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Evaluation of the antifungal effect of EDTA, a metal chelator agent, on *Candida albicans* biofilm

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Abstract. – OBJECTIVE: ***Candida albicans*** **biofilm is frequently found on artificial surfaces and the infections related to biofilm are difficult to eliminate, as they require the removal of artificial devices and treatment with antifungal drugs. Nowadays, fungal growth in biofilms is difficult to eradicate with conventional antifungal drugs such as fluconazole. Among chelating agents, disodium salt-Ethylene Diamine Tetraacetic Acid (EDTA) is known to have antifungal activity. In this study, we examined the *in vitro* activity of the EDTA and the antifungal drug fluconazole against *C. albicans* mature biofilm.**

MATERIALS AND METHODS: ***C. albicans*** **ATCC 20191, fluconazole-susceptible strain, was grown at an inoculum starter of 1 x 10^6 cells/ml for 72 h in 24-well microtiter plates and was further treated for 24 h with EDTA and/or fluconazole. Antifungal activities in biofilms were expressed as reduction in optical density (OD) determined by a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetric assay and compared to untreated biofilms.**

RESULTS: Colorimetric readings revealed that EDTA alone (at 25 and 2.5 mM) significantly reduced fungal metabolic activity in preformed biofilms. Also, EDTA combined with fluconazole significantly reduced the growth of biofilm when compared to biofilm treated with fluconazole alone (at 25 and 2.5 µg/ml).

CONCLUSIONS: Our data suggest that the employment of EDTA or other chemicals destabilizers of the biofilm matrix, in combination with antifungal drugs, could lead to the development of new strategies for the management of infections associated to *Candida* biofilm. Another relevant result of our study suggests that the initial cell concentration, probably through mechanisms of quorum sensing, affects the cellular viability during the process of biofilm formation.

Key Words:
Biofilm, *Candida albicans*, EDTA, Fluconazole, XTT assay.

Introduction

Biofilms are complex three-dimensional structures formed by bacterial and/or fungal cells and by an extracellular polymeric matrix primarily composed of polysaccharides. The matrix may vary in composition and structure according to the organisms present[1].

Biofilm formation occurs when microorganisms attach on several surfaces including living tissues and abiotic medical surfaces. Therefore, its production appears to be a risk factor for the development of infections in patients with indwelling and medical devices, such as intravenous catheters, vascular prostheses, cerebrospinal fluid shunts, urinary catheters, orthopedic prostheses, biliary tract stents, dentures, endotracheal tubes, and others[2,3]. Also, periodontitis, chronic lung infection in cystic fibrosis, native valve endocarditis and chronic wound infections have also been acknowledged to be associated with biofilms[2,4].

Candida albicans is the most commonly isolated fungal species among the fungal pathogens involved in the formation of biofilms, but other species such as *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida dubliniensis* are also capable of producing biofilms[5-7].

The biofilm-forming ability of *C. albicans* contributes, most likely, to the high resistance of biofilms to antimicrobials and to high rates of nosocomial infections (up to 40%).

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The structural and protective role of *C. albicans* biofilm matrix has been described by many authors[8,9] and several studies suggest that beta-1,3-glucan synthesis during biofilm growth might prevent antifungal drug activity[10]. Further, a recent work[11] reported that about 87% of polysaccharides in the matrix included alfa-1,6 and alfa-1,2 branched mannans. These polysaccharides are associated with unbranched beta-1,6-glucans in a mannan-glucan complex. Moreover, other components of extracellular matrix are described such as beta-1,3-glucan, glycoproteins (55%), lipids (~15%), and nucleic acids mostly non-coding extracellular DNA (eDNA)[11,12].

To date, both beta-1,3-glucan and eDNA have been linked to poor antifungal activity of drugs such as fluconazole against *Candida* biofilm[8,13]. All these findings suggest that the biofilm matrix appears to be a very complex and specialized structure.

