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Fluconazole and amphotericin-B resistance are associated with increased catalase and superoxide dismutase activity in *Candida albicans* and *Candida dubliniensis*

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ABSTRACT

Introduction: *Candida dubliniensis*, a new species of *Candida* that has been recovered from several sites in healthy people, has been associated with recurrent episodes of oral candidiasis in AIDS and HIV-positive patients. This species is closely related to *C. albicans*. The enzymatic activity of *C. dubliniensis* in response to oxidative stress is of interest for the development of drugs to combat *C. dubliniensis*. **Methods:** Fluconazole- and amphotericin B-resistant strains were generated as described by Fekete-Forgács et al. (2000). Superoxide dismutase (SOD) and catalase assays were performed as described by McCord and Fridovich (1969) and Aebi (1984), respectively. **Results:** We demonstrated that superoxide dismutase (SOD) and catalase activities were significantly higher ($p<0.05$) in the fluconazole- and amphotericin B-resistant strains of *C. dubliniensis* and *C. albicans* than in the sensitive strains. The catalase and SOD activities were also significantly ($p<0.01$) higher in the sensitive and resistant *C. albicans* strains than in the respective *C. dubliniensis* strains. **Conclusions:** These data suggest that *C. albicans* is better protected from oxidative stress than *C. dubliniensis* and that fluconazole, like amphotericin B, can induce oxidative stress in *Candida*; oxidative stress induces an adaptive response that results in a coordinated increase in catalase and SOD activities.

Keywords: Catalase. Superoxide dismutase. *Candida albicans*. *Candida dubliniensis*.

INTRODUCTION

Candida dubliniensis, a new emerging opportunistic species, was first described in 1995 in Dublin, Ireland. The cultures were obtained from human immunodeficiency virus (HIV)-positive patients with recurrent oral candidiasis. Although *C. dubliniensis* was initially recovered from the oral cavity, this species has also been reported at other body sites, including the respiratory tract, central nervous system, vagina, and skin, as well as in the blood, urine, and feces of both HIV-positive and HIV-negative patients[1-4]. Nunn et al.[5] also described the isolation of *C. dubliniensis* from nonhuman environmental sources. Thus, this species is not confined to humans[5].

Candida dubliniensis is phenotypically and genotypically similar to *C. albicans* and *C. stellatoidea*. Like these *Candida* species, *C. dubliniensis* forms chlamydospores and is germ tube-positive in phenotypic identification tests. The detection of unusual carbohydrate assimilation profiles, which can be identified using commercially available systems such as the API® ID 32C and API® 20C AUX kits, were one of the earliest indications that *C. dubliniensis* represented a new species within the *Candida* genus[4]. Studies have since documented a high prevalence of this organism in patients suffering from diabetes (3.6 to 18.2%), cystic fibrosis (11.1%), cancer (2 to 4.6%), vulvovaginal candidiasis (0.17 to 2.4%), and candidemia (0.5 to 7.9%)[6].

Candida dubliniensis is more susceptible to polyenes and azoles than *C. albicans*; however, a remarkable feature of *C. dubliniensis* is the rapid development of secondary resistance to fluconazole[7]. Azoles, such as fluconazole, act on the ergosterol biosynthesis pathway by inhibiting the enzyme C14-α-demethylase, a cytochrome P450-dependent enzyme[8]. Amphotericin B, a polyene antifungal agent, binds to sterols such as ergosterol and disrupts the osmotic integrity of the fungal membrane, resulting in the leakage of intracellular ions and metabolites[9]. Despite intense study of *C. dubliniensis*, the enzymatic activities of superoxide dismutase (SOD) and catalase in fluconazole-resistant and amphotericin B-resistant *C. dubliniensis* are unknown. In *Candida*, SOD plays an important protective role against attack by macrophages and neutrophils, which generate superoxide radicals[10]. Superoxide dismutase converts damaging superoxide radicals (O_2^-), one type of reactive oxygen species (ROS), to the less damaging

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hydrogen peroxide (H_2O_2), which can be converted into water by catalase[11,12]. Catalase has been implicated in amphotericin B resistance by inhibiting the oxidative damage produced by this polyene[13]. Ergosterol, a sterol present in the fungal plasma membrane, is the target of amphotericin B. The binding of amphotericin B to ergosterol produces channels that leak potassium ions, resulting in the disruption of the proton gradient[14,15]. The antioxidant enzymes SOD and catalase may act synergistically to protect cells from oxidative damage[16]. Some authors have suggested that the coordinated inactivation of ROS by the sequential action of superoxide- and hydrogen peroxide-inactivating enzymes is essential for the effective protection of cells against ROS toxicity[17].

