xylazine (5 mg/kg body weight) and ketamine HCl (50 mg/kg body weight) by intramuscular injection. We used *C. albicans* DSM pathogens (no. 70010; German Collection of Microorganisms, Braunschweig, Germany), which had shown a high virulence in previous experiments. To obtain sufficient infection, a suspension of 0.1 mL viable yeast cells in sterile glucose (2.5 × 10⁷ cells/mL) was injected into the central corneal stroma tangential to the corneal surface. In all animals, a corneal infiltrate developed of 8 to 11 mm diameter. After 48 hours, all inoculated eyes showed a similar corneal ulceration.

**Treatment**

Forty-eight hours after inoculation, each animal was assigned to one of seven groups (I-VII; Table 1). Topical antimycotic treatment of all infected eyes was started on day 3, with fluconazole (Diffucan, fluconazole 0.2% in NaCl; Pfizer, Karlsruhe, Germany) administered 10 times a day for 21 days at 1-hour intervals. Starting on days 3 (early), 9 (middle), and 15 (late), respectively, treatment groups II, III, and IV received additional prednisolone (10 mg prednisolone-21-acetate in 1% Inflanefran Forte Eye Drops; Allergan, Irvine, CA) therapy at 2-hour intervals (5 times a day), whereas groups V, VI and VII received prednisolone at 1-hour intervals (10 times a day). Animals treated with fluconazole alone acted as a control group (group I). To avoid bacterial infection, all animals received aqueous chloramphenicol (1% Thilocanol; Alcon, Freiburg, Germany) twice daily.

All animals were evaluated daily over a 24-day period by slit-lamp biomicroscopy for the following parameters: size of corneal infiltrates (in millimeters); size of the ulceration (in millimeters); level of corneal clouding; hypopyon (in millimeters); level of conjunctivitis; level of neovascularization, descemetocele, or corneal perforation; and extent of pathogen recultivation. Scoring of the different parameters was performed by masked observers.

For a standardized grading of neovascularization, the scale depicted in Figure 1 was used. For judgment and statistical comparison, it is important to take into account that central neovascularization in one or two quadrants is more severe than peripheral circular neovascularization. Therefore, the following two-digit system was developed: The first digit coded for the distance to the center of the cornea (from 1 to 3), and the second digit coded for the number of quadrants in which neovascularization occurred. This resulted in a numerical code that could be statistically compared (e.g., 21 coded for corneal neovascularization to the middle in one quadrant and 13 for peripheral neovascularization in three quadrants).

Corneal clouding was evaluated by the following scale: 0, clear cornea; 1, minor edema; 2, corneal clouding in more than two quadrants of the cornea; and 3, total corneal clouding. Conjunctivitis was evaluated by the degree of conjunctival hyperemia (low, 1; middle, 2; or high, 3) in each eye, as previously described.

In addition, standardized photographs of the eyes were taken on days 3, 8, 12, 16, 20, and 24. After 21 days of antmycotic treatment, animals were deeply anesthetized with an intramuscular injection of ketamine HCl (50 mg/kg body weight) and killed. Eyes were then cleaned with sterile balanced salt solution, and corneas were immediately removed. Corneas were divided by cutting them through the center of the ulceration. One half was stored on prepared blood agar, and the other was suspended in sterile glucose solution.

**TABLE 1. Experimental Groups**

<table>
<thead>
<tr>
<th>Drug</th>
<th>I (Control)</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
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<tbody>
<tr>
<td>Fluconazole (day 5)</td>
<td>10×</td>
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<tr>
<td>Prednisolone</td>
<td>—</td>
<td>5× (low)</td>
<td>5× (low)</td>
<td>5× (low)</td>
<td>10× (high)</td>
<td>10× (high)</td>
<td>10× (high)</td>
</tr>
<tr>
<td>Starting day*</td>
<td>—</td>
<td>5 (early)</td>
<td>9 (middle)</td>
<td>15 (late)</td>
<td>5 (early)</td>
<td>9 (middle)</td>
<td>15 (late)</td>
</tr>
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* Day after infection on which drug was administered, referred to as early (day 3), middle (day 9), and late (day 15).

