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Original Article

Evaluation of antifungal effect of amphotericin B in comparison with nystatin on Candida species derived from patients undergoing head-and-neck radiotherapy

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ABSTRACT

Background: There is ample evidence showing the development of nystatin-resistant strains in patients undergoing malignancy treatment. Amphotericin B is a polyene antifungal drug that combines with ergosterol to cause cell death and is more effective on fungal species than routine antifungals such as nystatin. This study aimed to compare the effect of nystatin and amphotericin B on fungal species isolated from patients before and during head-and-neck radiotherapy.

Materials and Methods: This *in vitro* experimental study was performed on samples isolated from patients undergoing head-and-neck radiotherapy before and during radiotherapy at Sayed al-Shohada Hospital in Isfahan, Iran. The isolates were identified by polymerase chain reaction-restriction fragment length polymorphism. Antifungal effects were determined by the microdilution method based on clinical and laboratory standards institute standards and minimum inhibitory concentration (MIC), minimum lethal concentration (MFC), drug sensitivity, and resistance were measured. The data were analyzed by SPSS version 22 (level of significance: 0.05).

Results: Before radiotherapy, all albicans strains were sensitive to nystatin, whereas 71.4% were sensitive to amphotericin B. After radiotherapy, *Candida albicans* strains were 100% sensitive to nystatin and 75% sensitive to amphotericin B.

Conclusion: The present study showed that before radiotherapy, all species isolated from patients, including *C. albicans, C. tropicalis, and C. glabrata*, were sensitive to nystatin, whereas a percentage of albicans species showed resistance to amphotericin B. In the 2nd week of radiotherapy, the same as before radiotherapy, all species isolated from patients were sensitive to nystatin, whereas a percentage of albicans species showed resistance to amphotericin B. In general, the current study showed that before and after radiotherapy, the antifungal effect of nystatin is greater than amphotericin B.

Key Words: Amphotericin B, antifungal effect, nystatin, oral candidiasis, radiotherapy

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INTRODUCTION

Radiotherapy may increase the incidence of mouth candidiasis through alternation in the volume and

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composition of the mucus.[1-4] From 1990 onwards, the *Candida* genera, which cause oral candidiasis in patients with malignant diseases, have changed. These patients presented an obvious shift from *Candida albicans* to non-*C. albicans* genera.[1,3] Radiotherapy can weaken the antimicrobial and cleaning effects of the saliva, and any change in the mouth's normal flora can increase the risk of oral candidiasis. Head-and-neck radiotherapy can increase the number of *Candida* colonies and alternation in the *Candida* genera.[5] The change in the *Candida* genera has led to the emergence of resistant strains.[6] Nystatin is a topical Polyene antifungal commonly used to treat fungal mouth diseases. The polyene group directly attaches to the ergosterol in the outer membranes of the fungi, leading to the development of pores, disruption of intracellular ion balance, alternations in the membrane, and ultimately the destruction of the cell.[7]

Resistance to nystatin is rare in *Candida* genera, but in recent studies, nystatin-resistant *Candida* has been reported,[8] which can be attributed to the recent change in the epidemiology of fungal infections and a shift toward resistant strains.[9,10] Mechanisms of polyene drug resistance include a general decrease in the ergosterol content of the cells, replacement of all or some of the estriol that binds to polyenes, and rearrangement or coverage of the existing ergosterols.[11] Amphotericin B is a polyene antifungal medication that attaches to the cell membrane ergosterol of the fungus and leads to the influx of the univalent ions such as Na, K, H, and Cl inside the cells and leads to the destruction of the fungal cell.[12,13] *In vitro* studies have demonstrated the susceptibility of different *Candida* strains, including albicans and nonalbicans, to amphotericin B.[14] Recent studies have shown that amphotericin B can be more effective against nonalbicans strains than traditional antifungals.[15-17] Considering the available evidence on the potential resistance to nystatin and the greater potential of amphotericin B in patients who have undergone head-and-neck radiotherapy, the objective of the current study was to evaluate and compare the efficacy of nystatin and amphotericin B on the fungal strains obtained before and during radiotherapy.