To further explore the antioxidant enzymatic activities and the coordinated inactivation of ROS by SOD and catalase in *C. dubliniensis* and *C. albicans*, we investigated the SOD and catalase activities in fluconazole- and amphotericin B-sensitive and resistant strains of *C. dubliniensis* and *C. albicans*.

METHODS

Strains

The *Candida albicans* and *Candida dubliniensis* strains used in this study [Ca 10, Ca 81, Ca 119, Ca 170, Ca ATCC 44373, Cd 1, Cd 2, Cd 3, Cd 4, Cd 5, Cd 7, Cd 9, Cd 11, and Cd 13] were isolated from patients with oral candidiasis at the University Hospital of Universidade Federal de Santa Maria (Brazil). Species identification was based on phenotypic and genotypic characteristics. The *C. dubliniensis* genotype was confirmed by random amplification of polymorphic deoxyribonucleic acid (DNA) using the primers CDU (5′ GCG ATC CCC A 3′) and B-14 (5′ GAT CAA GTC C 3′)[18,4]. The *C. albicans* strain ATCC 44373 from the American Type Culture Collection was used as a control strain.

Generation of fluconazole-resistant strains

The minimal inhibitory concentration (MIC) of fluconazole was first determined for *C. albicans* and *C. dubliniensis* according to the Clinical and Laboratory Standards Institute (CLSI) document M27-A3[19]. After the MIC determinations, the cultures were grown overnight in Sabouraud glucose broth (SDB), and the cells were added to flasks containing 20ml of media to achieve a final absorbance of 0.1 at 640nm. The cultures were incubated at 30°C for 10h, and fluconazole was then added to a final concentration of 8μg ml^{-1}. After 14h of further incubation, the cells of the fluconazole-containing culture were consecutively subcultured 3 times in fresh SDB medium containing 8μg ml^{-1} fluconazole; in each case, the cultures were incubated at 30°C with shaking for 24h. After the third incubation, the cells were added to flasks containing 20ml of SDB medium containing 8μg ml^{-1} fluconazole, and the turbidity of the cultures was adjusted to achieve a final absorbance of 0.1 at 640nm. After a 10h incubation, fluconazole was added at a final concentration of 16μg ml^{-1}, and after 14h, the cells from this culture were subcultured 3 times in fresh SDB medium containing 16μg ml^{-1} fluconazole; each subculture was incubated at 30°C with shaking for 24h. The fluconazole concentration was always duplicated under this procedure, and the final concentration was 64μg ml^{-1}. Cells from this culture were plated on Sabouraud glucose agar (SDA), and the colonies were designated *C. albicans* fluconazole resistant and *C. dubliniensis* fluconazole resistant[20].

Generation of amphotericin B-resistant strains

Induction of amphotericin B resistance was performed as described for the generation of the fluconazole-resistant strains, with some modifications. The time needed for cell growth between exposures to different amphotericin B concentrations was 48h. Resistant cells obtained at 2μg ml^{-1} were plated on SDA, and the colonies were designated *C. albicans* amphotericin B-resistant and *C. dubliniensis* amphotericin B-resistant.

Susceptibility tests

Antifungal agents: standard fluconazole (Pfizer Central Research) and amphotericin B (Bristol-Myers, Squibb Australia Pty Ltd) powders were obtained from their respective manufacturers. Stock solutions were prepared in water (fluconazole) and dimethyl sulfoxide (amphotericin B). Serial two-fold dilutions were prepared exactly as outlined in the CLSI document M27-A3[19]. Final dilutions were made in Roswell Park Memorial Institute (RPMI) 1640 medium buffered to pH 7.0 with 0.165mmol l^{-1} morpholinopropanesulfonic acid (MOPS). Aliquots (100μl) of each antifungal agent at a 2X final concentration were dispensed into the wells of plastic microdilution trays, which were sealed and frozen at -70ºC until use. The fluconazole dilutions ranged from 0.12 to 128μg ml^{-1}, and the amphotericin B dilutions ranged from 0.0313 to 16μg ml^{-1}.

