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Evaluation of the use of antifungals in mycotic keratitis by *in-vitro* efficacy study of natamycin, fluconazole and itraconazole

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Ophthalmic preparation of 1% fluconazole and 5% natamycin are available for the treatment of mycotic keratitis, which contain much higher concentrations of drugs than requires for inhibition of most isolates by *in vitro* study. Itraconazole is another prospective drug which is highly effective in systemic mycosis caused by similar fungi and also occasionally tried successfully by oral or 1% topical preparation, in mycotic keratitis. *In vitro* tests by broth dilution method were done using available preparations of three drugs, against 9 strains of *Aspergillus flavus* and 3 strains of *Fusarium solani* isolated from treatment refractory cases of mycotic keratitis along with 3 pre-treatment isolates of *Aspergillus flavus*. MIC range of itraconazole was 0.5 to 2 µg/ml for *Aspergillus* sp. and 2-8 µg/ml for *Fusarium* sp.: of fluconazole it was 2-8 µg/ml respectively and of natamycin 2-16 µg/ml and 4-32 µg/ml respectively. MIC values indicated that their treatment refractoriness was not due to drug resistance but due to poor penetration of drugs to the deeper stroma of avascular cornea. The superiority of itraconazole over other two was also supported by *in vitro* study results.

Key words : Mycotic keratitis, antifungals, sensitivity test

INTRODUCTION

Opportunist fungi from exterier environment can colonise on traumatised cornea leading to mycotic keratitis. Here fungi usually grow in varying depth of sub-epithelial stratum, rarely penetrate into deeper plane. Because cornea is an avascular structure, blood is not the prime source of nutrient or oxygen for fungi ; only minimum nutrient is derived from tears and damaged cells where as oxygen from atomosphere across the tears. Thus a slow growth of fungus is possible there. Absence of vascular defence mechanisms favours the opportunistic growth which in case of skin surface restricts easy invasion of such fungi.

The agents of mycotic keratitis are exclusively filamentous fungi, *Aspergillus* being the commonest species (Foster, 1992; Srinivasan *et al.*, 1989) followed by *Fusarium, Penicillium* and a number of other nondematiaceous or dematiaceous fungi. As there is best scope of direct application of drugs, ophthalmic antifungal preparations which are poorly absorbed from eye, are used in a high tolerable concentrations. Natamycin (5%) and fluconazole (1%) are two ophthalmic preparations available at present and contain at least 100 times higher concentrations than Minimum Inhibitory Concentrations(MIC) of most isolates. Yet failure of treatment with such drugs is not uncommon. So question arises about their efficacy at such mode of application. Soon after application the drug concentration falls steadily by dilution effect of tears with each blinking, so in many cases drugs may not penetrate into deeper stratum at a sufficient concentrations.

Considering the efficacy of itraconazole in systemic mycoses, it should be the drug of choice for most of the keratomycotic agents (Speight and Holford, 1997). Unfortunately topical preparation of itraconazole is not available. Yet some report of prospective trials (Thomas *et al.*, 1988) show greater success by use of oral itraconazole or 1% suspension (Abad and Foster, 2000).

Although sensitivity tests with filamentous fungi are not yet standardized (NCCLS Report, 1997) comparative efficacy of drugs can be studied against some treatment responsive and treatment refractory isolates. Results of *in vitro* drug

sensitivity tests often do not corroborate with *in vivo* efficacy of systemic mycoses caused by same group of fungi, yet such study may have some predictive value in keratitis, because drugs act here directly on fungi, remaining unmodified by host's system and also non-interfering host's defence system for their indirect mechanism of action (Perfect *et al.*, 1985).

MATERIALS AND METHODS

Organisms

The fungi used in the study were isolated from fungal keratitis cases attending Medical Mycology Department, Calcutta School of Tropical Medicine, for investigation, during May, 1999 to April, 2000. Out of 26 isolates, 9 *Aspergillus flavus* were taken, 3 each from natamycin, fluconazole and itraconazole non-responsive groups and 3 other isolates included from *Fusarium solani*, one each from three antifungal non-responsive groups. Three pre-treatment isolates of itraconzole non-responseve *A. flavus* were also included. Cases which did not show subjective improvement even after 2 weeks topical application of an antifungal, were considered as treatment refractory cases.

Inoculum

The inoculum was prepared from 10-14 days culture on Sabouraud's dextrose agar (SDA). Sterile distilled water containing 0.05% Tween 80, was added to the surface growth. Spores and hyphae were scraped off with a sterile bent wire loop. Fungal suspension was gently centrifused for 2 minutes at 500 r.p.m. The spore count in upper supernatant was done in counting chamber and final suspension was prepared by concentration of supernatant to a density which may contain about $1x10^6$ spores per ml, by centrifugation and resuspension.