MATERIALS AND METHODS

Candida spp. in this *in vitro* experimental study were isolated from 33 patients in Sayed al-Shohada Hospital in Isfahan, Iran, before and during head-and-neck radiotherapy (three-dimensional conformal). The isolated strains were identified by Polymerase Chain Reaction Restriction Fragment Length Polymorphism. *Candida* species studied in the current study were previously collected and stored at the Department of Medical Parasitology and Mycology, Isfahan University of Medical Sciences.[18] To prepare the fungal suspension, the *Candida* strains were first cultured on Sabouraud dextrose agar (SDA) and incubated at 35°C for 24 h.

A suspension adjusted to match the turbidity standard of 0.5 MacFarland was prepared for each isolated strain, and the light absorption of the prepared suspensions was then adjusted to 530 nm using a spectrophotometer WPA Biowave II wavelength (Biochrom UK).

The Clinical and Laboratory Standards Institute (CLSI) guidelines were used to prepare the primary stocks of the antifungal agents as 5.12 mg for nystatin (Sigma-Aldrich; Germany), and 1.6 mg for amphotericin B (Sigma-Aldrich; Germany) were separately dissolved in 1 mL dimethyl sulfoxide (DMSO; Merck-Germany) and incubated for 30 min at room temperature to obtain a homogenized stock.

Minimum inhibitory concentration (MICs) of both antifungal agents were determined by the microdilution inhibitory method. For this purpose, the enzyme-linked immunoassay 96-well microplates were utilized; 10 wells were used for 0.5–128 µg/mL of nystatin and 10 wells for 0.003–16 µg/mL for amphotericin B in Roswell Park Memorial Institute; two wells were also assigned to positive and negative controls. Then, 100 µL of the fungal suspension provided from each isolated strain was added to each well. Finally, 100 µL of the 1×10^3 fungal suspension plus 100 µL of postretirement medical insurance (PRMI) was added to the positive control well, and the negative control well was only filled with 100 µL of pure PRMI without drugs and microorganisms according to CLSI-M27.[9] After incubation at 35°C for 24 and 48 h, the turbidity in the wells was evaluated, and the first wells without turbidity after 24 and 48 h were considered as the MIC24 and MIC48, respectively (level of significance: 0.05).

To determine minimum fungicidal concentration (MFC), 20 µL of the suspension in

the MIC well and the following wells were added to SDA plates, and after swab culturing, the plates were incubated for 24–48 h at 35°C. Plates with <5 grown colonies were used to determine MFC.[13]

Data were analyzed by SPSS version 22(IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). To investigate the antifungal effects of nystatin and amphotericin B, their MIC24, MIC48, and MFC were separately measured, and the median, range, and mode were also determined and analyzed with Mann–Whitney test. To compare the antifungal effect of two drugs, drug sensitivity and drug resistance were determined for each of the two drugs, nystatin and amphotericin B. The breakpoint for amphotericin B was determined in such a way that MIC values <2 were considered sensitive and MIC values ≥2 as resistant. The breakpoint of nystatin was determined in such a way that MIC values ≤2 were considered sensitive and MIC values >2 as resistant.

RESULTS

The studied *Candida* samples from the mycological collection of the Department of Medical Parasitology and Mycology, Isfahan University of Medical Sciences, included 14 C. *albicans*, five *Candida tropicalis*, two *Candida glabrata* before radiotherapy, and 12 *C. albicans*, four *C. tropicalis*, two *C. glabrata*, one *Candida parapsilosis*, and one *Candida krusei* in the 2nd week of radiotherapy.

To evaluate the antifungal activity of nystatin and amphotericin B, their MIC24, MIC48, and MFC were separately measured for *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis*.

The frequency was not determined for two species of *C. krusei* and *C. parapsilosis*, since only one strain of each was isolated from the patients during radiotherapy. Furthermore, MIC24, MIC48, and MFC of nystatin and amphotericin B before and during radiotherapy could not be compared since neither of the species was isolated before radiotherapy from the patients.

