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ORIGINAL ARTICLE

High Glucose Concentrations Decrease the *in vitro* Antifungal Activity of Fluconazole against *Candida Albicans* as Proved by CLSI Broth Microdilution and Disk Diffusion Methods

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Key words:

C. albicans, Fluconazole, Glucose, MIC, Susceptibility

ABSTRACT

Background: *Glucose ≥ 1% was proved to decrease antifungal activity of voriconazole and amphotericin B against Candida spp. in varying degrees. This effect has not been studied with fluconazole.* ***Objectives:*** *Testing in vitro effect of glucose on fluconazole activity against C. albicans to investigate whether high blood glucose levels encountered in hyperglycemia can affect the response to fluconazole treatment in diabetics and whether adding glucose to culture media can affect the results of fluconazole susceptibility testing.* ***Methodology:*** *Clinical and Laboratory Standards Institute (CLSI) broth microdilution and disk diffusion methods were used for testing fluconazole against C. albicans ATCC 10231 and additional 5 clinical isolates in presence of wide range of glucose concentrations (0 mg/dl-8000 mg/dl). Fluconazole MIC ranges, MIC_{50}, MIC_{90} and inhibition zones (IZ) were determined.* ***Results:*** *The MIC ranges were 0.125-0.25 µg/ml, 0.125-0.50 µg/ml, 0.25-1 µg/ml, and 2-8 µg/ml for glucose concentrations 200 mg/dl, 500 mg/dl, 1000 mg/dl, 8000 mg/dl, respectively. MIC_{50} increased up to sixteen fold with glucose concentration 8000 mg/dl and MIC_{90} increased sixteen fold with glucose concentrations 4000 mg/ml and 6000 mg/dl, and thirty two fold with 8000 mg/dl concentration. The hyperglycemic glucose concentrations range (300 mg/dl to 600 mg/dl) showed maximum one fold increase of both MIC_{50} and MIC_{90}. All six tested strains showed decreases of the IZ with increase in glucose concentrations. These decreases were statistically significant in 2 strains with glucose concentrations 300 mg/dl to 600 mg/dl, in half of tested strains with glucose concentrations 700 mg/dl and 800 mg/dl, and with five strains for concentrations 1000 mg/dl and 2000 mg/dl. All tested strains expressed statistically significant decreases in IZ with concentrations 4000 mg/dl to 8000 mg/dl.* ***Conclusion:*** *This in vitro study showed decrease in antifungal activity of fluconazole against C. albicans by exposure to increasing glucose concentrations and this warrants special care for prescribing this antifungal drug for diabetics and caution during the interpretation of the results of its susceptibility testing against C. albicans when tested by using media containing glucose.*

INTRODUCTION

Candida albicans is responsible for a wide array of infections ranging from common superficial cutaneous and mucosal infections to systemic infections including candidemia[1, 2] and invasive deep seated infections[3, 4]. Candidal infections come on the top of opportunistic infections affecting immunocompromised patients[5-8].

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Nowadays, the increase in the immunocompromised states like diabetes mellitus, human immunodeficiency virus (HIV) infection, extensive use of immunosuppressive drugs and use of cancer chemotherapeutic agents result in increase of incidence, severity and invasiveness of such candidal opportunistic infections[9, 10].

Fluconazole is a triazole antifungal that works through inhibition of ergosterol synthesis in the fungal cell membranes by inhibition of cytochrome P-450-dependent 14α-sterol demethylase[11]. It is one of the most commonly and extensively used antifungals worldwide. It is available in the market long time ago, since 1990[12], and considered as the first line treatment for candidal infections in many countries. The response of *C. albicans* to antifungals is affected by many factors

either within the yeast cell itself or in the surrounding environment. Among the most important yeast endogenous factors, is the development of resistance mechanisms like modification of the targets of the drug or efflux pumps[13-16]. On the other hand, the environmental factors can provide inhibitors of the interaction between *Candida* and antifungal drugs. Moreover, exposure to certain environmental factors, such as glucose, can modulate gene transcription with expression of resistance genes and development of resistance to many stresses including the antifungal agent miconazole[17].

