Effect of Gentamicin to Mic Values of Fluconazole in Fluconazole Resistant Candida Spp.

Flukonazole Dirençli Candida Spp. İzolatlarında, Gentamisinin Flukonazolün Etkinliği Üzerine Etkisi

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ABSTRACT

Aim: Resistance to antifungal drugs contributes to the decrease in the effectiveness of antifungals. Therefore, the combined use of drugs in use with other molecules is considered to be a more realistic method than the discovery of new drugs. In this study, the effect of the combined use of fluconazole with gentamicin on the MIC value of fluconazole resistant Candida isolates of fluconazole was determined, and an ideal combination of fluconazole for the treatment of fluconazole-resistant candida infections was determined.

Method: It was conducted checkerboard assay for identifying the combined effect of gentamicin and fluconazole. In this method, combination efficacy was tested by comparing drugs on a 96-wells plate with this method.

Results: Of the 33 Candida albicans isolates included in our study, 14 (42%) were found to be resistant to fluconazole. Gentamicin alone has not been found to be effective on fluconazole-resistant C. albicans isolates. However, when gentamicin and fluconazole were used together, additive and synergistic effects were observed. At the end of this study, synergistic effect was detected in 2 of 14 samples and additive effect in 11 of them. No antagonistic effect of the drugs on the study isolates was detected.

Conclusion: The proper gentamicin concentrations to increase fluconazole susceptibility were calculated by identifying the effects of gentamicin on fluconazole efficiency in fluconazole-resistant C. albicans isolates, and gentamicin+fluconazole combinations were identified as an alternative.

Keywords: Fluconazole, gentamicin, checkerboard assay, Candida spp.

ÖZET

Amaç: Antifungal ilaçlara karşı gelişen direnç, antifungallerin etkinliğinin azalmasına katkıda bulunmaktadır. Bu nedenle kullanılmakta olan ilaçların başka moleküller ile kombine kullanımları, yeni ilaç keşfine kıyasla daha gerçekçi bir yöntem olduğu düşünülmektedir. Bu çalışmada, flukonazolün gentamisin ile kombine kullanılmasının, flukonazole dirençli kandida izolatlarının flukonazolün MİK değeri üzerine etkisi saptanarak, flukonazol direncini ortadan kaldıran ideal gentamisin konsantrasyonlarının hesaplanması ve böylece flukonazole dirençli kandida enfeksiyonlarının tedavisi için alternatif olabilecek bir flukonazol+antibiyotik kombinasyonu belirlenmesi amaçlanmıştır.

Yöntem: Flukonazol ve gentamisinin birlikte etkisininin belirlenmesi için dama tahtası testi yapılmıştır. Bu yöntemle, ilaçların 96 kuyucuklu plak üzerinde karşılaştırılarak kombinasyon etkinlikleri test edildi.

Bulgular: Çalışmamıza dahil edilen 33 adet C. albicans izolatının 14'nün (%42) flukonazole dirençli olduğu tespit edilmiştir. Flukonazol dirençli C. albicans izolatları üzerinde gentamisinin tek başına etkili olduğu gözlenmemiştir. Ancak gentamisin ve flukonazol birlikte kullanıldığında aditif ve sinerjik etkiler gözlemlenmiştir. Bu çalışmanın sonunda 14 örnekten iki tanesinde sinerjik etki 11 tanesinde aditif etki tespit edilmiştir. İlaçların, çalışmaya alınan izolatlar üzerine herhangi bir antagonistik etkisi saptanmamıştır.

Sonuç: Flukonazole dirençli C. albicans izolatlarında gentamisin'nin flukonazol etkinliği üzerindeki etkileri belirlenerek flukonazol duyarlılığını artıracak uygun gentamisin konsantrasyonları hesaplandı ve alternatif olabilecek gentamisin+flukonazol kombinasyonları belirlenmiştir.

Keywords: Flukonazol, gentamisin, dama tahtası yöntemi, Candida spp.