Inoculum standardization - inoculum suspensions of the yeast strains were prepared in sterile saline (0.85% NaCl) from 24h cultures grown on SDA at 35°C. The turbidity of the suspensions was adjusted to achieve a final absorbance of 0.1 at 530nm[8,19].

CLSI broth microdilution method (document M27-A3): microplates containing 100μl of two-fold serial dilutions of the antifungal drugs in standard RPMI 1640 medium (0.2% glucose) were inoculated with 100μl of inoculum containing 1×10^3 to 5×10^3 colony-forming units per milliliter (CFU/ml). Following inoculation, the reference microdilution plates were incubated at 35°C, and the Minimum Inhibitory Concentration MICs were determined after 24 and 48h. The fluconazole and amphotericin B reference MICs corresponded to the lowest drug dilutions that resulted in prominent growth inhibition (≥ 80%) or the absence of growth, respectively[19].

Enzyme assays

Candida albicans and Candida dubliniensis strains: strains were cultured for 48h in SDA containing 64μg ml^{-1} fluconazole or 2μg ml^{-1} amphotericin B. The yeast cells were added to sterile saline (0.85%) to achieve a final absorbance of 0.1 at a wavelength of 625nm. Aliquots (1ml) of this suspension were added to 50ml of SDB containing 4% glucose and 1% mycological peptone, and these cultures were incubated at 30ºC with shaking for 24h for the fluconazole experiments and for 48h for the amphotericin B experiments. After centrifugation, the cells were washed 3 times with sterile water, and a crude extract was prepared.

Preparation of cell-free extracts: the crude extracts were prepared using the glass bead lysis method. Cells that had been cultured in SDB supplemented with fluconazole or amphotericin B were washed 3 times with MilliQ water and resuspended in lysis buffer (50mmol l^{-1} potassium phosphate pH 7.0) containing 0.5g of 500-µm-diameter glass beads. The mixture was homogenized in a Teflon-glass homogenizer for 6 cycles of alternating homogenization for 20s and cooling on ice. The mixture was then centrifuged for 30 min in a refrigerated centrifuge to remove the cell debris and glass beads. The supernatant was used for enzyme assays.

Superoxide dismutase activity

Superoxide dismutase activity was assayed according to McCord and Fridovich[21]. The assay was performed in a total volume of 1ml containing 50mmol l^{-1} glycine buffer (pH 10), 60mmol l^{-1} epinephrine, and the enzyme. Epinephrine was added, and adrenochrome formation was recorded at 480nm with a ultraviolet-visible **(**UV-Vis) spectrophotometer for 4 min. One unit of SOD activity was equivalent to the amount of enzyme required to inhibit epinephrine oxidation by 50% under the experimental conditions. The assays were performed in triplicate.

Catalase activity

Catalase activity was determined in the cell-free extracts using the method of Aebi et al.[22]. H_2O_2 solution (10mM), enzyme extract, and 50mM phosphate buffer (pH 7) were pipetted into a cuvette. The reduction of H_2O_2 was followed at a wavelength of 240nm for 4 min against a blank containing 50mM phosphate buffer and enzyme extract. Catalase activity was expressed in $\Delta E\ min^{-1}$ (mg protein)$^{-1}$. The assays were performed in triplicate.

Protein analysis

Protein concentrations were measured using the Bradford method and Coomassie Blue; serum albumin was used as the standard[23].

Statistics

Paired t tests were used to compare the sensitive and resistant cells. Comparisons among the *C. albicans* and *C. dubliniensis* groups were made using unpaired t tests. The calculations were performed using the GraphPad InStat statistical program.

Ethical considerations

This protocol was approved by the Bioethics Committee of the Universidade Regional Integrada do Alto Uruguai e das Missões from Frederico Westphalen - RS, Brazil, under registration n° 061-2/PIH/04.

