Topical fluconazole for experimental candida keratitis in rabbits

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Abstract
Using a reproducible model of Candida albicans keratitis in rabbits we studied the effect of topical fluconazole, a new triazole. Candida albicans DSM 70010 (2.5×10^6 cells) was injected into the corneal stroma of both eyes of 21 rabbits. All eyes developed a corneal ulcer. Forty-eight hours after inoculation the animals were divided into three groups: (1) 14 eyes, received fluconazole (2 mg/ml) and the epithelium subsequently removed; (2) 14 eyes, received only fluconazole drops; (3) 14 eyes, received 0.9% NaCl: half of this group was also debrided. We applied one drop of either substance 10 times a day for 24 days. A further six rabbits were used to judge if the drug penetrated into the cornea and aqueous humour. There was a highly significant difference between the fluconazole groups (1, 2) and the control group (3) as to hypopyon and complications (descemetocoele, corneal perforation) as well as recultivation of C. albicans from corneal tissue. The difference between the fluconazole groups with and without debridement was not significant. The drug penetrated into the cornea and aqueous humour of both uninflamed and inflamed eyes.

Fungal infections of the cornea have markedly increased during the last decades. Apart from polyenes such as amphotericin-B, nystatin, and pimaricin (natamycin), azoles such as the imidazoles and ketoconazole have been applied to fungal keratitis. The azoles proved effective and were non-toxic to the eye. However, the treatment of clinical keratocymes is still inefficient.

Fluconazole (2-(2,4,-difluorophenyl)-1,3-bis (1 H-1,2,4-triazol-1-yl) propan-2-ol; UK 49-858) is a new triazole compound (molecular weight 306:3) which has certain properties suggesting its use for the treatment of fungal keratitis. It has been reported to be more efficient than ketoconazole in systemic candidosis of immuno-suppressed as well as normal mice. Moreover, fluconazole is the only antifungal azole derivative which penetrates into the cerebrospinal fluid.

Parenterally administered fluconazole under experimental conditions was shown to penetrate freely into ocular tissues.

This is the first report on the use of topical fluconazole in experimental Candida albicans keratitis in rabbits. We also studied penetration of the drug into the cornea and aqueous humour.

Material and methods
Bioavailability. One drop of aqueous fluconazole solution (2 mg/ml) was applied to both eyes of six rabbits every hour for a period of eight hours. On three animals the corneal epithelium had been gently removed with a hockey knife under a microscope. Fifty minutes after the last treatment the eye was irrigated with saline.

Aqueous humour samples (0-2 to 0-3 ml) were obtained by puncture of the anterior chamber with a 26 gauge needle. Corneal samples were excised at the limbus with scissors and ground in a tissue grinder (Ultra-Turrax, Janke and Kunkel, Staufen, Germany). These samples were centrifuged at 12,000 rpm.

Serial dilution tests of both the aqueous humour samples and the supernatant of the corneal samples were performed on microplates. A 100 μl candida suspension (10^6 blastoconidia/ml) from a 24 h culture in glucose peptone broth containing 50 μg/ml gentamicin sulphate was added to 50 μl of the samples. The plates were incubated for 24 hours at 37°C. The minimal inhibition concentration (MIC) of fluconazole was determined by serial dilution under comparable conditions.

Inoculum. We used Candida albicans DSM (German Collection of Micro-organisms) No 70010, which shows filamentous growth in aqueous humour. Yeast cells from a 24 h culture in Sabouraud agar were suspended in peptone broth, counted with a haemacytometer, and adjusted to a concentration of 2.5×10^6 cells/l.

Animals. Twenty-one male, pigmented, inbred rabbits, weighing approximately 2.5 kg each were used. No pretreatment was performed.

Inoculum procedure. This method has been described previously. The animals were anaesthetised with intramuscular xylazin and ketamine, 5 mg/kg body weight. To avoid preservatives no local anaesthetic was applied. Under an operating microscope a 27 gauge needle was inserted into the central corneal stroma to a depth of about one-half of the corneal thickness. A 10 μl inoculum, containing 2.5×10^6 cells, was injected in both eyes. No reflux of the inoculum was observed. If penetration into the anterior chamber occurred, the animal was removed from the study.

Therapy. Forty-eight hours after inoculation the animals were randomised into three groups (14 eyes of 7 animals each). In groups 1 and 2 aqueous fluconazole solution (2 mg/ml 0.9% NaCl) and in group 3 0.9% NaCl was administered. In addition, the epithelium of the eyes in group 1 was gently removed. To exclude any mechanical effect of debridement the same was done in half of those in the control group 3. This procedure was done every third day with a hockey knife under a microscope. All eyes received a daily treatment of 0.5% aqueous.
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gentamic sulphate (without preservatives) to avoid a bacterial infection.

Evaluation of ocular infections. All eyes were examined every day and a standardised photographic record was made. The occurrence of a descemetocele, corneal perforation, and hypopyon was noted.