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INTRODUCTION

Candida is the fungi which reproduce through monocellular gemmulation (with blastospores) and which can form pseudo/true hyphae (Cowen et al, 2015). They form generally broken white or cream-coloured, moist or dry, with the plane or creased surface, opaque or bright, and sour odorous colonies within 24 hours at room temperature or 37°C (Perlin et al, 2015). Candidiasis is a significant important opportunistic pathogen fungal infection for people with the weak immune systems.Candida albicans is the most common etiological agent of candidiasis (Liu et al, 2016). In recent years, the incidence of hospital candida infections has increased because of the increase in hospitalization due to cancer and other diseases which weaken the immune system, the increase in organ transplantation surgery, the increase in antibiotic use, the increase in the number of patients stayed in intense care units, and the interventional practices applied to the patients (Karabıcak et al, 2016; Pfaller et al, 2004). Candida types are on a high position among hospital infection factors (Pfaller 2001).

The proliferation of antifungal use due to the increases in fungal infection frequency and accordingly the mortality and morbidity rates can cause the emergence of resistant fungal strains. Azole group and fluconazole which has a broad effect spectrum have been used in the prevention and treatment of C. albicans infections. However, it causes an increase in fluconazole use, fluconazole resistance or poly-cross resistance incidence in azole derivatives (Pfaller et al, 2015; Chen et al, 2012; Kocoglu et al, 2005; Ener, 1998). For this reason, it is needed to develop new antifungal agents. Given the length of the process and the rapid resistance development, it is on the agenda to regain the drugs to treatment through the combined use of the existing molecules rather than to develop new drug molecules. It is especially focused on the combination of the antifungals with the non-antifungal molecules in combination studies.

Gentamicin is an antibacterial antibiotic that belongs to the aminoglycoside group and acts by inhibiting protein synthesis (Yücel and Kantarcıoglu, 1999). Gentamicin (GM) has also been reported to have an antifungal effect against Fusarium species (Miceli et al, 2011). However, it has been reported that gentamicin can show anti-candidal activity when used with azole group drugs (Lu et al, 2018). In this study, fluconazole was used in combination with gentamicin. We thus aimed to identify a fluconazole+antibiotic combination that could serve as an alternative for the treatment of fluconazole-resistant Candida infections through the identification of the effects of fluconazole-resistant Candida isolates on the minimum inhibitory concentration (MIC) of fluconazole and to calculate the ideal gentamicin concentrations to overcome fluconazole resistance. For this purpose, we aimed to research the synergic effects of gentamicin and fluconazole in Candida isolates, which were provided by Trakya University Health Research and Application Center.

MATERIAL and METHOD

Microorganisms

In our study, it was used C. albicans American Type Culture Collection (ATCC) 10231 and 50 Candida spp. isolates which were isolated from various clinical samples

that were sent to Trakya University Health Research and Application Center as the quality control strains recommended by CLSI M27-A3 (2008) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). 33 of the Candida specimens collected during the period when the study was planned could be revived and the study was continued with these specimens. 33 isolates which were testified as C. albicans through the germ tube test were stocked in cryovials with beads and were held at -80ºC.

Germ Tube Test

It is a rapid test used in the treatment of C. albicans. It is positive in 95-97% of C. albicans. In the microscopic examination of the germ tube preparation, it is seen that the head of short strains formed by C. albicans does not create any articulation in the intersection of blasconidium and the germ tube (Figure 1).

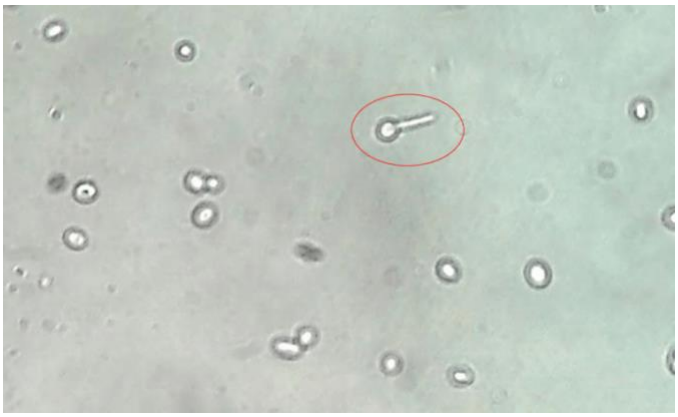

Figure 1. Germ tube formation of *C. albicans* (https://www.researchgate.net/figure/5-Germ-tube-formation-by-C-albicans-40X_fig5_326734793)

