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Original Article / *Klinik Çalışma - Araştırma*

The Effect of Combinations of Antimycotics on Systemic Candidiasis in a Mouse Model

Sistemik Fare Kandidoz Modelinde Antifungal Kombinasyonlarının Etkinliği

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Objective: This study was planned to investigate the therapeutic efficacy of terbinafine and interaction between terbinafine with amphotericin B, and fluconazole on candidiasis in a mouse model.

Material and Methods: Treatment with amphotericin B (1mg/kg/day intraperitoneally), fluconazole (100 mg/kg/day ip), terbinafine (100 mg/kg/day by oral gavage) and combinations of terbinafine with amphotericin B and terbinafine with fluconazole at the same doses began 24 h after infection and continued for 10 days, and kidney cultures were performed.

Results: No significant improvement in survival was not found between the control and the terbinafine group (p>0.05). With the addition of amphotericin B to terbinafine, significant improvement in survival was found compared with the survival of untreated controls (p<0.0001). When compared with the control group, the kidney culture results of the amphotericin B group were superior to those with two-fold reduction in CFU counts (p<0.05), but the difference between the terbinafine group and the control group was not significant (p>0.05). Terbinafine with amphotericin B and terbinafine with fluconazole combinations, when compared by fungal density reduction with amphotericin B, were less effective and the difference was significant (p<0.0001).

Conclusion: Terbinafine had no effect on controlling systemic candidiasis alone and a slight effect in combination with amphotericin B and fluconazole.

Key words: Candidiasis; combination; mice; terbinafine.

Amaç: Bu çalışma fare sistemik kandidoz modelinde terbinafin ve terbinafin- amphotericin B ve terbinafin-flukanazol etkinliğinin araştırılması için planlandı.

Gereç ve Yöntemler: Enfeksiyon oluşumundan 24 saat sonra amphotericin B (1mg/kg/gün ip), fluconazole (100 mg/kg/gün ip), terbinafin (100 mg/kg/gün oral gavaj) ve terbinafin ile amphotericin B ve terbinafine ile fluconazole'ün aynı dozlarda kombinasyonlarının uygulandığı 10 günlük tedavi başladı, ve böbrek kültürleri yapıldı.

Bulgular: Yaşamı sürdürme bakımından kontrol grubu ile terbinafin grubu arasında fark gözlenmedi (p>0.05). Terbinafin tedavisine amphotericin B eklenmesi ile kontrol grubu ile arada anlamlı fark oluştu (p<0.0001). Böbrek kültür sonuçlarının değerlendirilmesi sonucunda amphotericin B grubu kontrol grubu ile değerlendirildiğinde ∫n CFU sayısındaki iki kat düşüş ile en etkili grup (p<0.05) iken terbinafin ve kontrol grubu arasında fark gözlenmedi (p>0.05). Terbinafin-amphotericin B ve terbinafin-fluconazole kombinasyonlarında fungal yoğunluk düşüşü bakımından amphotericin b ile karşılaştırıldığında daha az etkili oldukları gözlendi (p<0.0001).

Sonuç: Terbinafinin sistemik kandidozda tek başına etkili olmadığı ve amphotericin B ve fluconazole kombinasyonları ile de ancak önemsiz sayılabilecek etkinliğe sahip olduğu gözlendi.

Anahtar sözcükler: Kandidoz; fare; terbinafin; kombinasyon.