Fungal recultivation. After 24 days the animals were killed by intravenous thiopentone sulphate. Aqueous humour samples were obtained from all eyes to study the penetration of fluconazole into inflamed eyes. Serial dilution tests for antifungal activity were performed as described above. The cornea was excised at the limbus and divided through the centre of the ulcer. The cut surfaces of both sections were pressed on to Sabouraud agar containing 50 µg/g gentamic sulphate and the plates incubated for one week at 21°C. Positive cultures were plated on blood agar and incubated for 48 hours at 37°C for further differentiation. These cultures were subjected to an auxanogram12 (API-20-C) to establish the presence of C. albicans.

Results
Bioavailability. Penetration of fluconazole into the cornea and aqueous humour of uninflamed eyes is shown in Table I. There is a moderate difference between eyes with and without corneal epithelium. In inflamed eyes (24 days after infection) the results of aqueous humour samples are the same as in non-inflamed eyes.

Course of infection and complications. All 42 eyes inoculated with Candida albicans DSM 70010 developed corneal infiltrates on day 2 with an average size of 3 x 3 mm. These infiltrates progressed to an ulcer between days 5 and 8. The incidence of hypopyon and complications (descemetocele or corneal perforation) is shown in Table II for each group. There was a marked difference between the therapy groups (1 and 2) and the control group (3a and 3b) in the occurrence of descemetoceles or perforations (p<0.01; χ² test). Figures 1-4 show typical corneal infections of each group on day 21.

The drug was well tolerated by all animals. No adverse effect could be observed.

Recultivation. Table III lists the results of Candida albicans recultivation. In group 1 no viable yeasts were recovered. In group 2, two of 14 samples showed candida growth. These results are highly significant compared with that of group 3a+b (p<0.01; χ² test).

Discussion
Most antifungal drugs are effective against superficial mycoses but are less successful in counteracting deep mycotic infiltrations. In particular, the treatment of keratomycosis is often frustrating owing to limited tissue penetration, narrow antimicrobial spectrum, and toxicity of the antifungal agents currently available.

The new triazole antifungal fluconazole, by virtue of its pharmacological properties, offers a fresh opportunity for the topical treatment of keratomycosis. The drug acts against all pathogenic Candida species except C. krusei13 and hence encompasses the majority of fungi which are notorious as causative agents of keratomycosis.14-16 Fluconazole is exceptionally hydrophilic, which is reflected by low protein binding and high penetration into cerebrospinal fluid, as well as by intestinal absorption and renal excretion.17,18 In rabbits fluconazole was shown to reach ocular tissues after intravenous administration.19 In addition fluconazole was shown to be non-mutagenic and less toxic than the other azoles.20

In the present study we used a rabbit model to investigate the potential of fluconazole in the therapy of deep keratitis due to C. albicans. The model is reproducible without the need of immunosuppressive pretreatment.1 Therapy is

Table I: Penetration of fluconazole into the rabbit cornea and aqueous humour of uninflamed eyes. Growth of C. albicans was inhibited by the indicated dilutions of corneal homogenate or aqueous humour.

<table>
<thead>
<tr>
<th>With debridement</th>
<th>Without debridement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal homogenate</td>
<td>1:4</td>
</tr>
<tr>
<td>Aqueous humour</td>
<td>1:8</td>
</tr>
</tbody>
</table>

Table II: Influence of topical fluconazole on hypopyon, descemetocele, or perforation in experimental candida keratitis.

<table>
<thead>
<tr>
<th>Eyes</th>
<th>Hypopyon</th>
<th>Descemetocele perforation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (fluconazole + debridement)</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Group 2 (fluconazole without debridement)</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>Group 3a (0.9% NaCl + debridement)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Group 3b (0.9% NaCl without debridement)</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Figure 1: Candida keratitis of group 1 (fluconazole + debridement) on day 21.

Figure 2: Candida keratitis of group 2 (fluconazole without debridement) on day 21.
started only on day 2, when stromal keratitis is manifest; hence the model more closely parallels human corneal candidiasis than a regimen which begins treatment one hour after infection.21

Our results demonstrate the efficacy of fluconazole in the topical therapy of experimental candida keratitis. As to complications such as descemetocele and corneal perforation, there was a highly significant difference between the treated eyes and the controls. The beneficial effect was confirmed because almost no recultivation of C. albicans was possible after 24 days of treatment.

However, no significant difference between the debridement and no-debridement groups was found. This finding contrasts with results from similar experiments using amphotericin B, which revealed a highly significant difference in favour of debrided eyes.22 The effect of debridement on the penetration of amphotericin B reflects its higher molecular weight (924-11), since the limiting size of molecules for diffusing into the normal cornea is about 500-00.23

We conclude that fluconazole proved highly effective in the therapy of experimental candida keratitis. If debridement of the corneal epithelium is undesirable, this drug seems to be superior even to amphotericin B.22