Way to Perform Germ Tube Test: The yeast cell is taken by tapping the pure colony with the needle. The inoculum is dispersed as 0.5 ml in human serum. It is incubated for 3 hours at 37 ºC. It is taken a drop from serum culture, put on the lam, and covered with lamella. It is examined in terms of germ tube forming firstly with scaling down, then with the immersion objective in the microscope (Abbasoglu, 2011).

Microdilution Method

In this method, it is used U-based 96 scrobiculate micro-dilution plates, RPMI 1640 medium, and double concentrated drug and yeast suspensions (CLSI, 2008). CLSI recommends visual assessment on 24th and 48th hours, and spectrophotometric assessment for the azoles. Thereafter, the concentration in the well in which turbidity is reduced significantly in the visual assessment for the azoles in comparison with the reproduction control, and the value in the well in which turbidity is reduced at the rate of 50% in comparison with the spectro-photometrical reproduction are accepted as the MIC value (Rex et al, 2001). For fluconazole, the isolates with 64 µg/ml and above MIC value are considered as resistant (R), the isolates with between 16-32 µg/ml MIC value as dose dependent-susceptible (DDS), and the isolates with8µg/ml and below MIC value as susceptible (S) (Pfaller et al, 2006).

In micro-dilution method, the stock solutions of gentamicin and fluconazole were prepared by dissolving in distilled water. In the study, it was used RPMI, SDA (Sabouraud dextrose agar) and SLM mediums (CLSI M27-A3 (2008)). After preparing RPMI medium by tamponing with MOPS, it was sterilized by filtering with Millipore filter. SDA and SLM mediums were sterilized by autoclaving for 15 minutes at 121°C. Antimicrobial susceptibility test was conducted in accordance with the recommendations of CLSI M27-A3. It was made passage to SLM medium from the yeast colonies produced in SDA plates, it was incubated for 24-48 hours at 35ºC, and the

turbidity of the culture was adjusted by adding liquid medium on it till it reaches the conformity to the 0.5 McFarland standard. Yeast suspension was used at 2,5x103 CFU/mL intensity by diluting at the rate of firstly 1:50 and then 1:20 before then it was adjusted at McFarland 0.5 intensity.

It was added L-glutamin tamponed to pH7 with 100 µL MOPS into all wells of the microplates, and it was added RPMI 1640 sodium bicarbonate-free liquid medium. After that, the substance concentration in the stock solution was double diluted by adding the stock solution at 100 µL volume into the first-rank wells of the prepared fluconazole and gentamicin micro-dilution plates.

It was continued the double dilution by using a multi-channel micropipette, and the substance concentration was reduced by half and half at each time in the following wells on the micro-dilution plates. As a result, it was obtained 512, 256, 128, 64, 32, 16, 8, 4,2,1,0.5,0.25 µg/mL concentrations of fluconazole and gentamicin in the germs.

After completing the dilution practice, it was conducted 10µL inoculation to each well in the micro-dilution plate from the prepared inoculum suspension. It was added the control wells which involve only medium and microorganism, and which involve only medium in every micro-dilution plate. Besides, it was checked its antimicrobial effects in the distilled water used as solvent. The yeast inoculated micro-dilution plates were left for incubation for 24-48 hours at 35ºC. At the end of the incubation process, MIC was identified as the lowest substance concentration by evaluating the wells which inhibited the reproduction of the microorganism in the micro-dilution wells as 50% or completely. Thus, the susceptibility of the isolates to gentamicin and fluconazole and the MIC values were identified.