Medium

6.7 g of Yeast Nitrogen Base (Difco) and 10 g of glucose were dissolved in 100 ml of distilled water. The solution was filter sterilized and storred at 40° C. Stock 100 ml Yeast Nitrogen Base Glucose (YNBG) solution was diluted with 900 ml sterile pH 7.0 phosphate buffer for working purpose.

Preparation of drug solutions

For the special purpose of testing usefulness of ophthalmic preparation of antifungals, topical 5% natamycin (Pimafucin, Elder), 1% fluconazole (Zocon, FDC) were taken as stock solution. Contents of itraconazole 100 mg capsule (Canditral, FDC) was dissolved in 10 ml Di-methyl sulphoxide (DMSO) and allowed to stand for 30 mins at room temperature to permit self sterilization. This was equivalent to 1% suspension used by some clinicians, mixing 100 mg capsule in 10 ml artificial tears and was used as stock 1% itraconazole solution. Working solutions were prepared diluting 4 ml of each stock solutions in 25 ml sterile diluted YNBG broth, then 4 ml of prepared solutions were again diluted with 25 ml sterile diluted YNBG broth. These working dilution of fluconazole and itraconazole contained 256 mg/ml drugs. Natamycin solution was further diluted five fold in YNBG broth to obtain 256 mg/ml concentration.

Test methods

Antifungal sensitivity tests were done using broth dilution method (Warnock, 1989). In 11 sterile capped tubes (110 × 16 mm) in rack (numbered 1 to 11) for each antifungal, diluted YNBG solution was added to each tube. One ml of 256 mg/ml drug solution was added to tube 1. Contents were mixed and transferred 1 ml to tube 2. The serial dilution process was repeated upto tube 9 and 1 ml was discarded from tube 9. This gave a set of doubling drug dilutions from 128 mg to 0.5 mg/ml. Fifty ml of the standardized inoculum was added to each of 1 to 10 tubes and contents were mixed. Tube 10 served as medium control and tube 11 as inoculum control. Tubes were incubated at 37^0C untill growth was visible in medium control. The lowest drug concentration at which there was no visible fungal growth, was taken as MIC of the drug in respect of test fungus. Thus MIC values of all three drugs for test strains were determined. Results were compared with *in vivo* efficacy.

RESULTS AND DISCUSSION

For several reasons, results of *in vitro* antifungal sesitivity tests for filamentous fungi are poor guide to anti-microbial usefulness. The reliability and reproducibility of the results are often greatly influenced by low solubility of drug in the medium and by composition of medium pH and inoculum size (Odds *et al.*, 1984). Yet MIC values of drugs for any pre and post treatment isolates, in identical

test conditions can be a good index for evaluation of therapy and test itself. MIC values of a particular drug with different test strains or such values of different drugs against a particular fungus can be compared for prediction of therapeutic usefulness. With this object therapeutic success or failure of three commonly used topical antifungals were evaluated analysing MIC values. Results are showen in Table 1.

Strippoli *et al.* (1988) determined 6.25 to 12.5 μg/ml concentration of itraconazole which completely inhibited spore germination in all test strains of *Aspergillus sp.* Grant and Clissold (1989) also reported same result. Conversely, in the agar dilution study of Espinel-Ingroff *et al.* (1984), MIC_{90} of itraconazole for *Aspergillus sp.* was 0.13 μg/ml. MIC range of itraconazole as determined in brain-heart infusion broth (Van Cutsem, 1989) was 0.001 to 1 μg/ml for *Aspergillus sp.* and 0.01 to 100 μg/ml for *Fusarium* sp. In our study MIC ranges were 05 to 2 μg/ml and 2 to 8 μg/ml itraconazole for *Aspergillus* and *Fusarium sp.* respectively, while 2 to 8 μg/ml and 4 to 8 mg/ml respectively for fluconazole and a range of 2 to 16 and 4 to 32 μg/ml respectively for natamycin. Surprisingly three pre-treatment and post-treatment isolates of *Aspergillus flavus* showed no great difference of their corresponding MIC values. Probably their treatment refractoriness was due to poor drug permeability to the target than to acquired resistance of the drug.