High concentrations of glucose (equal and more than 1%) were found to have variable effects on response of *Candida* spp. to different antifungals as these concentrations resulted in the decrease of sensitivity of *Candida albicans* and *Candida tropicalis* to voriconazole and amphotericin B but no effect on anidulafungin activity was found[18].

The previously mentioned studies[17, 18] explained the inhibitory effect of glucose on voriconazole, miconazole and amphotericin B either by expression of resistance genes in the tested yeast or direct interaction between the drugs and glucose by hydrogen bonding which varied according to the affinity of glucose to different antifungals. The fluconazole has not been studied in this regard, and because fluconazole is not identical to miconazole or voriconazole in chemical composition, affinity to cytochrome P-450 enzymes, pharmacokinetic properties or therapeutic spectrum[12,19,20], so it may behave differently from these two previously studied azole agents. This study aimed at application of both Clinical and Laboratory Standards Institute (CLSI) broth microdilution and disk diffusion methods for *in vitro* testing of the effect of a wide range of glucose concentrations (including different blood glucose levels of hyperglycemia occurring in diabetic patients) on response of *C. albicans* to fluconazole to investigate two issues; the first one is of a clinical importance; if the high blood glucose in diabetic patients could affect antifungal activity of fluconazole, which used extensively in treatment of candidal infections in such patients. The second issue is of a laboratory importance; if the glucose added to culture media used for cultivation of *Candida* could affect its susceptibility results to fluconazole.

METHODOLOGY

1. Candida strains:

The study tested six *Candida albicans* strains including the reference strain *Candida albicans* ATCC 10231 (Becton Dickinson, France) (was considered strain 1 in this study) and five clinical strains; three isolated from three patients of oral candidal thrush and two isolated from two high vaginal swabs. These clinical strains were isolated by culturing on Sabouraud dextrose agar (SDA) (Himedia, India) and identified by gram stain, germ tube formation and formation of chlamydospores on corn meal agar (Himedia).

Inoculum preparation:

All six test strains were subcultured on SDA and incubated for 24 hours at 35 °C. Five colonies were picked and resuspended in 5 ml sterile normal saline and vortexed well for 15 seconds, then the turbidity was adjusted, by adding sterile saline when appropriate, to 0.5 McFarland by using spectrophotometer at 530 nm, this gave cell suspension of $1\text{-}5\times10^6$ cells/ml that was directly used in disk diffusion method. For the broth microdilution method, this suspension was diluted 1:500 and 1:1000 by RMPI 1640 (GIBCO, Invitrogen, Auckland, NZ) to prepare 4× and 2× the desired final standardized inoculum ($0.5\text{-}2.5\times10^3$ cells/ml), respectively. Hemocytometer examination under light microscope was used to check cell counts in all of these prepared cell suspensions.

2. Antifungal agent:

Fluconazole (Pfizer) was used as a test antifungal. It was prepared according to CLSI guidelines. First, it was dissolved in sterile distilled water and serially diluted using sterile RPMI 1640 to obtain 2× of the desired final concentrations ranging from 0.125 µg/ml to 64 µg/ml, and then one hundred microliters volumes of each of these double strength concentrations were dispensed in the wells of 96-well sterile rounded bottom microwell plates (Nunclon Surface, Nunc A/S, Kamstrupvej, Roskilde, Danmark). Ten columns were containing the drug while two columns were left without drug containing RPMI 1640 only to be used later for positive and negative growth controls. The plates were kept at -80 °C till used.