Furthermore, upregulation of efflux pumps and decreased cell membrane ergosterol content, described respectively in early and mature phases of *Candida* biofilm, may explain the poor activity of antifungals, targeting ergosterol (e.g., fluconazole and amphotericin B)[14-16]. Meanwhile, divalent ions such as calcium, magnesium and iron appear to stabilize the structure of the exopolymeric matrix through electrostatic interactions[17-19].

It is also known that anticoagulant and calcium and magnesium chelator EDTA (Ethylene Diamine Tetraacetic Acid) may have antimicrobial activity against several Gram-negative bacteria, staphylococci and *Candida* spp.[18-20]. In fact, EDTA has been shown to affect the structural integrity of the biofilm through its chelation of calcium, iron, and magnesium in *Staphylococci* and *Candid*a spp.[21] and to inhibit *C. albicans* biofilm formation through its inhibitory effect on filamentation[22,23].

The reported capability of EDTA to take over divalent cations is effective at both preventing biofilm formation and at treating established biofilm.

The observation that extracellular matrix represents a diffusion barrier for drugs and antibodies led us to investigate the *in vitro* toxicity of EDTA on the structure of biofilm.

It is known that both *in vitro* and *in vivo* biofilm development occurs in three stages: early (0-11 h), intermediate (12-30 h), and mature (31-72 h and over)[6,14]. Based on previous results, we hypothesized that the antibiofilm effect of EDTA, by disintegrating the extracellular matrix of the mature biofilms, may contribute to an increased diffusion of antibiotics as well as to an increased activity of antifungal drugs.

The aim of this study is to evaluate the *in vitro* antifungal activity of EDTA, alone or combined with fluconazole, on mature *C. albicans* biofilms (72 h).

Materials and Methods

Organisms and Growth Conditions

The yeast strain from American Type Culture Collection (ATCC) *Candida albicans* ATCC 20191 was used in this study. The reference strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as a quality control in antifungal susceptibility test. All strains were maintained at 4°C on Sabouraud Glucose Agar (SGA) slants (Difco, Becton Dickinson and Company, Sparks, MD, USA). For the experiments, all strains were subcultured on SGA slants at 37°C for 24 h. Then, yeast cells were seeded in assay media.

Antimicrobial Agents

Fluconazole powder was obtained from MP Biomedicals Europe (Asse-Relegem, Belgium). Stock solutions of fluconazole (1.0 mg/ml) were prepared in sterile saline and frozen at -80°C; 0.5 M disodium salt-Ethylene Diamine Tetraacetic Acid (EDTA) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and stored at room temperature.

Antifungal Susceptibility Tests

C. albicans ATCC 20191 was tested for *in vitro* susceptibility to fluconazole according to the European Committee on Antimicrobial Susceptibility Testing method (EUCAST Edef 7.2, 2012)[24-26]. Briefly, 96-well flat-bottom microtiter plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) containing serial dilutions of fluconazole (100 µl/well, range from 256 to 0.03 µg/ml) in Roswell Park Memorial Institute (RPMI) 1640 medium with 2.0% glucose, an inoculum of 0.5 to 2.5 × 10^5 cells/ml, and incubation at 35°C were used. Drug- and yeast-free controls were also prepared and quality control was ensured by testing *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

MIC values were determined spectrophotometrically (at 490 nm), after 24-h incubation, as

the lowest concentration of drug that resulted in ≥ 50% inhibition of growth about that of the growth control. The yeasts were classified as susceptible if the MIC was ≤ 2 µg/ml, and resistant if the MIC was > 4 µg/ml.

Biofilm Development and Optimization of Yeast Cells Inocula

C. albicans ATCC 20191 cells were inoculated in Yeast Extract-Peptone-Dextrose (YPD) broth (Difco, Becton Dickinson and Company, Sparks, MD, USA) and grown for 24 h at 28°C with shaking. The cell culture was harvested by centrifugation (2,500 *g* for 10 min at 4°C), washed twice with Phosphate Buffered Saline (PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO, USA) and diluted to final concentrations of 1, 2, 4, 8, 16, 32 × 10^6 cells/ml in RPMI 1640 medium containing L-glutamine, without sodium bicarbonate (Sigma-Aldrich) and buffered to pH 7.0 with 165 mM 3-[N-Morpholino]-propanesulfonic acid (MOPS; Sigma-Aldrich). The cell suspensions (500 µl) were dispensed in duplicate into each flat-bottom wells of sterile, polystyrene 24-well microtiter plates (Costar, Corning Incorporated, NY, USA) and incubated for 96 h at 37°C. RPMI medium without cells was also added as a negative control. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by washing the biofilms 3 times with 250 µl/well of sterile PBS. The viability of cells was then tested by a 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma) reduction assay, as described below.

