ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2015, 00, 1–5
doi: 10.1093/mmy/myv036
Advance Access Publication Date: 0 2015
Short Communication

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Combination of fluconazole with silver nanoparticles produced by *Fusarium oxysporum* improves antifungal effect against planktonic cells and biofilm of drug-resistant *Candida albicans*

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Received 21 January 2015; Revised 22 April 2015; Accepted 25 April 2015

Abstract

Silver nanoparticles (AgNPs) have been extensively studied because of their antimicrobial potential. Here, we evaluated the effect of biologically synthesized silver nanoparticles ($AgNP_{bio}$) alone and in combination with fluconazole (FLC) against planktonic cells and biofilms of FLC-resistant *Candida albicans*. $AgNP_{bio}$ exhibited a fungicidal effect, with a minimal inhibitory concentration (MIC) and fungicidal concentration ranging from 2.17 to 4.35 μg/ml. The combination of $AgNP_{bio}$ and FLC reduced the MIC of FLC around 16 to 64 times against planktonic cells of all *C. albicans*. There was no significant inhibitory effect of $AgNP_{bio}$ on biofilm cells. However, FLC combined with $AgNP_{bio}$ caused a significant dose-dependent decrease in the viability of both initial and mature biofilm. All concentrations of $AgNP_{bio,}$ alone or in combination with FLC, were not cytotoxic to mammalian cells.

The results highlight the effectiveness of the combination of $AgNP_{bio}$ with FLC against FLC-resistant *C. albicans*.

Key words: fluconazole resistance, biological nanoparticles, fungicidal effect.

Introduction

Candida albicans is the leading cause of opportunistic mycoses worldwide [1]. Crucially, this species is frequently associated with biofilm formation on biotic surfaces and implanted medical devices [2], which can contribute to a high mortality rate among infected patients [2]. Besides, the emergence of fluconazole (FLC)-resistant isolates has been observed in the last decades [1].

Currently, several approaches have been examined to overcome reduced susceptibility of *Candida* to FLC [3]. Recent advances in nanotechnology have stirred interest in the application of metallic nanoparticles in medicine. Because of their antimicrobial properties [4,5], silver nanoparticles (AgNPs) have been investigated alone or in combination with other compounds [6,7].

Here, the antifungal potential of biologically synthesized AgNPs ($AgNP_{bio}$) alone or in combination with FLC was investigated against planktonic cells and biofilm of FLC-resistant *C. albicans*.

Materials and methods

Microorganisms and culture conditions

Candida albicans ATCC 26790 and oral FLC-resistant *C. albicans* isolates from healthy (isolate E) and diabetic (isolate 122) individuals seen in a primary healthcare unit in Maringá city, Paraná, Brazil in 2011 were used in this study. Minimum inhibitory concentration (MIC) of FLC (Sigma Chemical Co, USA) was previously determined by standard broth microdilution method, using M27-S4 recommendations [8]. All yeasts were maintained at −80°C in Sabouraud dextrose broth (Himedia, India) containing 30% glycerol. The protocols were approved by the Ethics Committee of Uningá (CEP no. 0017/11).

Synthesis of $AgNP_{bio}$

$AgNP_{bio}$ were freshly biosynthesized as previously described [9]. Briefly, *Fusarium oxysporum* (strain 551) biomass (10 g) was added to 100 ml of distilled water and incubated for 72 hours at 28°C. The culture was filtered, and the filtrate was mixed with $AgNO_3$ (1.0 mM; Sigma-Aldrich, USA) and kept at 28°C for 28 hours. $AgNP_{bio}$ were characterized by scanning electron microscopy and energy-dispersive spectroscopy [9].