3. Glucose:

The study investigated the effect of glucose on the activity of fluconazole against *C. albicans*. In broth microdilution method, different weighted amounts of glucose powder (Sigma-Aldrich) were dissolved completely in sterile RPMI 1640 using vigorous vortexing to prepare solutions with concentrations 4× of the final desired concentrations to be tested that were: 200 mg/dl, 300 mg/dl, 400 mg/dl, 500 mg/dl, 600 mg/dl, 700 mg/dl, 800 mg/dl, 900 mg/dl, 1000 mg/dl, 2000 mg/dl, 4000 mg/dl, 6000 mg/dl, and 8000 mg/dl, taking into consideration the amount of glucose already incorporated in the RPMI 1640 which is 2 g/L (or 200 mg/dl), so that no glucose was added to prepare the first desired final concentration (200 mg/dl) as RPMI 1640 was used alone (plain), while glucose powder was added to prepare the other mentioned concentrations. All of these solutions were further sterilized by filtration using sterile 0.22 µm diameter MILLEX GV syringe filters (MILLIPORE, Ireland). In disk diffusion method, the needed amounts of glucose powder were weighted and added during preparation of the test medium, which was the Muller-Hinton (MH) agars, to be incorporated

in test medium to prepare MH agar plates containing the final glucose concentrations: 0 mg/dl, 100 mg/dl, 200 mg/dl, 300 mg/dl, 400 mg/dl, 500 mg/dl, 600 mg/dl, 700 mg/dl, 800 mg/dl, 900 mg/dl, 1000 mg/dl, 2000 mg/dl, 4000 mg/dl, 6000 mg/dl, and 8000 mg/dl. The 0 mg/dl concentration was taken as glucose free control.

4. Antifungal susceptibility testing:

a- Broth microdilution method: was carried out according to CLSI M27-A3 guidelines[21]. In the previously prepared 96-well plates, for each strain, the first 2 rows were inoculated with 100 µl of prepared 2× inoculum without adding glucose to act as 200 mg/ml glucose concentration (which is the concentration of glucose incorporated in the plain RPMI 1640 and this was taken as a baseline glucose control), while 50 µl of prepared 4× inoculum and 50 µl of prepared 4× glucose solutions were added to wells of the other rows (two rows for each glucose concentration) thus each well of the 96-well plate contained total 200 µl volume, resulting in dilution of antifungal drug, test strain and glucose to the desired final concentrations mentioned above. Each test *Candida* strain was tested in duplicates with each glucose concentration with running positive and negative growth controls. The plates were incubated for 48 hours at 35°C. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of fluconazole that caused ≥50% inhibition of the growth compared to that of the drug-free positive growth control (CLSI M27-A3). The test was repeated three times on three different occasions, the MICs range, MIC_{50} and MIC_{90} were determined for all strains (MIC_{50} and MIC_{90} were the lowest fluconazole concentrations inhibited 50% and 90% of the tested strains, respectively).

***b- Disk diffusion method*:** was performed according to CLSI standard antifungal disk diffusion susceptibility testing method of yeasts[22] with slight modifications. Briefly, MH agars prepared earlier containing the different concentrations of glucose were inoculated by sterile swabs using the previously prepared inoculum $1\text{-}5\times10^6$ cells/ml. Each test strain was tested in duplicates with all concentrations of glucose. Plain MH agars (without glucose added) were included as 0 mg/dl concentration of glucose (taken as glucose free controls). Sterile 5 mm diameter discs prepared from Whatman filter papers were applied onto the surfaces of the inoculated MH agars then impregnated with 12.5 µl of 2 mg/ml solution of fluconazole to deliver 25 microgram of fluconazole for each disc. The plates were incubated at 35 °C for 24 hours (and extended for 48 hours in cases of insufficient growth after 24 hours). The inhibition zones (IZ) around the fluconazole discs were measured in millimeters (mm). The test was repeated in three different times and the means of IZ were calculated for each test strain with each tested glucose concentration.

5. Data analysis:

The results of the three broth microdilution assessments were expressed in forms of MIC ranges, MIC_{50} and MIC_{90} for comparison between the different tested glucose concentrations by use of number fold increases of fluconazole concentrations tested. In regards to the results of the three repeats of disk diffusion tests, the inhibitory zones (IZ) were measured in mm, expressed as means±SD and analyzed by one-way ANOVA to study statistical variation with each tested strain, where the fluconazole IZ and different glucose concentrations were considered as study factors. Post Hoc multiple comparisons were carried out using Tukey test. The significant statistical difference was considered when P value < 0.05. The IBM SPSS Statistics 20 program (IBM Corporation, New York, USA) was used for data entry and analysis.