The dilution exhibiting the highest biofilm metabolic activity was used for testing our antimicrobial agents.

XTT Reduction Assay

XTT reduction assay was performed on mature biofilms, which were obtained as previously described[27]. Briefly, a saturated solution of 0.5 g/l of XTT (Sigma) was prepared in PBS buffer. The solution was filter-sterilized through a 0.22 µm pore-size filter (Millex-GS, Carrigtwohill, Ireland), aliquoted, and stored at -70°C until use. Before each assay, an aliquot of this XTT stock was thawed, and menadione (Sigma-Aldrich; 10 mM stock solution in acetone, stored at -70°C) was added to obtain a final concentration of 1 µM. A 250 µl aliquot of the XTT-menadione solution was added to each prewashed biofilm and negative control wells to measure the background levels of XTT reduction. The plates were incubated in the dark for 3 h at 37°C. Following incubation, 100 µl of the obtained solution were transferred to a polystyrene flat-bottom 96-well microtiter plate (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). The colorimetric change in the XTT reduction assay, which directly correlates with the metabolic activity of the biofilm, was measured at 490 nm in a microtiter plate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA).

Antimicrobial Agents and Candida Biofilm

The antimicrobial activity of the divalent cation chelator EDTA and the antifungal fluconazole against *Candida* biofilms was tested by transferring 500 µl of a standardized cell suspension (1 × 10^6 cells/ml) in RPMI medium into each well of the polystyrene 24-well microtiter plates (Costar), and by incubating for 72 h at 37°C to allow the yeast to adhere to the surface of the wells. As negative controls, two wells of each plate were filled with the same volume of RPMI medium without cells. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by washing the biofilms three times with PBS (250 µl/well).

Then, each chemical agent (250 µl/well) diluted in RPMI medium was added to the biofilms: fluconazole was tested at final concentrations of 250, 25, and 2.5 µg/ml, and EDTA was tested at final concentrations of 25 and 2.5 mM. EDTA and fluconazole were tested alone and in combination, and RPMI 1640 broth without antimicrobials was added to a series of wells containing biofilm for controls. The plates were then incubated for a further 24 h at 37°C. Then, the medium was aspirated and all the wells were washed three times with PBS. To quantify anti-*C. albicans* biofilm activity the XTT colorimetric assay was used.

Light Microscopy

Microscopic examination of the mature biofilms in microtiter plates was performed by light microscopy (Olympus TH4-200 inverted microscope).

Statistical Analysis

All data were entered into an EXCEL database (Microsoft, Redmond, WA, USA) and the analysis was performed using the Statistical Package for the Social Sciences Windows, version 15.0 (SPSS Inc., Chicago, IL, USA). Descriptive sta-

tistics consisted of the mean ± standard deviation for parameter with Gaussian distribution (after confirmation with histograms and the Kolgomorov-Smirnov test) or occurrence frequencies, expressed as percentages. Comparison of Gaussian variables was performed with ANOVA one-way and multiple comparisons by Bonferroni test. A p-value of < 0.05 was considered statistically significant.

Results

Determination of MIC for Candida

Table I summarizes the *in vitro* susceptibilities of *C. albicans* ATCC 20191 and quality control strains to antifungal drug fluconazole as determined by EUCAST method. In this study, EUCAST broth microdilution method was used to evaluate the MICs. All strains were classified as susceptible (MIC ≤ 2 μg/ml) or resistant (MIC > 4 μg/ml), according to breakpoints recommended by EUCAST.