Currently, there are two independent standards for antifungal susceptibility testing of fluconazole against Candida: the broth microdilution (BMD) method developed by the Clinical and Laboratory Standards Institute (CLSI) and the BMD method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The two methods are similar in that both use BMD, RPMI 1640 broth as the base medium, and a prominent inhibition (50% relative to the growth control) MIC endpoint criterion (CLSI, 2008 and EUCAST, 2018)

Checkerboard Assay

It was conducted checkerboard test for identifying the combined effect of gentamicin and fluconazole. In this method, different drugs and their concentrations are compared on a 96-wells plate and their combination efficiencies are tested. The MIC values which were obtained through drug combinations are compared with the single MIC values, and the fractional inhibitor concentration (FIC) is obtained. After that, the FIC values of the drugs in the combination are summed up and the FIC index (FICI) is calculated. The FIC value of each antimicrobial substance is obtained by dividing the lowest antimicrobial substance concentration in the non-reproductive well into the MIC value of that substance identified alone towards the same strain (Ozseven et al, 2012).

In the study, in the checkerboard assay which will be conducted in 96-wells U based microplates; it was distributed fluconazole serial dilutions were to In this studyleft-to-right first 10 wells of the microplates and gentamicin serial dilutions to top-to-bottom first 8 wells

of another microplate, and the contents of these two plates was integrated on another microplate (Figure 2). The MIC values obtained from the drug combination was compared with the alone MIC values, and the fractional inhibitor concentration (FIC) was found. After that, the FIC values of the drugs in the combination were summed up, and the fractional inhibitor concentration index (FICI) was calculated (Figure 2). It was tried many different combinations of the fluconazole and gentamicin concentration rates by applied to the checkerboard assay. The most suitable concentration in which they are influential jointly was identified (Ozseven et al, 2012; Dösler and Gürler, 2006).

Synergic Effect; Two drugs strengthen the effects of each other.

AdditiveEffect; The effect of drugs is equal to the sum of the effects observed when they are used solely.

Antagonistic Effect; The effect of drugs is lower than the effect observed when they are used solely (Ozseven et al, 2012; Dösler and Gürler, 2006).

Figure 2. C. albicans isolate reproduced in 96-wells microplate in which there are different concentrations of fluconazole and gentamicin.

FINDINGS

Microdilution Method Results

The MIC values obtained as a result of the susceptibility test conducted with microdilution method were examined by considering the EUCAST and CLSI limit values, and MIC results were shown in Figure 2. When it is examined by following per under EUCAST recommendations, it was identified that 14 of 33 C. albicans isolates are fluconazole-resistant (Table 1).

Table1. FluconazoleMIC limit values and the number of susceptive and resistant isolates of *C. albicans* according to EUCAST and CLSI (CLSI, 2008, The European Committee on Antimicrobial Susceptibility Testing, 2018)

	EUCAST				CLSI			
	MIClimit values (µg/mL)		Number of Isolates		MIClimit values(µg/mL)		Number of Isolates	
	Susceptive (S)	Resistant (R)	S	R	Susceptive (S)	Resistant (R)	S	R
Fluconazole	≤2	>4	20	14	≤ 8	≥ 64	23	11

The effect of gentamicin was researched and it was not observed any antimicrobial effect of gentamicin on C. albicans isolates. The susceptibility results of C. albicans isolates to fluconazole and gentamicin are given in Table 2. In the study, the checkerboard results, FIC values and interaction type are given in Table 3.

Table 2.Susceptibility results of *C. albicans* isolates to fluconazole and gentamicin

Isolate No:	Minimum Inhibitor Concentration(MIC) (µg/mL)	
	Flukonazol	Gentamisin
1,2,4,5,7,11,13,15,16,18,21,22,27,31,53,56,59,61,64	≤ 0,25	>512
3	256	>512
8,17,49,63	128	>512
9,20,23	32	>512
10	512	>512
28,29,30	64	>512
48	8	>512
51	64	>512
C.albicans ATCC10231	1	>512

Table 3.MIC values, checkerboard results, FICI values and the interaction type of fluconazole and gentamicin