RESULTS

Broth microdilution method:

The susceptibility of six *C. albicans* strains were tested for fluconazole in the presence of different concentrations of glucose ranging from 200 mg/dl (taken glucose baseline control) to 8000 mg/dl. The MICs were gradually increased with increase in glucose concentration as shown in table 1. The MIC ranges were 0.125-0.25 µg/ml, 0.125-0.50 µg/ml**,** 0.25-0.50 µg/ml, 0.25-1 µg/ml, 0.50-2 µg/ml, 1-4 µg/ml, and 2-8 µg/ml for glucose concentrations 200 mg/dl and 300 mg/dl, 400 mg/dl to 600 mg/dl, 700 mg/dl and 800 mg/dl, 1000 mg/dl, 2000 mg/dl, 4000 mg/dl and 6000 mg/dl, 8000 mg/dl, respectively. In comparison to the baseline control, fluconazole MIC_{50} and MIC_{90} increased with different glucose concentrations, as MIC_{50} increased twofold for concentrations 300 mg/dl to 700 mg/dl, fourfold for concentrations 800 mg/dl to 1000 mg/dl, eightfold for concentrations 2000 mg/dl to 4000 mg/dl and sixteen fold for rest of tested concentrations. Moreover, the MIC_{90} also increased in parallelism, as glucose concentrations; 400 mg/dl to 800 mg/dl showed twofold increase, concentrations 900 mg/dl to 1000 mg/dl had fourfold increase, concentrations 2000 mg/dl had eightfold increase, while sixteen fold increase were found for glucose concentrations 4000 mg/ml to 6000 mg/dl and thirty two fold for 8000 mg/dl concentration. Glucose concentrations (300 mg/dl to 600 mg/dl), that mimics those concentrations encountered in the blood of hyperglycemic patients, had maximum one fold increase of both MIC_{50} and MIC_{90}.

Table 1: Fluconazole MIC ranges, MIC_{50}, MIC_{90} of the six *C. albicans* strains tested with different glucose concentrations

Glucose (mg/dl)	*Fluconazole (µg/ml)*		
	MIC range	**MIC_{50}**	**MIC_{90}**
200	0.125-0.25	0.125	0.25
300	0.125-0.25	0.25	0.25
400	0.125-0.50	0.25	0.50
500	0.125-0.50	0.25	0.50
600	0.125-0.50	0.25	0.50
700	0.25-0.50	0.25	0.50
800	0.25-0.50	0.50	0.50
900	0.25-1	0.50	1
1000	0.25-1	0.50	1
2000	0.50-2	1	2
4000	1-4	1	4
6000	1-4	2	4
8000	2-8	2	8

MIC, minimal inhibitory concentration which was defined as the lowest fluconazole concentration inhibited ≥50% of growth compared to that of the drug-free positive growth controls; MIC_{50}, MIC_{90}, the lowest fluconazole concentrations inhibited 50%, 90% of the tested isolates, respectively. The results expressed were representative for three assessments.

Disk diffusion method:

The susceptibility of six *C. albicans* strains was tested for 25 µg fluconazole disks on MH agar plates containing different concentrations of glucose ranged from 0 mg/dl (glucose free control) to 8000 mg/dl. The IZ of the control plates were 34.3±2.51, 36±2, 41±1, 33.7±3.21, 44.3±0.58, 41.3±1.15 for strain 1, strain 2, strain 3, strain 4, strain 5, strain 6, respectively, as shown in table 2. Strain 1 had IZ ranged from 34±2 to 19.3±1.53 with glucose concentrations 100 mg/dl and 8000 mg/dl, respectively. The smallest difference between the control and 8000 mg/ml glucose concentration was approximately 9.4 mm and was found with strain 4, while the biggest difference (17.6 mm) was obtained from strain 6. Overall, all tested six strains showed statistically significant decrease in susceptibility to fluconazole with the full ranges of glucose concentrations tested.