Antifungal susceptibility testing on planktonic cells

The MIC of FLC (range used: 0.25–128 μg/ml) and $AgNP_{bio}$ (range used: 0.28–8.7 μg/ml) was determined by the standard broth microdilution method with minor modifications [8]. In all antifungal susceptibility assays, the test medium was RPMI 1640 buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (RPMI). The effect of combinations of FLC (0.25–128 μg/ml) with nanoparticles (0.28–8.7 μg/ml) on planktonic cells was assessed by the checkerboard method [10]. Briefly, the yeast cell inoculum was set according to standard protocols [8], the dilution of each compound was placed in wells of microtiter plate to provide 80 combinations. In addition, MIC of compounds alone was also determined. The results were interpreted using the fractional inhibitory concentration index (FICI). FICI values classified the combination as: $\leq$0.5, synergy; >0.5 to 4.0, no interaction; $\geq$4.0, antagonism [11]. The MIC values of compounds alone or in combination were read visually at 24 hours of incubation and were defined as the lowest concentration that resulted in a visible decrease of turbidity compared to compound-free growth control. For time-kill curve analysis, 1×10^6 CFU/ml of all *Candida* yeasts were incubated with each compound at previously determined MIC values in RPMI, alone or in combination. At 0, 2, 4, 8, 16, 24 hour time points, aliquots were inoculated on SDA and incubated at 37°C for 24 hours. *Candida* growth without any compound was used as control (CTR). For viability analyses, 1×10^6 CFU/ml in RPMI were incubated with each compound at MIC values, alone or in combination for 2 hours, treated and untreated cells were washed with PBS and stained with FUN-1 dye using the LIVE/DEAD yeast viability kit (Molecular Probes, USA), following the manufacturer's recommendation. Cell viability was analyzed by fluorescence microscopy (Leica DM2000) using fluorescein filters. All assays were carried out in triplicate on three different occasions.

Inhibition of biofilm formation assay

Candida biofilms were formed in flat-bottomed 96-well plates as previously described [12]. After 1.5 and 24 hours of biofilm formation, the medium was aspirated off and each well was washed with PBS. RPMI (200 μl), containing $AgNP_{bio}$ (2.17 or 4.75 μg/ml) alone or in combination with serial twofold dilutions of FLC (128–2 μg/ml),

was added, and the plates were further incubated for 24 hours at 37°C. Viability of biofilms was determined using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay [12]. Briefly, a 100 µl aliquot of XTT-menadione (0.1 mg/ml XTT and 1 µM menadione, Sigma Chemical Co, USA) was added and the plates incubated in the dark for 2 hours at 37°C. The product formed was measured at 490 nm with microtiter plate reader (Synergy HT, Biotek, USA). All assays were carried out in triplicate on three different occasions.

Cytotoxicity assay

The cytotoxicity of $AgNP_{bio}$ alone or in combination with FLC was determined as described by Marcato et al. [13], except that the HEp-2 cell line was used and the $AgNP_{bio}$ concentration ranged from 2.17 to 8.7 µg/ml. Briefly, cells were cultured into 96 well culture plate (Techno Plastic Products, Switzerland) for 48 hours at 37°C, and 5% CO_2. Medium containing $AgNP_{bio}$ (2.17, 4.35, 8.7 µg/ml) alone or a combination of FLC (serial twofold dilutions of 1–128 µg/ml) and nanoparticle (2.17 or 4.35 µg/ml) was added, and cells were incubated for 24 hours. Cell viability was determined by the MTT [dimethylthiazol diphenyl tetrazolium bromide (Sigma Chemical Co., USA)] according to the manufacturer's recommendation. The concentration of the compounds needed to inhibit the viable cells up to 50% by regression analysis correspond the 50% cytotoxic concentration.

Statistical analysis

The results were analyzed by one-way ANOVA using Graphpad Prism version 6.0 (Graphpad Software). Comparative analysis was performed using Tukey's test. $P < 0.05$ was considered significant.

Results

In this study, $AgNP_{bio}$ produced by *F. oxysporum* were evaluated for their effect against FLC-resistant *C. albicans*. The MICs of FLC and $AgNP_{bio}$ alone or in combination against planktonic cells are shown in Table 1. All *C. albicans* strains were resistant to FLC with MIC values ranging from 64 to >128 µg/ml. $AgNP_{bio}$ exhibited an antifungal effect with MIC values ranging from 1.74 to 4.35 µg/ml. In addition, this effect on yeast was selective, since the cytotoxic concentration of $AgNP_{bio}$ for HEp-2 cells was higher than 8.70 µg/ml.

According to FICI, no synergistic effect was observed when $AgNP_{bio}$ and FLC were combined in this study.

Table 1. Effect of fluconazole and/or biological silver nanoparticles against fluconazole-resistant *Candida albicans*.