After 24 and 48 h of incubation, *C. albicans* ATCC 20191 (MIC ≤ 0.5 μg/ml) and *C. parapsilosis* ATCC 22019 (MIC ≤ 2 μg/ml) were fluconazole-susceptible, while *C. krusei* ATCC 6258 was fluconazole-resistant (MIC ≥ 64 μg/ml). The MIC values of the quality control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) fell within the established ranges published in the EUCAST guidelines.

Fungal Inocula and Efficiency of Forming Biofilm

C. albicans ATCC 20191 cells were evaluated for their capacity to form biofilm in polystyrene, flat-bottom 24-well microtiter plates (Costar). To determine the growth rate and the optimal concentration of metabolically active cells, a calibration curve was plotted.

The initial cellular concentrations for each well were: 1, 2, 4, 8, 16 and 32 × 10^6/ml. The plates were incubated for 96 h at 37°C, and colorimetric XTT reduction assay was used for quantification of *Candida* biofilm metabolic activity[27].

Notably, the change in cell number of the initial inocula significantly affects biofilm growth: the calibration curve in Figure 1 shows a significant increase in metabolic activity of mature biofilms obtained with a starter inoculum of 1 × 10^6 cells/ml. Moreover, increasing concentrations of initial inocula resulted in a proportional decrease of metabolic performance.

Effect of Antimicrobial Agents on Candida Biofilm

To test antimicrobial activity of EDTA and fluconazole, 72-h *Candida* biofilms were washed and overlaid with EDTA alone or fluconazole alone or EDTA combined with fluconazole, and incubated for an additional 24 h. The colorimetric change in the XTT assay, representing the ability of viable cells to metabolize tetrazolium salts, was then measured in a microtiter plate reader.

As shown in Figure 2, the treatment with EDTA at 2.5 mM and 25 mM promoted a significant decrease ($p < 0.05$) in the *Candida* biofilm

Table I. Fluconazole MICs at 24 and 48 h for *Candida albicans* ATCC 20191 strains used in this study, according to the EUCAST Edef 7.2 method.

Yeast strain	MIC (μg ml^{-1})	
	24 h	48 h
C. albicans ATCC 20191	0.5	0.5
C. krusei ATCC 6258	64	64
C. parapsilosis ATCC 22019	2.0	2.0

MIC (minimal inhibitory concentration) is the lowest fluconazole concentration at which a prominent decrease in turbidity is observed when compared with the drug-free control (50% growth inhibition). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 are quality control strains.

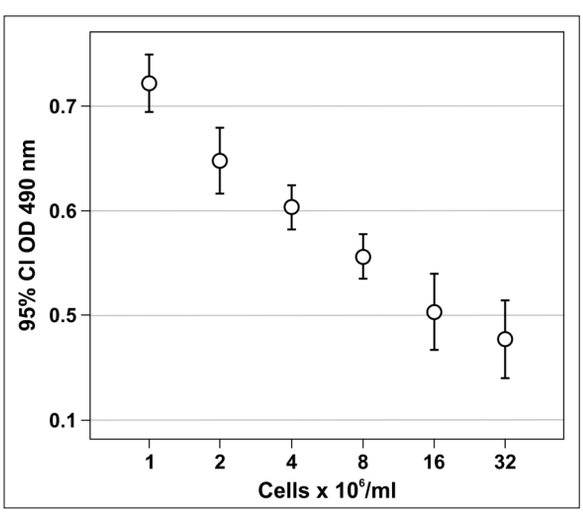

Figure 1. Growth profiles of *Candida albicans* biofilms after 96 h of incubation and at inocula starter from 1 to 32 × 10^6 cells/ml. Quantification of biofilms was performed by XTT reduction assay based on metabolic activity. Data in OD are means ± standard deviations of four independent experiments performed in duplicate (n = 54). A p value of < 0.05 was considered statistically significant. (*) = $p < 0.05$.