In comparison to the glucose free controls, the IZ decreased with hyperglycemic glucose concentrations range (300 mg/dl to 600 mg/dl) by approximately 5.6-7.6 mm, 5.7-7 mm, 0.3-1.7 mm, 1.7-3 mm, 1.6-3.3 mm, and 1.6-7.3 mm for strain 1, strain 2, strain 3, strain 4, strain 5, and strain 6, respectively. These decreases were statistically significant in strains 1 and 2 with glucose concentrations 300 mg/dl to 600 mg/dl, and in strain 6 with concentration 600 mg/dl and were non-significant in rest of strains. On the other hand, fluconazole activity showed significant decrease in half of tested strains with glucose concentrations 700 mg/dl and 800 mg/dl, and with five strains for concentrations 1000 mg/dl and 2000 mg, and in all six strains with concentrations 4000 mg/dl to 8000 mg/dl.

Table 2: Susceptibilities of the tested *C. albicans* strains for fluconazole in presence of different glucose concentrations as determined by disk diffusion method

Glucose mg/dl	*Fluconazole inhibition zone (mm) Means ± SD* [a]					
	Strain 1	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**	**Strain 6**
0 [b]	34.3±2.51	36±2	41±1	33.7±3.21	44.3±0.58	41.3±1.15
100	34±2	35.3±1.15	40.7±0.58	33.3±3.05	43.7±0.58	40.7±1.15
200	31±2	34.7±0.58	41±1	31.7±2.08	44±1	41±1
300	26.7±1.53**	30±2*	39.7±2.51	32±1.73	42.7±2.30	39.7±0.58
400	28.7±1.15*	30.3±2.51*	39.3±1.15	31.7±2.08	41.3±1.15	38.3±1.53
500	27.7±2.51**	29±1**	40.7±1.15	30.7±2.30	42±2	37.7±1.15
600	28±1**	30±1*	40.3±2.51	31±1	41±1	34±1.73**
700	26.3±1.52**	28±2.64**	38±2	31±1	40.7±0.58	35±1*
800	27±1**	26±1**	39±1	32±2.64	40±2.64	35.3±2.08*
900	26.7±2.08**	26±1.73**	35±1**	33±2	40±3.46	34.7±1.53**
1000	25.3±0.58**	26±2.64**	33.3±2.08**	30 ±2	38.7±1*	34±2.64**
2000	25±1**	27.7±1.53**	32.3±1.53**	27.7±2.88	38±1*	34±2**
4000	21±1.73**	27.7±2.08**	31±1.73**	25.7±1.15**	32.7±2.51**	31.3±3.21**
6000	20.7±0.58**	25.6±1.53**	28±1**	25.3±0.58**	33±2**	28.3±2.88**
8000	19.3±1.53**	24±1**	29±1**	24.3±1.53**	31.7±2.08**	23.7±2.08**
P value [c]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

[a] SD, standard deviation, [b] 0 mg/dl, contained no glucose and was taken as glucose free control. The results expressed are representative for three experiments. * P<0.05 and ** P<0.01 (express the statistical differences between each concentration separately and the corresponding glucose free control). [c] P value, was the overall value of statistical difference within and between means for each tested strain with all tested glucose concentrations.

DISCUSSION

The current study investigated the *in vitro* effect of glucose on the activity of fluconazole against *C. albicans* to test two issues; the first was the anticipated impact of high blood glucose on response to antifungal treatment using fluconazole in hyperglycemic patients, and second was the effect of incorporation of glucose in culture media used for growing, isolation and *in vitro* antifungal susceptibility testing of *C. albicans* on response of this yeast to fluconazole.

Both broth microdilution and disk diffusion methods used in our study agreed and reveled that the increases in glucose concentrations reduced the response to fluconazole for all tested *C. albicans* strains. These results came in concordance with Mandal et al.[18] who found 4-fold and 2-fold decreases in activity of voriconazole and amphotericin B, respectively, in presence of 2% glucose in test media. The same result was reported for miconazole when tested against *C. albicans* in the presence of 1% of glucose for one hour contact, as the tested *Candida* showed significant resistance to the cidal effect of miconazole[17].