	Minimal inhibitory concentration (in µg/ml)			
C. albicans	FLC[#]	$AgNP_{bio}$	FLC/$AgNP_{bio}$	FICI[†]
ATCC26790	>128	4.35	2 / 2.17	0.510
Isolate E	64	1.74	4 / 1.09	0.689
Isolate 122	128	1.74	8 / 1.09	0.689

Note: MIC values of compounds were read visually at 24 hours of incubation. [#]MIC: ≤2.0 µg/ml, susceptible; 4.0 µg/ml, susceptible dose dependent; ≥8 µg/ml, resistant [8]. [†]FICI: ≤0.5, synergy; >0.5 to 4.0, no interaction; ≥4.0, antagonism [11].

However, the combinations caused a marked reduction in FLC MIC (around 16 to 64 times) for all *C. albicans* strains (Table 1). Interestingly, the effect of $AgNP_{bio}$ on planktonic cells of *C. albicans* appeared to be time dependent and fungicidal in contrast to the fungistatic effect of FLC. In combination with FLC, a significant reduction in CFU count was observed after 2 hours, and no CFU were detected after 4 hours. With $AgNP_{bio}$ alone, total cell death was observed after 24 hours (Fig. 1A). Microscopy showed that yeasts exhibited a green-fluorescent staining, reflecting dead cells after 2 hours in the presence of FLC and $AgNP_{bio}$ combined, which corroborated the effects on CFU counts (Fig. 1B). FLC-resistant *C. albicans* isolates (E and 122) also presented similar effects (data not shown).

When $AgNP_{bio}$ was tested on biofilm, no significant effect at 2.17 or 4.35 µg/ml $AgNP_{bio}$ was observed, but the combination with FLC caused a significant ($P < .001$) FLC dose-dependent decrease in viability of these cells after 24 hours. For both concentrations, the effect was more pronounced during the initial phases of biofilm formation (Table 2). Comparable effects on FLC-resistant *C. albicans* isolates were obtained (data not shown). No $AgNP_{bio}$ and FLC combination showed a cytotoxic effect on HEp-2 cells, as judged by the cell viability remaining higher than 50% after 24 hours (data not shown).

Discussion

Here, an eco-friendly $AgNP_{bio}$ produced by *F. oxysporum* was shown to have fungicidal effect against FLC-resistant *C. albicans*, with no cytotoxicity to mammalian cells. This inhibitory effect of $AgNP_{bio}$ on FLC-susceptible *C. albicans* had been previously described [14,15]. Similar to our results, Ishida et al. [5] reported a fungicidal effect of $AgNP_{bio}$ produced by *F. oxysporum* on