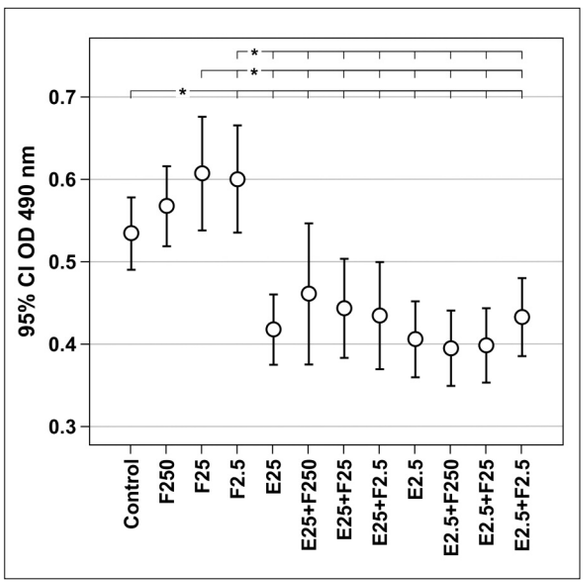

Figure 2. Effect of EDTA and fluconazole alone and in combination on *Candida albicans* mature biofilms (72 h) after 24 h of drug exposure (final stage of biofilm = 96 h). Control: growth of biofilm in the absence of antimicrobial compounds. Abbreviations: E25 (Ethylene Diamine Tetraacetic Acid 25 mM); E2.5 (Ethylene Diamine Tetraacetic Acid 2.5 mM); F250 (fluconazole 250 micrograms/ml); F25 (fluconazole 25 micrograms/ml); F2.5 (fluconazole 2.5 micrograms/ml). Data in OD are means ± standard deviations of four independent experiments performed in duplicate (n = 112). A p value of < 0.05 was considered statistically significant (*). $p < 0.05$: E25 *vs*. Control; E2.5 *vs*. Control; E25+F25 vs. F25; E25+F2.5 *vs*. F2.5; E2.5+F250 *vs*. F250; E2.5+F25 vs. F25; E2.5+F2.5 *vs*. F2.5.

viability compared to the control (*Candida* alone), whereas the growth in response to fluconazole alone (250, 25 and 2.5 µg/ml) was higher than the control, even though not statistically significant. Relating to combination treatment (EDTA plus fluconazole), the exposure to EDTA at 2.5 mM plus fluconazole (250, 25 and 2.5 µg /ml) resulted in OD similar to that of EDTA at 2.5 mM ($p < 0.05$ *vs*. control or *vs*. fluconazole alone). Also, a significant difference was observed after exposure to EDTA at 25 mM plus fluconazole at 25 and 2.5 µg/ml ($p < 0.05$ *vs*. control or *vs*. fluconazole at 25 and 2.5 µg/ml). However, the combination of EDTA at 25 mM and fluconazole (250 µg /ml) did not show significant differences *vs*. control or *vs*. fluconazole at 250 µg/ml.

Biofilm Images

Microscopic images of biofilms revealed that cell density decreased when biofilm cells were treated with EDTA alone or in combination with fluconazole. These data confirm XTT readings. A representative example of four experiments is shown in Figure 3.

Discussion

Biofilm formation by *Candida* spp. is a key factor for colonizing and causing disease in humans. *C. albicans* biofilm development is a multistep process starting with an adherence phase followed by proliferation of attached cells. Subsequently, a maturation phase is associated with the formation of pseudo-hyphae and hyphae, and the secretion of extracellular matrix components[28].

Nowadays, *C. albicans* biofilms are highly resistant to most of the antifungal agents including a commonly used drug such as fluconazole. In particular, it is known that several components of extracellular matrix, upregulation of efflux pumps, decreased cell membrane ergosterol content and persister cells are implicated in the high tolerance and/or resistance of biofilm to azole drugs[8,13,14,29-32].