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Recently, it was reported that 10% of all nosocomial bloodstream infections were due to fungi, particularly *Candida albicans*.[1] Amphotericin B has traditionally been considered the cornerstone of therapy for deep-seated fungal infections and fungemia, but it has toxic side effects.[1-4] Despite advances, antifungal therapy with amphotericin B, the azoles or flucytosine has not been uniformly successful. Problems with toxicity, the emergence of resistant strains of *Candida spp.* and the lack of activity have been recognized.[5-7] This has increased the interest in using new drugs and drug combinations to enhance the efficacies of the drugs.[3,7-9]

Terbinafine is the first oral antimycotic in the allylamines class that inhibits ergosterol synthesis at the stage of squalene epoxidation.[10-12] Terbinafine demonstrates excellent in vitro activity against many fungal species.[10] Barchiesi et al.[6,8] demonstrates in two studies that in vitro activities of terbinafine in combination with amphotericin B, fluconazole, and itraconazole showed excellent activities. However, other authors maintained that clinical studies were necessary to elucidate further the potential utility of these combination therapies. Howeve, a few reports have suggested that terbinafine may be effective for the treatment of systemic fungal infections. Sorensen et al.[13] tested terbinafine and fluconazole in the treatment of experimental coccidioidal meningitis in a rabbit model. They found a slight effect on survival, histology, and reduction of the numbers of CFU in tissue, but these effects were not significant, even though the authors found *in vitro* susceptibility of the organism to terbinafine. *In vitro* data have documented the occurrence of enhanced activity of amphotericin B and triazoles when combined with terbinafine, but *in vivo* correlation has not been established.[7]

In the study described here, we used a well-described murine model of invasive candidiasis to evaluate potential interactions between terbinafine by amphotericin B and terbinafine by fluconazole in the treatment of invasive candidiasis. In this mouse model, a clinical isolate of *C.albicans* from the blood of a neutropenic patient was used.

MATERIALS AND METHODS

Female BALB/c mice (age, 10 to 12 weeks; weight, 20 to 25g) were raised at the Animal Research Facility of the Erciyes University, Hakan Çetinsaya Experimental Research Laboratories (Kayseri, Turkey) They were housed in cages at four or five animals per container. They received food and water ad libitum. All animal experimentation procedures were approved by and conducted in the quidelines of the Institutional Animal Care and Use Committes of the Erciyes University Medical Faculty.

A clinical isolate of *C.albicans* which was isolated from the blood of a neutropenic patient was used. The organism had been identified by standard methods.[14] When needed for an experiment, blastoconidia were grown 48 h on fresh Sabouraud dextrose agar slants.[15] Blastoconidia were harvested and washed three times with sterile saline, and counted in a hemocytometer.[14-16] The dose was adjusted to ~$4x10^6$ CFU / mouse in 0.25 ml of sterile saline for the study.[15] The mice were infected by injection of this inocula into a lateral tail vein.[2,3,15]

Amphotericin B (Fungizone, Bristol-Myers Squibb), and fluconazole (Triflucan 100mg, Pfizer) were used, and prepared for injection as recommended by the manufacturer and terbinafine (Lamsil, Novartis) dissoluting in polyethylene glycol 200 and diluted in sterile distilled water.[17,18]

The MICs for amphotericin B, fluconazole and terbinafine were determined by the broth microdilution method described by the National Committee for Clinical Laboratory Standards M27 A.[19] The median MIC of fluconazole, amphotericin B, and terbinafine after 48 h incubation were 4, 0.5, and 8 μg / ml respectively.

The animals were divided into 6 groups, each of the group containing 10 mice. Beginning 1 day after infection, one group remained untreated (the control group), three other groups were treated with fluconazole (Pfizer) at 100mg / kg / day in 0.2 ml i.p., with amphotericin B 1 mg / kg / day ip, and with terbinafine 100mg / kg / day by oral gavages.[1,2,13,17] The remaining two groups were treated with terbinafine with amphotericin B and terbinafine with fluconazole at the same doses. Treatment was continued up to day 10. For survival, the study groups were observed through day 12. For studies of tissue burden, groups were sacrified on day 12 after infection. Animals death before day 12 were not considered in the quantitation of tissue cultures. The lack of tissue cultures for these animals in order to determine the pathogens out of candia was the limitation of this study. The right kidney was removed aseptically, homogenized in 2 ml of saline, in a specified homogenizer, and serial 10-fold dilutions plated on SDA slants containing 80 mg of chloramphenicol / ml and 100 IU penicillin / ml to eliminate bacterial croos-contamination, incubated at 30°C for 48 h, and then colonies were counted.[1,2,5,9] The culture-negative plates were counted as having 0 CFU / g. The entire organ was plated when we achieved very low counts with serial dilutions.[16]