Table 1 shows MIC24, MIC48, and MFC for nystatin and amphotericin B against *C. parapsilosis* and *C. krusei.*

Table 2 shows medians and ranges of MIC24, MIC48, and MFC for nystatin and amphotericin B against *C. albicans, C. glabrata,* and *C. tropicalis.*

Table 1: Minimum inhibitory concentration 24, minimum inhibitory concentration 48, and minimum lethal concentration of nystatin and amphotericin B of *Candida parapsilosis* and *Candida krusei*

Antifungal activity	Strain			
	Candida parapsilosis		*Candida krusei*	
	Nystatin	Amphotericin B	Nystatin	Amphotericin B
MIC24	0.5	0.06	1	3
MIC48	0.5	2	1	2
MFC	1	2	1	2

MFC: Minimum lethal concentration; MIC: Minimum inhibitory concentration

For the statistical comparison MIC24of, MIC48, and MFC indicators of two drugs amphotericin B and nystatin in *Candida* strains, statistical analysis of these indicators was done separately before and after treatment with Mann–Whitney *U* statistical analysis. The results showed that before radiotherapy in the *C. albicans* strain of all three indicators in the nystatin group, mean rank was significantly higher than the amphotericin B group [Table 3].

Similarly, after the radiotherapy treatment, all three mentioned indicators in the nystatin group had a higher mean rank than the amphotericin B group [Table 3].

Different from the *C. albicans* strain in *tropicalis* strain MIC48 before and after treatment in the nystatin group with amphotericin B was not significant (P = 1.000). However, in MIC24 and MFC indices, both before and after treatment, the mean rank of nystatin was mysteriously higher than amphotericin B [Table 3].

In the *glabrata* strain, all the indices before and after the treatment in the two groups of nystatin and amphotericin B had significant differences so that the mean rank in the nystatin group was higher than that of amphotericin B, and only in the MFC index before the treatment of the two groups of nystatin and amphotericin B; the difference was not significant [Table 3].

Considering that all the samples from all three species of albicans, tropicalis and glabrata were 100% sensitive to Nystatin, statistical analysis was not performed to compare the percentage of sensitive and resistant samples to two drugs.

The percentage of resistant, dose-dependent and sensitive species is shown separately in Table 4.

Table 2: Median and range of minimum inhibitory concentration 24, minimum inhibitory concentration 48, and minimum lethal concentration of amphotericin B and nystatin on species isolated from radiotherapy patients before and during radiotherapy

Antifungal activity	Strain					
	Candida albicans		*Candida tropicalis*		*Candida glabrata*	
	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy
MIC24 amphotericin B, median (range)	0.2 (<0.003–>2)	0.09 (0.003–>2)	0.25 (0.03–0.25)	0.06 (0.06–0.12)	0.003 (<0.003–0.06)	0.0165 (0.003–0.03)
MIC24 Nystatin, median (range)	1 (<0.5–2)	0.75 (0.5–1)	1 (0.5–1)	1 (0.5–1)	1 (1–1)	0.5 (0.5–0.5)
MIC48 amphotericin B, median (range)	2 (>0.06–<2)	2.5 (0.12–>2)	2 (0.25–>2	1.65 (0.06–>2)	0.12 (<0.003–0.25)	0.075 (0.03–0.12)
MIC48 Nystatin, median (range)	1 (0.5–4)	1.5 (0.5–2)	2 (0.5–2)	1.5 (1–2)	2 (1–2)	0.5 (0.5–0.5)
MFC amphotericin B, median (range)	2 (0.025–>2)	2 (0.25–>2)	1 (1–>2)	1.5 (1–>2)	1 (0.25–1)	1.5 (1–2)
MFC Nystatin, median (range)	1 (0.5–32)	1 (<0.05–1)	1 (0.5–1)	1 (0.5–8)	1 (0.5–2)	0.5 (0.5–0.5)

MFC: Minimum lethal concentration; MIC: Minimum inhibitory concentration

Table 3: *P* value of minimum inhibitory concentration 24, minimum inhibitory concentration 48, and minimum lethal concentration of amphotericin B and nystatin on species isolated from radiotherapy patients before and during radiotherapy

P value of antifungal activity	Strain					
	Candida albicans		*Candida tropicalis*		*Candida glabrata*	
	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy
MIC24	0.008	0.000	0.000	0.000	0.000	0.002
MIC48	0.013	0.004	1.000	1.000	0.000	0.002
MFC	0.031	0.000	0.002	0.319	0.258	0.002