RESULTS

Fluconazole- and amphotericin B-resistant *C. dubliniensis* and *C. albicans* strains were obtained by exposing sensitive isolates to increasing concentrations of these antifungal agents. For the sensitive *C. dubliniensis* cells, the MICs ranged from 0.06 to 0.5µg ml^{-1} for fluconazole and from 0.0312 to 0.125µg ml^{-1} for amphotericin B; for the sensitive *C. albicans* cells, the MICs ranged from 0.5 to 4.0µg ml^{-1} for fluconazole and from 0.0625 to 0.25µg ml^{-1} for amphotericin B. After the induction of resistance, tests of the susceptibility to fluconazole and amphotericin B demonstrated that resistance developed in all strains assayed.

Table 1 shows the SOD and catalase activities in the cell-free extracts of the fluconazole-sensitive and fluconazole-resistant

TABLE 1 - Superoxide dismutase and catalase activities of the fluconazole-sensitive and fluconazole-resistant *Candida dubliniensis* strains.

	Mean SOD activity [U mg protein^{-1}]		Mean catalase activity [$\Delta E\ min^{-1}$ (mg protein)$^{-1}$]	
Strains	sensitive[a]	resistant[b,c]	sensitive[a]	resistant[b,d]
01	13.4 ± 6.0	48.8 ± 2.8	0.5 ± 0.2	1.6 ± 0.1
02	56.4 ± 17.7	113.6 ± 23.6	1.9 ± 0.4	3.7 ± 0.1
03	27.1 ± 3.6	21.9 ± 5.2	0.8 ± 0.2	1.1 ± 0.1
04	5.1 ± 2.9	10.3 ± 2.5	0.5 ± 0.1	0.6 ± 0.1
05	17.2 ± 11.8	130.2 ± 33.7	1.5 ± 0.3	3.4 ± 0.5
07	14.0 ± 2.2	17.7 ± 3	1.1 ± 0.05	1.7 ± 0.4
09	28.2 ± 2.9	38.8 ± 12.2	0.6 ± 0.05	0.9 ± 0.2
11	7.4 ± 1.2	47.2 ± 6.1	1.9 ± 0.6	1.6 ± 0.7
13	83.2 ± 5.8	67.9 ± 6.2	1.5 ± 0.4	1.7 ± 0.7

The data are the mean ± SD of triplicate experiments. U: unit of SOD; ΔE: absorbance change at 240nm over 240s. [a]Fluconazole-sensitive *C. dubliniensis;* [b]Fluconazole-resistant *C. dubliniensis* (64µg/ml^{-1}); [c]Statistical analyses revealed significant differences between the SOD activities of the fluconazole-resistant and fluconazole-sensitive *C. dubliniensis* groups ($p < 0.01$); [d]Statistical analyses revealed significant differences between the catalase activities of the fluconazole-resistant and fluconazole-sensitive *C. dubliniensis* groups ($p < 0.01$). SOD: superoxide dismutase; SD: standard deviation; *C.: Candida.*

C. dubliniensis strains. In general, the mean SOD activity was 1.97-fold greater for fluconazole-resistant *C. dubliniensis* than for the sensitive cells. The difference between the 2 groups was significant ($p < 0.01$). The catalase activity of the fluconazole-resistant cells was 1.58-fold greater than that of the fluconazole-sensitive cells; this difference was significant ($p < 0.01$).

Table 2 shows the SOD and catalase activities of the *C. dubliniensis* strains resistant to 2μg ml^{-1} amphotericin B. The mean SOD activity of the *C. dubliniensis* strain resistant to 2μg ml^{-1} amphotericin B was 1.97-fold greater than that of the sensitive cells; this difference was statistically significant ($p < 0.01$). The mean catalase activity of the group resistant to 2μg ml^{-1} amphotericin B was 2-fold greater than that of the sensitive group. Statistical analyses showed significant differences ($p < 0.01$) between the *C. dubliniensis* catalase activities of the cells resistant to 2μg ml^{-1} amphotericin B and the sensitive cells.

The SOD and catalase activities of the fluconazole-sensitive and fluconazole-resistant *C. albicans* cells are shown in **Table 3**. The mean SOD activity of the fluconazole-resistant *C. albicans* was 1.3-fold greater than that of the sensitive cells; this difference was statistically significant ($p < 0.01$). The mean catalase activity of the fluconazole-resistant group was 1.57-fold greater than that of the fluconazole-sensitive group; this difference was statistically significant ($p < 0.01$).