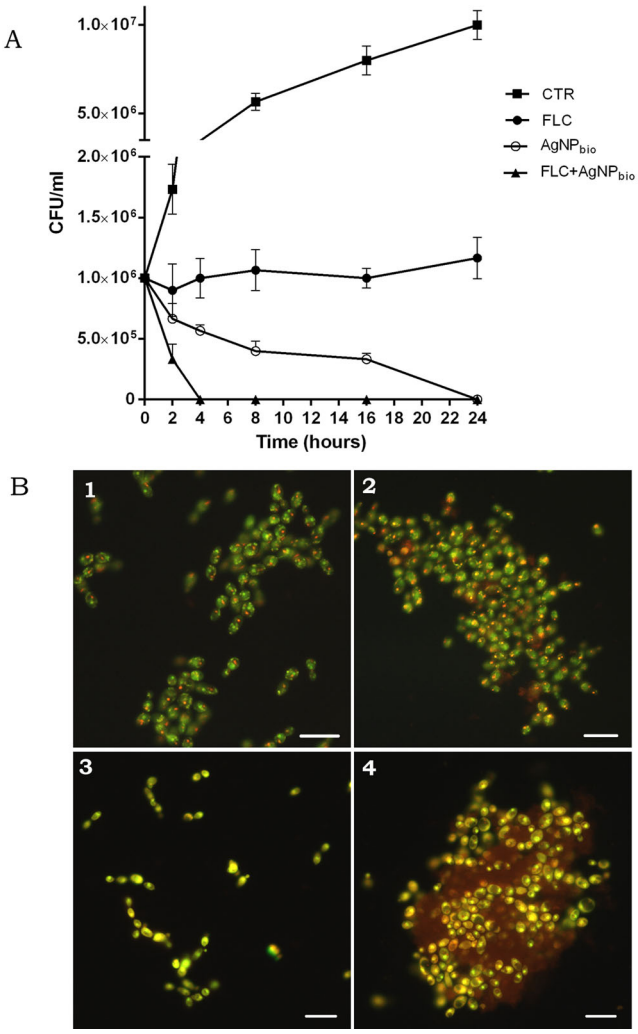

Figure 1. Effect of fluconazole and biological silver nanoparticles alone or in combination against FLC-resistant *Candida albicans*. A. Time-kill curve, 1×10^6 yeast of all *Candida* strains were incubated at 37°C with or without the two compounds alone or in combination at MIC values and at the time point 0, 2, 4, 8, 16, 24 hours, aliquots were plated in SDA. Values are the mean ± SD representative of three independent experiments. B. Fluorescence microscopy analyses after *Candida* yeast staining with FUN-1 for viability analysis. 1×10^6 yeast of all *Candida* were incubated with or without the two compounds alone or in combination at MIC values for 2 hours, and visualized by fluorescence microscopy. Shaded cells characterize dead cells with diffuse yellow-green fluorescence and unshaded cells represent metabolically active yeasts that contain red-fluorescent structures in their vacuoles. Untreated viable cells (1) and cells treated with FLC (2), $AgNP_{bio}$ (3) and $AgNP_{bio}$/FLC (4). Bar: 5 μm. Only *C. albicans* ATCC 26790 results are shown.

C. albicans, with MIC and MFC values of 1.68 and 3.40 μg/ml, respectively. Cytotoxic concentration of $AgNP_{bio}$ found for HEp-2 cells was similar to Marcato et al. [13]. Besides, Lima et al. also showed noncytotoxicity up to a concentration of 10 μg/ml of silver biogenic nanoparticles on 3T3 cells [16].

Interestingly, our results highlight the decrease of FLC MIC of FLC-resistant *C. albicans* when combined to $AgNP_{bio}$. The resistance to FLC in *C. albicans* is strongly associated with overexpression of genes encoding efflux pumps or lanosterol 14α-demethylase [17]. On the other hand, the mechanisms of *C. albicans* death induced by AgNPs are not completely understood. AgNP-treated *C. albicans* exhibits a disrupted cell wall and cytoplasmic membrane [15]. In addition, AgNPs cause an increase in reactive oxygen species and hydroxyl radical production, which can also contribute to cell membrane damage [18]. Since $AgNP_{bio}$ can alter the permeability of the cell membrane, we can hypothesize that they may facilitate the entry of FLC, which interferes with ergosterol biosynthesis.

Regarding the effect of $AgNP_{bio}$ effect against biofilm, previous studies have shown an antifungal effect of chemically produced AgNPs in initial stages of biofilm formation of *C. albicans* [4], in contrast to our results. In addition, $AgNP_{bio}$ was not able to inhibit the mature biofilm of yeasts at the concentrations tested here, but their combination with FLC reduced the viability of biofilm in a dose-dependent manner. Importantly, silver nanoparticles produced by *F. oxysporum* are stable for several months due to protein capping, which occurs in the biogenic process, as observed by transmission electron microscopy [19]. Therefore, these results indicate the potential of $AgNP_{bio}$ for the development of new strategies for the treatment of FLC-resistant *C. albicans* infections. Further studies are needed to establish the mechanism of yeast death and the usefulness of $AgNP_{bio}$ in medicine.

Acknowledgments

The authors thank the technician Ediel C. Costa for their help with laboratory maintenance and Dr. C. Nozawa for providing the HEp2 cells. This work is supported by grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant No. 402728/2013-0) and Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina. CL, ATM received fellowships from CNPq and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES). Dr. A. Leyva helped with English editing of the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Table 2. Effect of biological silver nanoparticles alone and fluconazole-combined on viability of *Candida albicans* (ATCC 26790) biofilms.

$AgNP_{bio}$	Biofilm	Fluconazole concentration in each combination (μg/ml)							
(μg/ml)	(hours)	0	2	4	8	16	32	64	128
2.17	1.5	0	0	0	0	0	0	22* (9.35)	73* (10.35)
	24	0	0	0	0	6* (3.5)	8* (10.32)	13* (9.39)	15* (4.03)
4.35	1.5	0	0	0	0	0	0	43* (10.18)	77* (5.51)
	24	0	7* (2.24)	8* (4.25)	9* (12.47)	11* (2.07)	14* (11.63)	15* (11.63)	20* (9.11)

Note: The values are expressed as percentage (±standard deviation) of decrease in viability of treated compared to untreated-biofilms (*statistical significance of $P < 0.001$, one-way ANOVA, Tukey's test).

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