Table 1 : MIC valuses of itraconazole, fluconazole and natamycin against keratomycotic agents

Fungal strains	Treatment before isolation	MIC values (μg/ml) I	F	N	Drug responded
Aspergillus flavus 1 (a)	Nil	1	4	8	—
Repeat isolate : 1 (b)	I	1	4	16	N
A. flavus, 2 (a)	Nil	0.5	8	4	—
Repeat isolate : 2 (b)	I	0.5	4	8	N
A. flavus, 3 (a)	Nil	1	2	4	—
Repeat isolate : 3 (b)	I	2	4	8	N
A. flavus, 4	N	1	8	16	I
A. flavus, 5	N	1	4	2	I
A. flavus, 6	N	0.5	4	8	I
A. flavus, 7	F	0.5	2	4	I
A. flavus, 8	F	0.5	8	2	I
A. flavus, 9	F	1	8	8	I
Fusarium solani, 1	F	2	8	4	I
F. solani, 2	N	8	8	32	I
F. solani, 3	I	4	4	4	N

I = Itraconazole, F = Fluconazole, N = Natamycin.

Because in the treatment of mycotic keratitis drug concentration at the target site is not related with plasma concentration of the drug, the term "sensitive" or "resistant" should be considered in term of efficacy at the highest tolerable concentration of the drug. Available topical preparation of fluconazole contains 10000 μg/ml drug and natamycin contains 50000 μg/ml, which are so high that theoretically all common keratomycotic agents should be inhibited by direct application of such drugs. Their therapeutic failure can not be explained by pharmacodynamics but can be explained by pharmacokinetics.

Eradication of fungus from affected cornea essentially depends upon permeation of antifungals into deeper stratum of cornea. Most topical antifungals can cross intact epithelium poorly and very slowly diffuse into stratum. So therapeutic success partly depends upon extent of pithelial breach and depth of fungal invasion. 0' Day *et al.* (1986) showed better penetration of amphotericin B and natamycin by daily scraping of epithelium and anterior stroma. The fungal growth within layers of stroma are very slow, because tissue environment itself is inhibitory, having no steady supply of nutrient or oxygen from blood. So the rate of sterol synthesis by fungus during growth within cornea is much lower than rate of sterol synthesis in any high nutrient test media. This can be a reason for therapeutic success of most sterol synthesis-inhibitors, even at a much lower corneal concentration of drugs than MIC values.

In the treatment of experimental candida-endophthalmitis (Savani *et al.*, 1987) itraconazole was as effective as fluconazole, despite poorer penetration of itraconazole in ocular tissues than excellent penetration of fluconazole. The workers evaluated penetration of itraconazole by bioassay in rabbits giving 80 mg orally. Serum levels were 2.3 μg/ml and levels of 0.05 mg per g in the cornea, 0.92 μg/ml in aqueous humor and 0.22 μg/ml in the vitreous body were obtained in the inflamed eye. Itraconazole was not detected in the tissues of the uninflamed eye, apart from 0.05 μg per g in the cornea. This indicates that following systemic use, the drug probably concentrates at avascular cornea through tears. This view is also supported by prospective study of oral itraconazole in human

trials. Thomas *et al.* (1988) treated 40 patients with oral itraconazole 200 mg once daily and 80% cure was achieved in aspergillus-keratitis. In human volunteers achievable plasma concentration of the drug following 100 to 200 mg daily oral dose was 0.34-0.5 µg/ml (Cauwenbergh, 1988). So a sustained application of drug through tears even at much lower concentration than topical drugs can also bring almost equal therapeutic success. Such informations obviate usefulness of *in vitro* sensitivity tests for mycotic keratitis.

Results of *in vitro* efficacy study only should be the guideline for selection of appropriate drug. Considering the superiority of itraconazole over fluconazole in the treatment of systemic mycosis caused by common filamentous fungi, prospect of itraconazole in the treatment of mycotic keratitis should be evaluated, particularly when oral itraconazole has shown promising results. Simultaneous use or oral and topical itraconazole can effect prompt recovery of such cases and can also guard inapparent deeper infection beyond cornea. Use of appropriate adjuvant which can promote permeation of drugs into deeper stroma, may prove useful in the treatment of such cases. Hyaluronidase can be a prospective adjuvant for such use.

Reuben *et al.* (1989) showed poor inhibitory and fungicidal activity of itraconazole against 44 clinical isolates of *Fusarium sp.*. Forster *et al.* (1975) found natamycin to be effective in 80% of fusarium infection and 60% of infection caused by other nonpigmented filamentous fungi. As fusarium infection is less common in India, oral and topical itraconazole can be the first line therapy of mycotic keratitis here, and as soon as fungal identity will be disclosed or nonresponsive to therapy will be suspected, topical natamycin can be started.

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