Comparisons of colony counts among the different treatment groups were performed by one-way ANOVA test correction with multiple comparisons followed by Post Hoc Scheffe procedure. Differences in survival were assessed by Kaplan-Meir analysis. A p value of <0.05 was considered statistically significant.

RESULTS

The survivals of the various treatment groups are shown in Fig 1. Overall, three of ten untreated mice survived to the end of the study, whereas four of ten, ten of ten, ten of ten, ten of eight, ten of six mice survived in groups treated by terbinafine, amphotericin B, fluco-

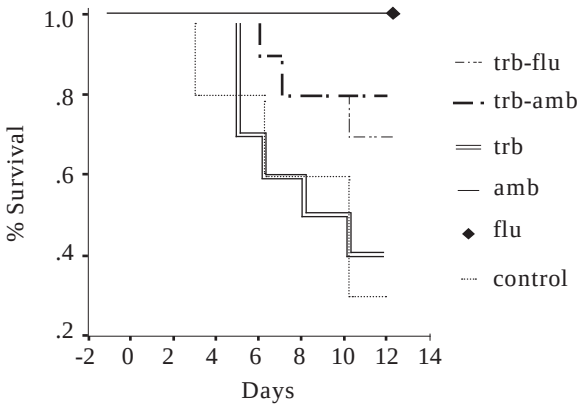

Figure 1. *Survival was not affected by Trb monotherapy (p>0.05). AmB and Flu as monotherapy were the most active regimens (p>0.05). The combinations of Trb+AmB, and Trb+ Flu were superior to Trb monotherapy, and controls (p<0.0001), but when compared with the treatment of AmB alone, significant improvement was not found (p>0.05).*

nazole, terbinafine with amphotericin B and terbinafine with fluconazole, respectively. Survival was not affected by terbinafine treatment, and there was only a suggestion of efficacy (p>0.05). Amphotericin B and fluconazole as monotherapy were the most active regimens. The combinations of terbinafine with amphotericin B, and terbinafine with fluconazole were superior to terbinafine monotherapy, and controls (p<0.0001), but when compared with the treatment of amphotericin B alone significant improvement was not found (p>0.05).

The same nonsignificant suggestion of efficacy seen from the survival of terbinafine treated animals was also seen in the reduction of *C. albicans* in the kidneys (p>0.05), (Table 1). A maximum effect was obtained with amphotericin B which reduced twofold ∫n CFU counts in the kidney, relative to the control group (p<0.0001) (Table 1). However, terbinafine with amphotericin B and terbinafine with fluconazole were less effective in reducing fungal density, relative to amphotericin B (p<0.0001), and also the difference between these combinations in fungal density reduction with controls was statistically significant, too (p<0.0001). The difference between combinations; amphotericin B with terbinafine and fluconazole with terbinafine were statistically not significant (p>0.05).

DISCUSSION

We investigated the *in vivo* interactions of fluconazole and amphotericin B with the most active allylamine derivative, terbinafine. Terbinafine alone was ineffective in our mouse model. The effects of terbinafine with amphotericin B and terbinafine with fluconazole were demonstrable by measuring survival over a 12 day period, and cultures of the major organ in this model, the kidney.