Among synthetic chelating agents, EDTA shows a high activity against fungal and bacterial biofilms. Thus, we examined the *in vitro* effects of EDTA alone and in combination with fluconazole on *Candida* preformed biofilms. In our study we employed a colorimetric method (XTT) to test biofilm growth. With this method, the measured growth rates represent the real metabolic activity of viable cells: we observed that, after 96 h of incubation, higher biofilm metabolic activity was obtained with an initial inoculum of 1×10^6 cells/ml. This result may suggest that, for biofilm generation, an optimal number of cells that cooperate to provide favorable environmental conditions for efficient growth is needed. For this purpose, it has been shown that a major process related to the population density of microorganisms may be the release of quorum sensing molecules (QS)[33]. In our study we demonstrated that the coordinated expression of QS molecules after the starter inoculum could modulate the growth rate of mature biofilm. Therefore, the progressively decreased viability of cells by augmenting the initial inocula, confirms that the development of biofilm during the maturation stage is a coordinated process in which a definite number of cells cooperates to maintain a viable biomass.

We also evaluated the antifungal activity of EDTA and the potential synergistic effect on

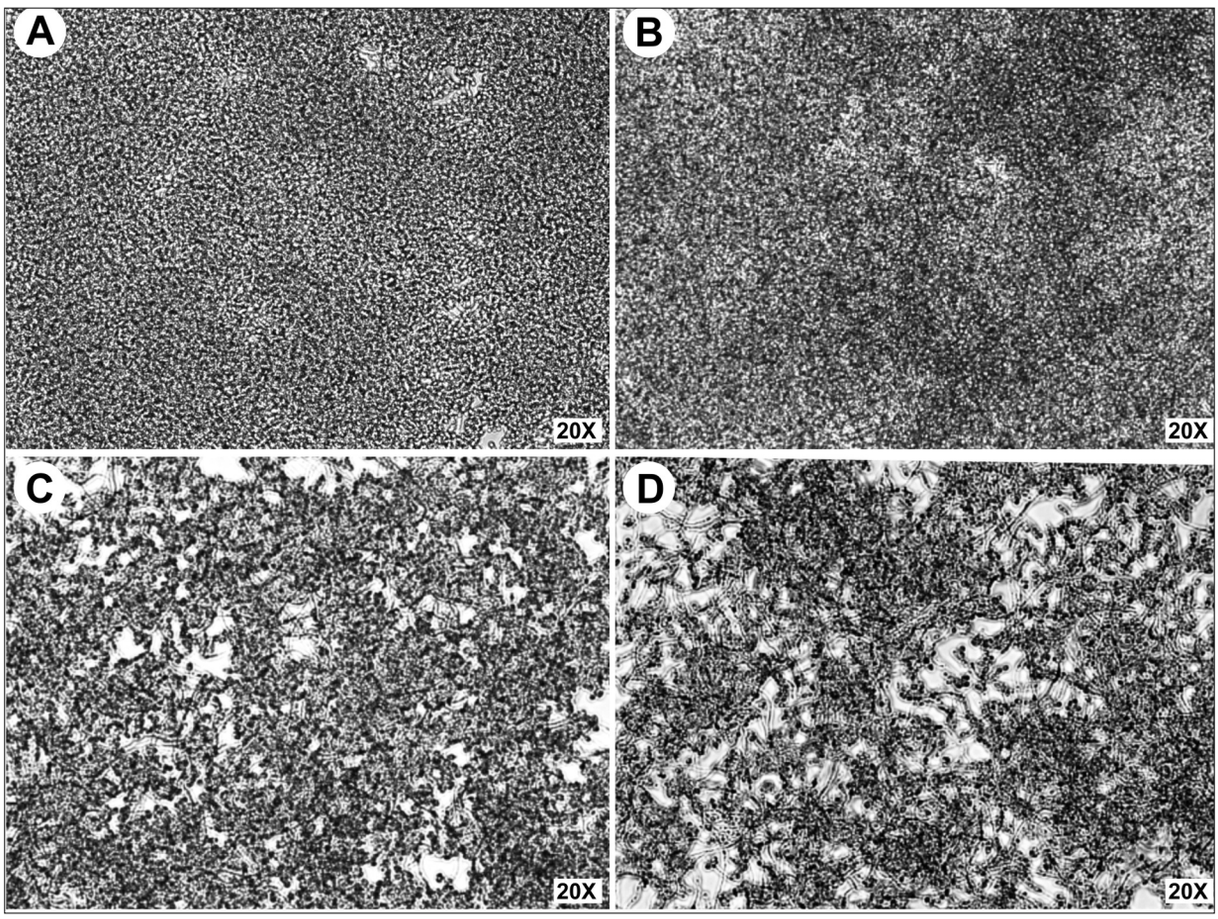

Figure 3. Microscopic images of *Candida albicans* biofilms in 24 - wells microtiter plates at 20× magnification. The images are a representative example of four experiments conducted in duplicate. *C. albicans* biofilms were grown for 72 h. Thereafter, the biofilms were treated for further 24 h with EDTA alone and in combination with fluconazole (final stage of biofilm = 96 h). ***A,*** *C. albicans* untreated; ***B,*** *C. albicans* + fluconazole 25 micrograms/ml; ***C,*** *C. albicans* + EDTA 25 mM; ***D,*** *C. albicans* + EDTA 25 mM plus fluconazole 25 micrograms/ml.