Table 4 shows the SOD and catalase activities of the *C. albicans* strains that were sensitive or resistant to 2μg ml^{-1}

TABLE 2 - Superoxide dismutase and catalase activities of the amphotericin B-sensitive and amphotericin B-resistant *Candida dubliniensis* strains.

	Mean SOD activity [U mg $protein^{-1}$]		Mean catalase activity [ΔE min^{-1} (mg $protein)^{-1}$]	
Strains	sensitive[a]	resistant[b,c]	sensitive[a]	resistant[b,d]
01	15.5 ± 0.4	69.7 ± 20.7	0.6 ± 0.1	2.4 ± 0.5
02	15 ± 1.5	22.6 ± 3.0	0.8 ± 0.1	0.9 ± 0.1
03	13.5. ± 0.4	37.8 ± 6.8	0.9 ± 0.01	2.1 ± 0.1
04	27.2 ± 1.8	50.7 ± 5.0	1.1 ± 0.1	1.8 ± 0.01
05	22.1 ± 3.2	37.3 ± 3.5	1.0 ± 0.1	1.8 ± 0.2
07	15.7 ± 1.9	44.1 ± 13.3	1.1 ± 0.01	3.1 ± 0.01
09	47.2 ± 12.6	101.1 ± 24.1	1.6 ± 0.2	2.2 ± 0.3
11	57.9 ± 33.2	69.3 ± 4.7	1.7 ± 0.2	3.6 ± 0.6
13	35.5 ± 10.2	57.8 ± 8.1	0.8 ± 0.1	1.0 ± 0.1

The data are the mean ± SD of triplicate experiments; U: unit of SOD; ΔE: absorbance change at 240nm over 240s. [a]Amphotericin B-sensitive *C. dubliniensis*; [b]*C. dubliniensis* resistant to 2μg/ml^{-1} amphotericin B; [c]Statistical analyses indicated significant differences between the SOD activities of the *C. dubliniensis* cells resistant to 2μg/ml^{-1} amphotericin B and the sensitive *C. dubliniensis* cells ($p < 0.01$). [d]Statistical analyses indicated significant differences between the catalase activities of the *C. dubliniensis* cells resistant to 2μg/ml^{-1} amphotericin B and the sensitive *C. dubliniensis* cells ($p < 0.01$). SOD: superoxide dismutase; SD: standard deviation; *C.: Candida.*

TABLE 3 - Superoxide dismutase and catalase activities of the fluconazole-sensitive and fluconazole-resistant *Candida albicans* strains.

	Mean SOD activity [U mg $protein^{-1}$]		Mean catalase activity [ΔE min^{-1} (mg $protein)^{-1}$]	
Strains	sensitive[a]	resistant[b,c]	sensitive[a]	resistant[b,d]
Ca 10	177.4 ± 22.1	300.5 ± 2	2.8 ± 0.2	3.5 ± 0.6
Ca 81	230.4 ± 5.1	273.1 ± 2.8	2.2 ± 0.1	2.3 ± 0.4
Ca 119	269.6 ± 5.9	289.7 ± 21.3	1.5 ± 0.2	2.6 ± 0.3
Ca 170	139.2 ± 6.2	238.3 ± 16.9	1.6 ± 0.4	4.1 ± 0.2
Ca ATCC 44373	188 ± 2.5	205.3 ± 7	2.3 ± 0.2	3.8 ± 0.9

The data are the mean ± SD of triplicate experiments. U: unit of SOD; ΔE: absorbance change at 240nm over 240s. [a]Fluconazole-sensitive *C. albicans;* [b]Fluconazole-resistant *C. albicans* (64μg/ml^{-1}); [c]Statistical analyses revealed significant differences between the SOD activities of the fluconazole-resistant and fluconazole-sensitive *C. albicans* groups ($p < 0.01$); [d]Statistical analyses revealed significant differences between the catalase activities of the fluconazole-resistant and fluconazole-sensitive *C. albicans* groups ($p < 0.01$). SOD: superoxide dismutase; SD: standard deviation; *C.: Candida.*

TABLE 4 - Superoxide dismutase and catalase activities of the amphotericin B-sensitive and resistant *Candida albicans* strains.