The decreased sensitivity of *C. albicans* to fluconazole could be explained, as previous studies proved with other antifungals, by either direct protective effect of glucose by binding with the antifungal through hydrogen bonds and preventing it from binding to its targets in the *Candida* cell membranes[18] or induction of transcription of resistance genes at the level of *Candida* genome, with subsequent increased resistance to antifungal and this effect was more prominent when *Candida* was incubated in high glucose containing media before exposure to the antifungal[17].

By comparing the current study to that of Rodaki et al.[17], there were differences in the methodology including; the inoculum size ($0.5\text{-}2.5\times10^3$ and $1\text{-}5\times10^6$ cells/ml versus 8×10^6 cells/ml), the method used (broth microdilution with total 200 µl volumes in microwells and disk diffusion, versus broth based method with lager volume of 4 ml in tubes), antifungal tested (fluconazole versus miconazole), tested drug concentration (wide array from 0.125 µg/ml to 64 µg/ml versus fixed 10 µg/ml), glucose concentration tested (wide range from 0 mg/dl to 8000 mg/dl versus 0% and 1%). However, the results of both studies agreed in the regard of decreased activity of the tested antifungal and this can support that the incubation of the candida-drug-glucose mix for 24 to 48 hours in the current study could easily affects transcriptome of the *C. albicans* with subsequent development of resistance to antifungals.

This study gives an example for modulation of one of the most important *C. albicans* virulence factors, which is the antifungal resistance, by the surrounding environmental factors and this correlates with Santana et al.[23] who found that dietary carbohydrates have a modulating effect on the biofilms formed by *C. albicans*.

Testing fluconazole against *C. albicans* with glucose concentrations like those occurring in the hyperglycemic patients (300 mg/dl to 600 mg/dl) showed decreases in the *in vitro* activity of this antifungal in comparison to glucose baseline control (200 mg/dl) by one fold increase in MIC_{50} and $MIC_{90,}$ as found in broth microdilution methodology, and up to 7.6 mm decrease in IZ in comparison to glucose free control (0 mg/dl) by disk diffusion testing, and these decreases were statistically significant with half of tested strains with many concentrations of the hyperglycemic range, and this responds on the first issue of this study by the possibility of occurrence of a decrease in the therapeutic effect of fluconazole by the high blood glucose levels encountered in hyperglycemic patients which necessitates special care in prescribing this antifungal in such patients and proper follow up to monitor its therapeutic effects when in use.

As regards the second issue, which was the effect of glucose supplementation to culture media on the results of the *in vitro* antifungal susceptibility testing, most media used with yeast and fungi (e.g. RPMI 1640, Sabouraud dextrose agar, potato dextrose agar) were found to contain from 0.2% to 4% glucose which is essential as carbon and energy source. For this reason, the study tested a wide range of glucose concentrations to include those found in formula already used and even higher concentrations to study more if the behavior of *Candida* could be changed with these higher glucose concentrations. The current results showed that most of these concentrations were found to have statistically significant decreasing effect on fluconazole sensitivity with all tested strains. So that, with referral to the results of the current study and studies of Rodaki et al. and Mandal et al.[18] either isolation and/or antifungal susceptibility testing of *C. albicans* on a culture media containing such glucose concentrations could act as potentials for giving false low susceptibility results which need a special attention in interpretation of such results.

In conclusion, the overall results showed decrease in the *in vitro* activity of fluconazole when tested against *C. albicans* in the presence of increasing concentrations of glucose which may be of a clinical impact that warrants special care for diabetic patients treated with fluconazole and caution during the interpretation of the results of antifungal susceptibility testing for this commonly used drug when tested against *C. albicans* on culture media containing glucose. Moreover, more extended *in vivo* study carried out on hyperglycemic patients having *Candida* infections and on fluconazole regimens is needed to test the real global biochemical and molecular events and interactions that may occur with simultaneous exposure of *Candida* to both fluconazole and high glucose to include other factors that may affect these interactions like plasma,

blood pH, blood cells, immune defenses, hormones, electrolytes and other factors found in the *in vivo* environment, and this will help in accurate monitoring of the therapeutic effects of fluconazole in those patients.

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