Candida biofilms when combined with fluconazole. In our experimental model, we employed the *C. albicans* ATCC 20191 strain, fluconazole-susceptible. Our data indicate that the biofilm viability was significantly reduced by EDTA alone (at 2.5 and 25 mM). This effect may be explained by the chelating activity of EDTA on the biofilm matrix. On the contrary, fluconazole at concentration five, fifty and five hundred times the MIC (2.5, 25 and 250 μg/ml) did not show any ability to inhibit biofilm growth. We suggest that the cell viability of biofilms treated with fluconazole could be due to a selection of drug-resistant cells or to the proliferation of preexisting persister cells: specific gene expression patterns in the biofilm mass, after fluconazole treatment, may be implicated. Finally, we did not detect synergistic activity of EDTA plus fluconazole, since the biofilm viability was similar to that observed using EDTA alone.

We speculated that EDTA could have some effect on fluconazole activity, as compared with fluconazole alone; however, we observed that the combination of EDTA at 25 mM plus fluconazole at 250 μg/ml did not reduce biofilm metabolic activity. One possible explanation of this result is that the complex geometry of the biofilm could preclude the access of chemicals in a concentration-dependent way. Further studies are necessary to elucidate the mechanisms underlying this effect.

Conclusions

Our data may suggest that experimental models employing EDTA in combination with suit-

able antifungal drugs are promising to eradicate biofilm formation on implanted biomaterials. The results of this study can open an encouraging research direction for the management of *Candida* infections frequently associated with contamination of indwelling medical devices.

Declaration of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and the writing of this article.