Isolate No.	FLZ MIC(µg/mL)	GM MIC(µg/mL)	CombinationFLZ/GMConcentration	FICI	Interaction Type
3	512	1024	512/8	1,0078125	additive
8	128	1024	64/128	0,625	additive
9	32	1024	32/8	1,00781	additive
17	256	1024	512/8	2,0078	additive
20	32	1024	32/16	1,015625	additive
23	16	1024	16/64	1,0625	additive
28	32	1024	8/256	0,5	**synergic**
29	32	1024	32/8	1,0078125	additive
30	64	1024	16/8	0,25781	**Synergic**
48	8	1024	4/8	0,5078125	Additive
49	128	1024	64/32	0,53125	Additive
51	128	1024	128/8	1,0078125	Additive
63	256	1024	256/512	1,5	Additive

DISCUSSION

Candidiasis, which threatens patients with weak immune systems, has recently been increasing. However, the number of antifungal drugs in the market is limited compared to the number of existing antibacterial drugs. This condition necessitates the development of new treatment strategies because of the increase in infections caused by resistant fungi. Combination drug treatment is one of the most common and effective strategies used to solve this problem (Liu et al, 2016).

Extensive use of fluconazole, an azole antifungal, has increased the incidence of fluconazole resistance and cross-resistance to multiple azole derivatives. Other classes of antifungal drugs exist but have limited availability, and for this reason, toxicity, maintenance costs, and resistance have continued to pose a serious problem (Liu et al, 2016; Guo et al, 2013).

Of the 33 C. albicans isolates included in our study, 14 (42%) were resistant to fluconazole. Gentamicin can have anti-candidal activity when it is used with azole antifungals (Lu M, 2018). In our study, the effects of gentamicin on C. albicans isolates with fluconazole resistance were researched, and no effects were observed. However, when gentamicin and fluconazole were used together, additive and synergistic effects were observed. At the end of this study, synergistic effects were detected in 2 of 14 samples, and additive effects were detected in 11 samples. No antagonistic effects of the drugs on the study isolates were detected.

The discovery and development of antifungal drugs is slower than that of antibacterial drugs. The efficiency of antifungals decreases because of the resistance that develops against antifungal drugs (Cui J, 2015). For this reason, it is thought that the combination of existing drugs with other molecules is a more realistic strategy than the discovery of new drugs.

Liu et al (2016) researched the synergic effects of fluconazole and calcium channel blockers on resistant C. albicans isolates (Liu et al, 2016) and showed similar results to the results of our study. The MIC values of the calcium channel blockers alone were>512 μg/mL, and the calcium channel blockers had no antifungal effects. However, when these two drugs were used together, Liu et al observed a synergic effect.

Lu et al (2018) researched the synergic effects of gentamicin on Candida isolates with azole resistance. The MIC values of gentamicin for all strains used in this study were>512 μg/mL (Lu et al, 2018). In our study, the gentamicin MIC values of all isolates included in the study were also>512 μg/mL. This result indicates that gentamicin did not have any antifungal effects. However, when gentamicin and fluconazole were used in combination, they had synergic effects on C. albicans (Kayaalp O, 2013; Rex et al, 2001) isolates (FIC28=0.5 and FIC30=0.25781), and gentamicin significantly increased the fluconazole susceptibility of resistant C. albicans isolates. To use of gentamicin might be lead of bacterial antibiotic resistance which is very common mix infection with C. Albicans.

LIMITATIONS OF THE STUDY

The study was conducted in a single centre, and the number of cases is limited.

CONCLUSİON AND SUGGESTİON

In this study, the proper gentamicin concentrations to increase fluconazole susceptibility were calculated by identifying the effects of gentamicin on fluconazole efficiency in fluconazole-resistant *C. albicans* isolates, and gentamicin+fluconazole combinations were identified as an alternative. We obtained an important finding that it may be appropriate to use multiple drug combinations as alternatives for resistant fungal infections. While these findings are encouraging, clinical research on the *in vivo* and *in vitro* compatibility of these results after re-conducting this study with many more isolates is needed. It is recommended to conduct molecular studies to research the source of resistance in isolates that are fluconazole-resistant and have reduced MIC values with gentamicin.

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CONFLİCT OF İNTEREST

The authors declare that there is no conflict of interest.

AUTHORSHIP CONTRIBUTION STATEMENT

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