Barchiesi et al.[8] tested fluconazole, itraconazole and amphotericin B combined with terbinafine *in vitro* against thirty clinical isolates of *C. albicans*. These authors found that terbinafine enhances the activities of amphotericin B and triazols against *C. albicans in vitro*. They also emphasised that clinical studies are warranted to further elucidate the potential utility of these combination therapies. In this study we found that *in vivo* amphotericin B and fluconazole enhance the activity of terbinafine against *C. albicans* at these doses. Conti et al.[20] tested terbinafine in a rat model of *Pneumocystis carinii* pneumonia. These authors found significant clearance of *P.carinii* when terbinafine was given at 80mg / kg / day. Sorensen et al.[13] tested terbinafine and fluconazole in the treatment of experimental coccidioidal meningitis in a rabbit model. They found a slight effect on survival, histology, and reduction of the numbers of CFU in tissue, at the dose of 200mg / kg / day. However these effects were not significant, even though the authors found *in vitro* susceptibility of the organism to terbinafine. We found a modest

Table 1. Quantitative kidney culture results of the mice treated by AmB, Flu, Trb, Trb+Amb, and Trb+Flu after 12 days of treatment a. The difference between controls and Trb group was statistically not significant (p>0.05). A maximum effect was obtained with AmB monotherapy, which reduced twofold ∫n CFU counts in the kidney, relative to the control group (p< 0.0001). However, Trb+AmB and Trb+Flu were less effective in reducing fungal density, relative to AmB (p<0.0001) and also the difference between these combinations in fungal density reduction with controls was statistically significant (p< 0.0001).

Drug	Dose (mg / kg / day)	Delivery route	Survivors	Organ load Ln Cfu / g
AmB	1	ip	10 / 10	4.05±0.62
Flu	100	ip	10 / 10	4.98±0.21
Trb	100	po	10 / 4	8.39±0.18
Trb+Amb	100	ip / po	10 / 8	6.81±0.13
Trb+Flu	100	ip / po	10 / 6	7.06±0.37
Control	-	-	10 / 3	8.71±0.24

[a]Amphotericin B (AmB) intraperitoneally (ip), fluconazole (Flu) intraperitoneally (ip), terbinafine (Trb) oral gavage (po).

effect on survival, and a reduction in the numbers of CFU in tissue, at the dose of 100 mg/kg/day, however these effects are not significant. It may because 100 mg/kg/day terbinafine was toxic, even if an 80 mg/kg/day dose was not. Concerning this, Walzer et al.[17] tested terbinafine in mouse and rat models of *Pneumocystis carinii* pneumonia in doses of 20-50-150 mg/kg/day in mice and 50-250 mg/kg/day in rats. The authors found that these therapies were ineffective.

Our data showed that amphotericin B, and fluconazole enhances the activity of terbinafine, in our murine model of systemic candidiasis but not as effective as amphotericin B or fluconazole monotherapy. Furthermore, terbinafine has a quite different distribution from fluconazole and amphotericin B in the body, so that the partners are not present in the internal organ (kidney) in appropriate concentrations. This may be adjusted by the selection of the dose.

In conclusion, careful consideration should be given before these combinations are used in the treatment of patients with candidiasis.

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Conflict of Interest

No conflict of interest declared by the authors.