**FIGURE 1. Standardized grading of neovascularization. A two-digit system was developed: The first digit coded for the distance to the center of the cornea (1 to 3), and the second digit coded for the number of quadrants (1 to 4) in which neovascularization occurred. This resulted in a numerical code that could easily be compared (e.g., 21 coded for corneal neovascularization to the middle in only one quadrant and 13 for peripheral neovascularization in three quadrants). This system takes into account that central neovascularization in one or two quadrants is more severe than peripheral circular neovascularization.
The plates and suspension were incubated for up to 72 hours at 37°C. If any growth occurred, they were replated on Sabouraud agar and incubated for an additional 48 hours at 37°C. The growth of single colonies was identified with an auxanogram (API ID 32C; Fa. bioMérieux, SA, Marcy-l’Etoile, France). The identification codes were compared with the DSM culture 70010 and then assessed for positive pathogen recultivation; only the recultivation of *C. albicans* cultures with the same identification code as DSM culture 70010 was considered positive. In the event of positive recultivation, possible resistance of *C. albicans* to fluconazole was tested with a kit (Fungitest; Sanofi Diagnostics Pasteur, Marnes-la Coquette, France), which allows the determination of the sensitivity of yeasts to antifungal agents, according to a standardized method.

Statistics

At the experimental end point (day 24), the following parameters were analyzed by χ² test: recovery rate, incidence of corneal clouding, conjunctivitis, neovascularization, and hypopyon. In addition, over the experimental time course, the shape and the degree of corneal clouding, conjunctivitis, neovascularization, and hypopyon were tested for statistical significance with the Wilk lambda test.

Results

All animals showed development of a corneal infiltrate of 8 to 11 mm diameter after inoculation. After commencement of drug treatment (48 hours), a similar corneal ulceration was observed in all experimental eyes. During the follow-up treatment period, only one eye exhibited a descemetocele (in group IV at day 12 after inoculation of the pathogen), which declined, and no perforation occurred. Statistical analysis (χ² test and the Wilk lambda test) revealed no significant differences between treatment groups for the parameter hypopyon.

Recovery Rates

As previously mentioned, the recultivation of *C. albicans* was considered positive only when the auxanogram identification codes were identical with the DSM culture 70010. No other organisms were recovered during the course of the experiment. Early application of prednisolone (groups II and V, independent of the dose) resulted in a significantly higher recultivation rate than all other groups when assessed after 24 days (*P* < 0.01 and *P* < 0.025, respectively; χ² test). The middle and late application of prednisolone (groups III, IV, VI, and VII; low and high dose) showed no significant difference from the control and revealed no negative influence on the recultivation rate of *C. albicans* (Fig. 2). Furthermore, no fluconazole-resistant *C. albicans* cultures were recultivated, as demonstrated by the fungus test kit (Fungitest; Sanofi Diagnostics Pasteur).

Conical Clouding

At the experimental end point in most groups treated with adjunct prednisolone (groups II, III, IV, VI, and VII), significantly less corneal clouding was observed compared with the control group (*P* < 0.001, *P* < 0.02, *P* < 0.05, *P* < 0.005, *P* < 0.005, respectively; χ² test). With early high-dose prednisolone, an increase of corneal clouding was detected after 2 weeks (Fig. 3A). At the experimental end point, no statistical difference was found (*P* > 0.136; χ² test).

In general, the control group showed the highest level of corneal clouding, and those that received early low-dose prednisolone (group II) showed the lowest level (Fig. 3). If prednisolone treatment was started after 9 or 15 days, both concentrations (groups III, IV, VI and VII) significantly decreased the level of corneal clouding compared with the control group. Symptoms of corneal clouding decreased most effectively with additional treatment of prednisolone from day 9 (*P* < 0.004, Wilk lambda test; Fig. 3B). If prednisolone was applied at day 15 (Fig. 3C), both low- and high-dose prednisolone still significantly reduced corneal clouding (*P* < 0.05, Wilk lambda test).

Neovascularization

At the test's end, a significantly lower rate of neovascularization was detected in almost all groups treated with adjunct prednisolone (groups II, III, VI, and VII; *P* < 0.001, *P* < 0.02, *P* < 0.005, *P* < 0.005, respectively; χ² test). In contrast, neither late low-dose nor early high-dose prednisolone (groups IV and V) had an effect at the end point when compared with the control group (*P* > 0.464, *P* > 0.269, respectively; χ² test).