	Mean SOD activity [U mg protein^{-1}]		Mean catalase activity [ΔE min^{-1} (mg protein)$^{-1}$]	
Strains	sensitive[a]	resistant[b,c]	sensitive[a]	resistant[b,d]
Ca 10	22.1 ± 8.5	178.4 ± 15	3.1 ± 0.3	4.4 ± 0.3
Ca 81	116.2 ± 6.4	93 ± 4.8	2 ± 0.2	2.9 ± 0.3
Ca 119	106.6 ± 11.7	298.2 ± 21.3	1.5 ± 0.2	2.9 ± 0.8
Ca 170	186.1 ± 3.2	195 ± 6.9	1.3 ± 0.4	3.9 ± 0.1
Ca ATCC 44373	82.4 ± 4	269.3 ± 10.1	2.8 ± 0.1	4.8 ± 1.1

The data are the mean ± SD of triplicate experiments. U: unit of SOD. ΔE: absorbance change at 240nm over 240s. [a]Amphotericin B-sensitive *C. albicans*; [b]*C. albicans* resistant to 2 μg ml^{-1} amphotericin B; [c]Statistical analyses revealed significant differences between the SOD activities of the *C. albicans* cells resistant to 2μg/ml^{-1} amphotericin B and the sensitive *C. albicans* cells ($p < 0.01$); [d]Statistical analyses revealed differences between the catalase activities of the *C. albicans* cells resistant to 2μg/ml^{-1} amphotericin B and the sensitive *C. albicans* cells ($p < 0.05$). SOD: superoxide dismutase; SD: standard deviation; *C.: Candida.*

amphotericin B. The mean SOD activity of the *C. albicans* strain resistant to 2μg ml^{-1} amphotericin B was 1.97-fold greater than that of the sensitive group; this difference was statistically significant ($p < 0.01$). The mean catalase activity of the cells resistant to 2μg ml^{-1} amphotericin B was 1.77-fold greater than that of the sensitive group; this difference was statistically significant ($p < 0.05$).

DISCUSSION

Since the recognition of *C. dubliniensis* as a new species in 1995, many studies have investigated the differences between this species and *C. albicans*. Studies of the responses of these two species under identical conditions have uncovered phenotypic as well as genotypic differences between these two species. Enzymes involved in virulence, such as aspartyl proteinases, phospholipases, chondroitin sulfatase, and hyaluronidase, have been thoroughly studied. De Bernardis et al.[24] and Linares et al.[25] demonstrated that the level of aspartyl proteinase was significantly higher in *C. albicans* than in *C. dubliniensis*, while no differences in phospholipase, chondroitin sulfatase, and hyaluronidase activities were observed[24-26]. In this study, we investigated the effect of fluconazole and amphotericin B resistance on the SOD and catalase activities of *C. albicans* and *C. dubliniensis*. These enzymes are not related to virulence factors but play important roles in the oxidative stress response in both species[27]. SOD activity is involved in the detoxification of free superoxide radicals, such as the radical produced by phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[29]. Phagocytic cells have 2 antimicrobial systems that are responsible for the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). To counter these antimicrobial systems, microorganisms such as *Candida* have multiple defense responses that can be adjusted according to the nature and amount of ROS produced by the host or environment[30]. Our results revealed that SOD activity was significantly increased in *C. dubliniensis* and *C. albicans* strains that were resistant to amphotericin B and fluconazole compared to sensitive strains. The increase in SOD activity in response to amphotericin B may be due to the production of oxidative damage in *Candida* through lipid peroxidation[15]. In our study, yeast cultures were exposed to increasing concentrations of amphotericin B, up to a maximum of 2 μg ml^{-1}. In general, at this concentration, amphotericin B kills *Candida* by inducing oxidative damage through lipid peroxidation; however, we determined that *Candida* SOD activity was activated in response to amphotericin B exposure. The mean SOD activity of the *C. dubliniensis* and *C. albicans* strains resistant to 2μg ml^{-1} amphotericin B was 1.97-fold greater than that of the sensitive strains. This SOD activation likely occurred due to the induction of an adaptive response upon exposure of *Candida* to sublethal concentrations of peroxide or superoxide. This adaptive response results in a several-fold increase in catalase, SOD, and glucose 6-phosphate dehydrogenase (G6PDH) activity[29,31,32]. In addition, the elevation of SOD activity in the yeast strains resistant to amphotericin B may contribute to an increased resistance to phagocytic cell attack, making the yeast cells more resistant to host defenses. We also observed that the SOD activities of the *C. albicans* strains that were sensitive to fluconazole and amphotericin B were significantly ($p < 0.01$) higher than the SOD activities of the sensitive *C. dubliniensis* strains. When SOD activity was compared among the resistant groups, *C. albicans* also expressed significantly ($p < 0.01$) higher levels of SOD than *C. dubliniensis*.