References

1) Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol 2006; 55: 999-1008.
2) Wu H, Moser C, Wang HZ, Høiby N, Song ZJ. Strategies for combating bacterial biofilm infections. Int J Oral Sci 2015; 7: 1-7.
3) Vandecandelaere I, Coenye T. Microbial composition and antibiotic resistance of biofilms recovered from endotracheal tubes of mechanically ventilated patients. Adv Exp Med Biol 2015; 830: 137-155.
4) Stewart PS, Costerton JW. Antibiotic resistance of bacteria biofilms. Lancet 2001; 358: 135-138.
5) Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother 2002; 46: 1773-1780.
6) Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. Adherence and biofilm formation of non-*Candida albicans Candida* species. Trends Microbiol 2011; 19: 241-247.
7) Villar-Vidal M, Marcos-Arias C, Eraso E, Quindós G. Variation in biofilm formation among blood and oral isolates of *Candida albicans* and *Candida dubliniensis*. Enferm Infecc Microbiol Clin 2011; 29: 660-665.
8) Nett JE, Sanchez H, Cain MT, Ross KM, Andes DR. Interface of *Candida albicans* biofilm matrix-associated drug resistance and cell wall integrity regulation. Eukaryot Cell 2011; 10: 1660-1669.
9) Vediyappan G, Rossignol T, d'Enfert C. Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. Antimicrob Agents Chemother 2010; 54: 2096-2111.
10) Mitchell KF, Taff HT, Cuevas MA, Reinicke EL, Sanchez H, Andes DR. Role of matrix β-1,3 glucan in antifungal resistance of non-*albicans Candida* biofilms. Antimicrob Agents Chemother 2013; 57: 1918-1920.
11) Zarnowski R, Westler WM, Lacmbouh GA, Marita JM, Bothe JR, Bernhardt J, Lounes-Hadj Sahraoui A, Fontaine J, Sanchez H, Hatfield RD, Ntambi JM, Nett JE, Mitchell AP, Andes DR. Novel entries in a fungal biofilm matrix encyclopedia. MBio 2014; 5: e01333-14.
12) Lopez-Ribot JL. Large-scale biochemical profiling of the *Candida albicans* biofilm matrix: new compositional, structural, and functional insights. MBio 2014; 5: e01781-14.
13) Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, Oliveira R. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. Mycopathologia 2010; 169: 323-331.
14) Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. Infect Immun 2003; 71: 4333-4340.
15) d'Enfert C. Biofilms and their role in the resistance of pathogenic *Candida* to antifungal agents. Curr Drug Targets 2006; 7: 465-470.
16) Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of *Candida* biofilm drug resistance. Future Microbiol 2013; 8: 1325-1337.
17) Raad I, Hanna H, Dvorak T, Chaiban G, Hachem R. Optimal antimicrobial catheter lock solution, using different combinations of minocycline, EDTA, and 25-percent ethanol, rapidly eradicates organisms embedded in biofilm. Antimicrob Agents Chemother 2007; 51: 78-83.
18) Percival SL, Kite P, Eastwood K, Murga R, Carr J, Arduino MJ, Donlan RM. Tetrasodium EDTA as a novel central venous catheter lock solution against biofilm. Infect Control Hosp Epidemiol 2005; 26: 515-519.
19) Cavaliere R, Ball JL, Turnbull L, Whitchurch CB. The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable *Hemophilus influenzae* biofilms to treatment with ampicillin and ciprofloxacin. Microbiologyopen 2014; 3: 557-567.
20) Gil ML, Casanova M, Martínez JP. Changes in the cell wall glycoprotein composition of *Candida albicans* associated to the inhibition of germ tube formation by EDTA. Arch Microbiol 1994; 161: 489-494.
21) Raad I, Chatzinikolaou I, Chaiban G, Hanna H, Hachem R, Dvorak T, Cook G, Costerton W. In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. Antimicrob Agents Chemother 2003; 47: 3580-3585.
22) Banin E, Brady KM, Greenberg EP. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl Environ Microbiol 2006; 72: 2064-2069.

23) Al-Akeel RA, El-Kersh TA, Al-Sheikh YA, Al-Ahmadey ZZ. Heparin-benzyl alcohol enhancement of biofilms formation and antifungal susceptibility of vaginal *Candida* species isolated from pregnant and nonpregnant Saudi women. Bioinformation 2013; 9: 357-362.

24) Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMI European Committee for Antimicrobial Susceptibility Testing (EUCAST). EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect 2008; 14: 398-405.

25) Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W, EUCAST-AFST. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 2012; 18: E246-E247.

26) Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope WW, European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST). EUCAST technical note on *Candida* and micafungin, anidulafungin and fluconazole. Mycoses 2014; 57: 377-379.

27) Pierce CG, Uppuluri P, Tristan AR, Wormley FL Jr, Mowat E, Ramage G, Lopez-Ribot JL. A simple and reproducible 96 well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 2008; 3: 1494-1500.

28) Polke M, Hube B, Jacobsen ID. *Candida* survival strategies. Adv Appl Microbiol 2015; 91: 139-235.

29) LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother 2006; 50: 3839-3846.

30) Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 2007; 5: 48-56.

31) Iñigo M, Pemán J, Del Pozo JL. Antifungal activity against *Candida* biofilms. Int J Artif Organs 2012; 35: 780-791.

32) Casalinuovo IA, Di Francesco P, Garaci E. Fluconazole resistance in *Candida albicans*: a review of mechanisms. Eur Rev Med Pharmacol Sci 2004; 8: 69-77.

33) Hogan DA. Talking to themselves: autoregulation and quorum sensing in fungi. Eukaryot Cell 2006; 5: 613-619.