Generally, the control group showed the highest level of neovascularization, whereas the early low-dose prednisolone group showed only traces of neovascularization (Fig. 4). When low- and high-dose prednisolone treatment was started at day 9 (groups III and VI), a significantly lower level of neovascularization was observed compared with the groups treated with...
fluconazole alone ($P < 0.0007$; Wilk lambda test). After 2 weeks, early high-dose prednisolone (group V) resulted in an increase of corneal neovascularization comparable with that of the control group (Fig. 4A). In the groups treated with low- and high-dose prednisolone from day 9 (groups III and VI), neovascularization significantly declined ($P < 0.002$, Wilk lambda test; Fig. 4B).

No significant differences in the time course were found after additional prednisolone treatment when evaluating the groups that received late combined treatment (groups IV and VII) against the control group, according to the Wilk lambda test (Fig. 4C). However, analysis of the experimental end point data alone showed a significant effect of prednisolone in the group that received late high-dose prednisolone (group VII; $P < 0.005$; $\chi^2$ test).

**Conjunctiva**

All experimental eyes showed development of conjunctival infection shortly after inoculation. The infection decreased
FIGURE 4. Time course of development of corneal neovascularization: days 3 (A), 9 (B), and 15 (C). Arrows: start of treatment.
over time until no trace was detected in almost all animals (Fig.
5). Minor conjunctivitis remained only in the control group and
those treated early with high-dose prednisolone (group V; \( P <
0.068 \), \( \chi^2 \) test).

Significant differences were found in the time course when
groups receiving middle and late combined treatment were
compared with control animals that did not receive pred-
nisolone. For the time period of days 9 to 15, a significant
difference (\( P < 0.05 \); Wilk lambda test) in conjunctivitis symp-
toms was also observed in groups treated with prednisolone
from day 9 (groups III and VI) compared with animals treated
until day 15 with fluconazole alone. Furthermore, from day 15
to the final experimental day, a significant difference (\( P <
0.0043 \); Wilk lambda test) in conjunctival symptoms was de-
tected in those receiving the late administration of pred-
nisolone (low- and high-dose) compared with the control.

These results illustrate the accelerated anti-inflammatory
effect of prednisolone over the time course if administered at
days 9 and 15. However, as pointed out, at the test’s end, no
significant difference was detected between the groups.

**Figure 5.** Resolution of conjunctivi-
tis over the course of the experi-
ment: days 3 (A), 9 (B), and 15 (C).
Arrows: start of treatment.
DISCUSSION

The treatment of fungal keratitis remains a serious and unresolved problem. In recent decades, many experimental and clinical studies, including our own, have shown fluconazole to be effective against deep keratitis.\textsuperscript{15–17} Fluconazole has a low protein-binding property, is exceptionally hydrophilic in nature, and has a predominantly renal excretion. Fluconazole is effectively distributed throughout all tissues, including a high penetration into cerebrospinal fluid,\textsuperscript{13,14} and has also been shown to be nonmutagenic and less toxic than the other azoles.\textsuperscript{29}

The application of corticosteroids to reduce the nonspecific inflammatory processes remains controversial in the treatment of fungal keratitis. Many studies have shown a more pronounced recultivation rate of remaining pathogens after additional treatment with corticosteroids in fungal infections. In these studies, this observation was restricted to a time point immediately before or immediately after inoculation of the pathogen.\textsuperscript{21–25} The infection was developed under immunosuppressive conditions, where both the pathogen and corticosteroids were applied simultaneously. Such study design is not realistic, however, because patients generally receive therapy some days after infection starts. In contrast, another study showed that the application of low-dose corticosteroids a few days after pathogen inoculation may be superior to antifungal single-dose treatment of fungal keratitis.\textsuperscript{25} However, the study was performed in the early 1960s, and a systematic investigation as a basis of modern clinical treatment seems to be justified and necessary. It may be beneficial to intervene with immunosuppressive treatment a few days after infection to allow the antifungal drug and the body’s immune system to respond to the infection. During this phase, corticosteroids may reduce the anti-inflammatory overreactions of the host without influencing the recultivation rate. At a later stage, reaction to the infection (corneal clouding, neovascularization) may be too pronounced to be altered by corticosteroids. Safety studies have previously demonstrated the absence of ocular toxicity after administration of steroids to the rabbit eye.\textsuperscript{30}

In our present study, we used a well-established rabbit model of fungal keratitis\textsuperscript{27,31} to investigate the influence of the timing and dose of corticosteroids in combination with fluconazole. Therapy was started 2 days after inoculation with \textit{C. albicans}, when stromal keratitis had become manifest. For 48 hours, there was a progression of the infection without any treatment (therefore, start of treatment began at day 3 with fluconazole alone in the control animals and combined therapy in the appropriate groups; see also Table 1).