As previously demonstrated for other enzymes (e.g., proteinases), SOD activity was higher in *C. albicans* than in *C. dubliniensis*. This study is the first to evaluate the effect of fluconazole resistance on SOD activity in *C. albicans* and *C. dubliniensis*. Our results suggest that fluconazole has an oxidant effect on *Candida,* resulting in the activation of its antioxidant system, including SOD. The mean SOD activities of the fluconazole-resistant *C. albicans* and *C. dubliniensis* strains were 1.93- and 1.97-fold higher, respectively, than those of the sensitive strains. If fluconazole induces SOD activity, like amphotericin B, then this reaction to fluconazole would contribute to the increased resistance of *Candida* to phagocytic cell attack.

Similar to SOD activity, catalase activity was elevated in the fluconazole- and amphotericin B-resistant *C. albicans* and *C. dubliniensis* cells compared to the sensitive groups. The enzyme catalase converts hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O)[16]. In *Candida*, catalase has been suggested to be involved in the resistance to amphotericin B and is involved in hypha formation[15,28]. Catalase activity has not been previously studied in fluconazole-resistant *C. albicans* and *C. dubliniensis* strains. Here, we demonstrated that the catalase activities of the amphotericin B-resistant *C. dubliniensis* and *C. albicans* strains were 2- and 1.77-fold higher, respectively, than those of the sensitive groups. The catalase activities of the fluconazole-resistant *C. albicans* and *C. dubliniensis* groups were 1.58- and 1.57-fold higher, respectively, than those of the sensitive groups. These findings demonstrate that similar changes in catalase and SOD activities occur in response to exposure to fluconazole and amphotericin B under the same conditions and corroborate the hypothesis that an adaptive response occurs when *Candida* is exposed to oxidative stress[32]. Based on the literature, we expected that these enzymes would have similar responses because the product of SOD is a substrate for catalase[11]. Here, we confirmed this enzymatic profile in *C. albicans* and *C. dubliniensis*. Similar to SOD, catalase activity was significantly ($p < 0.01$) higher in the sensitive and resistant *C. albicans* strains compared to the respective *C. dubliniensis* groups. In combination with the increased susceptibility of *C. dubliniensis* to hydrogen peroxide and macrophage killing, this result suggests that *C. albicans* is better protected from oxidative stress than *C. dubliniensis*, in accordance with the higher virulence of *C. albicans*[33,34,35].

The results obtained in this study also demonstrate a synergism between SOD and catalase in the *C. dubliniensis* and *C. albicans* strains that were resistant to fluconazole and amphotericin B, in which protection by SOD generates hydrogen peroxide but not other ROS, such as lipid hydroperoxides or the reactive nitric intermediate (RNI) peroxynitrite, which would be more detrimental to the cells due to the absence of defenses against these types of compounds. Because the hydrogen peroxide generated by SOD activity is still toxic to the cell, it must be eliminated by catalase, which converts this toxic compound into water[14].

In conclusion, this study is the first to determine the effect of fluconazole and amphotericin B resistance on catalase and SOD activities in *C. albicans* and *C. dubliniensis*. The stress-inducing effect of fluconazole on *Candida* has not been previously described, and we have demonstrated that catalase and SOD activities are increased in fluconazole-resistant cells. We also confirmed that *C. albicans* has more active antioxidant pathways than *C. dubliniensis*.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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