MFC: Minimum lethal concentration; MIC: Minimum inhibitory concentration

Table 4: The percentage of sensitive, resistant, and susceptible dose-dependent *candida* spp. to nystatin and amphotericin B before and during the 2nd week of radiotherapy

Antifungal activity	Strain					
	Candida albicans		*Candida tropicalis*		*Candida glabrata*	
	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy	Before radiotherapy	During radiotherapy
Sensitive (S)						
Nystatin	100%	100%	100%	100%	100%	100%
Amphotericin B	71.4%	75%	100%	100%	100%	100%
SDD						
Nystatin	-	-	-	-	-	-
Amphotericin B	-	-	-	-	-	-
Resistant (R)						
Nystatin	-	-	-	-	-	-
Amphotericin B	28.6%	25%	-	-	-	-

SDD: Susceptible dose dependent

DISCUSSION

The current study was designed to evaluate and compare the antifungal efficacy of nystatin and amphotericin B, and the resistance rate of different *Candida* strains to them. The current study's findings generally demonstrated that 100% of the isolated *Candida* were sensitive to nystatin before radiotherapy. The current study demonstrated that the sensitivity of *C. albicans* to the two mentioned medications was not different before and after radiotherapy, and the sensitivity to nystatin was higher than amphotericin B before and during radiotherapy. One of the possible reasons that we did not observe any change in drug

resistance to *C. albicans* after radiotherapy may be the environmental adaptation of the Candida. Several studies have demonstrated that environmental changes can lead to fast changes in gene expression, which enables it to adapt to environmental changes.[13] This can justify the observation that although radiotherapy can change the mouth environment (including changes in the volume and composition of the saliva), the decrease in the volume and composition of the saliva has not led to a change in drug resistance of this strain.

Contrary to our findings, in Bulacio *et al*. and Freitas *et al*.'s studies, 100% of the isolated Candida strains were sensitive to amphotericin B after the radiotherapy.[15,16] In our study, however, we observed 25% resistance to amphotericin B. Recently, several studies have demonstrated increased resistance in different fungal strains, including *Candida*.[17,19] The underlying mechanism of resistance to amphotericin B is a defect in the ERG3 gene, which is involved in the biosynthesis of ergosterol. Following this gene defect, other sterols accumulate in the cell membrane of the fungus, which is resistant to polyenes such as amphotericin B.[20] As shown in this study, contrary to the studies mentioned above, the sample was also taken before radiotherapy and its resistance and sensitivity were evaluated. The possible reason for the difference noted between the current study and those of Freitas *et al.*[15] and Bulacio *et al.*[16] was the initial (before the radiotherapy) resistance of *C. albicans* to amphotericin B, which had continued after the radiotherapy. In the current study, the initial sampling (before the radiotherapy) was in patients with head-and-neck malignancies, and malignancy is one of the causes of resistance to antifungal medications.[21] Therefore, the initial resistance of the *C. albicans* strain may be due to patients' underlying malignancy. In addition to malignancy, there has been an increasing number of reports of clinically significant amphotericin B resistance in fungal pathogens, including *C. albicans*.[22]

Contrary to our findings, in a study conducted by Karbach *et al*., the amphotericin B MIC spectrum for *C. albicans* was reported as 0.38–0.5 μg/mL,[12] whereas we recorded it as >0.03–2 μg/mL. This discrepancy may be due to the difference in the sampling time. In Karbach's study, sampling was conducted at least 180 days after the radiotherapy, whereas in the current study, samples were obtained 2 weeks after the radiotherapy sessions. Moreover, different phases of the salivary gland's functions after radiotherapy can be another reason for the discrepancy in the results.

Contrary to the mentioned studies, clinical *(in vivo)* findings of Finlay *et al*.'s study demonstrated that 72% of patients with *Candida* who underwent head-and-neck radiotherapy were sensitive to amphotericin B.[23] The sensitivity of the isolated *C. albicans* was 75% in the current *in vitro* study after radiotherapy. Ramla *et al*. demonstrated that the production of hydrolytic enzymes (such as phospholipase and proteinase) by the fungus could increase following radiotherapy,[3] which can cause alternations in the efficacy of antifungal medications following radiotherapy. However, as these enzymes only function in the host's tissues, they do not affect the *in vitro* susceptibility of the strain; however, they may be effective in the mouth *(in vivo)* environment.