REFERENCES

1. Loui A, Banerjee P, Drusano GL, et al. Interaction between fluconazole and amphotericin B in mice with systemic infection due to fluconazole-susceptible or –resistant strains of Candida albicans. Antimicrob Agents Chemother. 1999;43:2841-7.
2. Santangelo R, Paderu P, Delmas G, Chen ZW, Mannino R, Zarif L, et al. Efficacy of oral cochleate-amphotericinB in a mouse model of systemic candidiasis. Antimicrob Agents Chemother. 2000;44:2356-60.
3. Sugar AM, Hitchcock CA, Troke PF, Picard M. Combination therapy of murine invasive candidiasis with fluconazole and amphotericin B. Antimicrob Agents Chemother. 1995;39:598-601.
4. Zarif L, Graybill JR, Perlin D, Najvar L, Bocanegra R, Mannino RJ. Antifungal activity of amphotericin B cochleates against Candida. Antimicrob Agents Chemother. 2000;44:1463-9.
5. Anaissie EJ, Karyotakis NC, Hachem R, Dignani MC, Rex JH, Paetznick V. Correlation between ın vitro and in vivo activity of antifungal agents against Candida species. J Infect Dis. 1994;170:384-9.
6. Barchiesi F, Di Francessco LF, Scalise G. In vitro activities of terbinafine in combination with fluconazole and itraconazole against isolates of Candida albicans with reduced suspectibility to azoles. Antimicrob Agents Chemother. 1997;41:1812-4.
7. Koç AN, Evrensel N, Gökahmetoglu S, Oguzkaya M. The in vitro effects of antifungal agents against Candida albicans. Antibiyotik ve Kemoterapi Dergisi. 2000;14:15-20.
8. Barchiesi F, Di Francesco LF, Compagnucci P, Arzeni D, Giacometti A, Scalise G. In vitro interaction of terbinafine with amphotericin B, fluconazole and itraconazole against clinical isolates of Candida albicans. J Antimicrob Chemother. 1998;41:59-65.
9. Cacciapuoti A, Loebenberg D, Corcoran E, Menzel F Jr, Moss EL Jr, Norris C, et al. In vitro and in vivo activities os SCH 56592 (posaconazole), a new triazole antifungal agent, against Aspergillus and Candida. Antimicrob Agents Chemother. 2000;44:2017-22.
10. Abdel-Rahman SM, Nahata MC. Oral terbinafine: a new antifungal agent. Ann Pharmacother. 1997;31:445-56.
11. Balfour JA, Faulds D. Terbinafine a review of its pharmacodynamics and pharmacokinetic properties, and therapeutic potential in superficial mycoses. Drugs. 1992;43:259-84.
12. Perea S, Gonzalez G, Fothergill AW, Sutton DA, Rinaldi MG. In vitro activities of terbinafine in combination with fluconazole, itraconazole, voriconazole, and posaconazole against clinical isolates of Candida glabrata with decreased susceptibility to azoles. J Clin Microbiol. 2002;40:1831-3.
13. Sorensen KN, Sobel RA, Clemons KV, Calderon L, Howell KJ, Irani PR, et al. Comparative efficacies of terbinafine and fluconazole in treatment of experimental coccidioidal meningitis in a rabit model. Antimicrob Agents Chemother. 2000;44:3087-91.
14. Warren NG, Hazen KC. Candida, Cryptococcus, and other yeasts of medical importance, In Manuel of Clinical Microbiology, 7th edn (P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, & R.H. Yolken, Eds), pp. 1184-1199. American Society for Microbiology, Washington, DC. 1999.
15. Sugar AM, Liu XP. Interactions of itraconazole with amphotericin B in the treatment of murine invasive candidiasis. J Infect Dis. 1997;177:1660-3.
16. Graybill JR, Najvar LK, Luther MF, Fothergill AW. Treatment of murine disseminated candidiasis with L-743, 872. Antimicrob Agents Chemother. 1997;41:1775-7.
17. Walzer PD, Ashbaugh A. Use of terbinafine in mouse and rat models of Pneumocyctis carinii pneumonia. Antimicrob Agents Chemother. 2002;46:514-6.
18. Ghannoum MA, Elewski B. Successfull treatment of fluconazole-resistant oropharyngeal candidiasis by a combination of fluconazole and terbinafine. Clin Diag Lab Immunol. 1999;6:921-3.
19. National Committee for Clinical Laboratory Standarts. Reference method for broth dilution antifungal susceptibility testing of yeast. 1997. Approved standart. Document M27-A. National Committee for Clinical Laboratory Standarts, Wayne, P.
20. Conti C, Angelici E, Canipari R. Structural changes in rat Pneumocystis carinii surface antigens after terbinafine administration in experimental P. carinii pneumonia. J Antimicrob Chemother. 1999;43:301-4.