Our results demonstrate that the combination of corticosteroids and antifungal therapy is not contraindicated and that clinical success depends on timing and dose. The early combination of prednisolone and fluconazole (starting from the first day of treatment, independent of the steroid dose) leads to a significantly higher recultivation rate of \textit{C. albicans} after 24 days than treatment with fluconazole only. Clearly, early additional administration of corticosteroids (day 3) has a negative influence on the body’s immune system. As also demonstrated

\textbf{FIGURE 6.} Representative photographs of animals treated with fluconazole alone showing clinical appearance at days 3 (A), 8 (B), 12 (C), and 24 (D) after inoculation with the pathogen. At day 24, central corneal clouding and neovascularization toward the center of the cornea were evident. No recultivation of pathogen was observed in this case.
in other studies, these groups (low and high dose) show the same effect with immediate administration before or after inoculation of pathogens. Those studies confirmed these findings and concluded that corticosteroid application is contraindicated.

Nevertheless, if low- or high-dose steroid application was begun at day 9 (middle) or 15 (late), no significant difference was observed in the recultivation rate of *C. albicans* compared with that seen in the control group. This leads to the conclusion that antifungal treatment is effective, even during corticosteroid therapy.

In most of the animals receiving combined treatment in our study, significantly lower levels of corneal clouding and neovascularization were detected compared with the control group treated with fluconazole alone (Fig. 6). It is possible that overreactions of the immune system could be reduced with a later additional application of prednisolone. In this regard, additional prednisolone administration (low and high dose) at day 9 was most effective, although if low- and high-dose prednisolone was applied at day 15, it still significantly reduced corneal clouding. Late combined treatment also showed a significantly lower grade of neovascularization at the last experimental day, although the difference was less than at day 9. We therefore conclude that starting the combined treatment after approximately 1 week is best, to modulate the response of the immune system. After approximately 2 weeks, the additional treatment is not sufficient to have an effect on overreactions of the immune response.

A previous study has shown that low-dose corticosteroid does not influence isolate recovery rates or efficacy of antifungal drugs, when the additional subconjunctival injection of low-dose corticosteroid commenced at day 3 after infection. However, our present study does not completely confirm those findings. In our investigation, early low- and high-dose prednisolone showed significantly greater pathogen recultivation. We found no differences in recultivation of the remaining pathogen with additional low- or high-dose corticosteroid administration when applied at days 9 or 15 after inoculation.

Early high-dose prednisolone treatment resulted in an immediate reduction in corneal clouding and neovascularization. After 2 weeks, an increase was observed in these parameters (Fig. 7) that was comparable with the level seen in the control group (treatment with fluconazole alone). These results correspond with those of O’Day et al., who demonstrated that inflammation was equivalent or significantly less with early corticosteroid administration until day 10 after infection. After day 15, inflammation in corticosteroid-treated corneas was significantly worse than in animals without corticosteroid application.

Early prednisolone treatment is therefore not recommended, although anti-inflammatory responses initially decrease. However, in the time course in these groups, the pathogens remained, as positive recultivation revealed. Furthermore, inflammatory responses, corneal clouding, and neo-
vascularization almost reached the levels exhibited in eyes treated with fluconazole alone.

In conclusion, the administration of additional prednisolone starting at day 9 after inoculation was clearly the most effective treatment to reduce corneal clouding and neovascularization in this rabbit model (Fig. 8). Most important, in these groups, the recultivation of pathogens was comparable with that of the control group treated with fluconazole alone. Overall, high-dose prednisolone was more effective in our experimental study.

Based on these experimental data, the combination of immediate antimycotic therapy with delayed corticosteroid may be beneficial in the treatment of fungal keratitis in human studies. However, clinical application in patients should be determined individually in all cases.

References

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