In this experiment, the *C. tropicalis* genus was completely susceptible (100%) to both nystatin and amphotericin B before and after radiotherapy. In the current study, in the studies carried out by Schelenz *et al*. and Bulacio *et al*., the complete susceptibility of the strain to both nystatin and amphotericin B was confirmed.[4,16] Contrary to our findings, the results of Al-Abeid *et al*. demonstrated that the tropicalis species was more susceptible to amphotericin B rather than nystatin.[24] The difference between Al-Abeid *et al*.'s study and ours may be caused by the fact that patients with chemotherapy and surgery were included in their study, but we only included those with head-and-neck radiotherapy. Studies by Karimi *et al*. demonstrated that neutropenia could lead to antifungal resistance.[25] Therefore, the difference between the studies may be due to drug resistance as a result of neutropenia in patients who received chemotherapy in Al-Abeid *et al*.'s study.

In the current study, *C. glabrata* was completely susceptible (100%) to both amphotericin B and nystatin. *C. glabrata* was formerly considered a nonpathogenic fungal strain, but with the increase in the application of immunosuppressive agents, mucosal and systemic infection with glabrata have increased. Innate resistance to azoles is one of the main obstacles to controlling *C. glabrata* infection. Amphotericin B is considered one of the major antifungal treatments in immunosuppressed individuals.[26] The outcomes of the current study, by those of Kurnatowski *et al*.'s study, demonstrated that *C. glabrata* isolated both

before and after radiotherapy were susceptible to both nystatin and amphotericin B.[27] Because hitherto, nystatin resistance has not been reported in the *C. glabrata* in the recipients of radiotherapy, and as this medication is only used in the topical form and has fewer side effects, it is considered a treatment of choice.

In this study, the only *C. krusei* specimen was isolated from a patient after radiotherapy. Before radiotherapy, this strain was detected in no patient. In the current study, the isolated strain was sensitive to nystatin and resistant to amphotericin B (MIC of nystatin and amphotericin B were 1 μg/mL and 2 μg/mL, respectively). In contrast with the current study, Bulacio *et al*. and Bansal *et al*. demonstrated that resistance of *C. krusei* to nystatin was 100%, whereas the sensitivity to other antifungal medications, including amphotericin B, was similar.[16,21] One of the main potential mechanisms of the resistance of this strain can be a mutation in ERG11 and FSK1 genes due to environmental conditions. Because head-and-neck radiotherapy can lead to the formation of amphotericin B-resistant *Krusei* strains, it seems that among antifungals of the polyene group, nystatin can be considered superior for the treatment of *C. krusei* infection.

Similar to the *Krusei* strain, *C. parapsilosis* was isolated from only one patient after radiotherapy. Immunosuppressed patients are exposed to a higher risk of hospital infections, and invasive *candida* is one of the most common hospital-acquired fungal systemic infections. In recent years, the prevalence of *non*albicans strains has increased, and *C. parapsilosis* is the second or third common strain isolated from these patients.[28] The only *parapsilosis* strain in the current study was completely sensitive to both nystatin and amphotericin B (MIC of nystatin and amphotericin B on *parapsilosis* were 0.5 μg/mL and 0.06 μg/mL, respectively). However, despite the very low MIC of nystatin on this strain in immunosuppressed individuals (such as patients with malignancy), it may not be considered a suitable candidate for *parapsilosis* and systemic (intravenous) amphotericin B may be a more suitable alternative for candidemia prophylaxis.

CONCLUSION

In general, the results of the present study showed that before radiotherapy, all species isolated from patients, including *C. albicans, C. tropicalis, and C. glabrata*, were sensitive to nystatin, whereas a percentage of albicans species showed resistance to amphotericin B. In the 2nd week of radiotherapy, the same as before radiotherapy, all species isolated from patients were sensitive to nystatin, whereas a percentage of albicans species showed resistance to amphotericin B.

In general, the current study showed that before and after radiotherapy, the antifungal effect of nystatin is greater than amphotericin B.

Data availability

The data used to support the findings of this study are included in the supplementary information files.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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