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Adhesion of *Candida* biofilm cells to human epithelial cells and polystyrene after treatment with silver nanoparticles

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ABSTRACT

This study investigated the adhesion to human epithelial cells and polystyrene surface of viable yeasts recovered from *Candida* biofilms treated with silver nanoparticles (SN). Biofilm resuspended *Candida* cells were added to HeLa cells or to empty wells of microtiter plates and the adhesion was verified using crystal violet staining. The adhesion of *Candida* cells was significantly reduced, mainly when biofilms were pretreated with 54 μg/mL SN. These new findings allow to conclude that SN may induce changes in viable yeasts, which can decrease the dissemination of *Candida* infections, mainly in susceptible patients.

1. Introduction

Candida albicans followed by *Candida glabrata* are the major commensal pathogens that cause fungal infections in compromised patients, such as oral or gastrointestinal candidiasis, urinary tract infections (mainly vulvovaginal candidiasis) [1] and systemic or bloodstream infections [2]. The pathogenicity of these microorganisms is related to several virulence factors, including their ability to adhere to host epithelial cells and/or inanimate substrates, which is an important precondition for colonization, infection and biofilm formation [3,4]. Additionally, *Candida* biofilms are resistant to a diversity of antimicrobial agents [4], and the fungal cells released from biofilms and which return to the planktonic state may act as a continuous source of disseminated infections.

In the light of these considerations, there is a significant interest in the use of alternative antifungal agents which might decrease or inhibit fungal adhesion capacity, preventing the colonization and infection by *Candida* species. Our research group verified that silver nanoparticles (SN) were more effective in inhibiting biofilm formation when applied in prophylaxis than on pre-formed *Candida* biofilms [5], and that the particle size and the type of stabilizing agent did not interfere in the antifungal activity of SN against those biofilms [6]. Thus, with the purpose of complementing these previous data, in this study we tested the hypothesis that *C. albicans* and *C. glabrata* viable cells recovered from *Candida* biofilms treated with SN exhibit significantly reduced adhesion capacity to human epithelial cells and polystyrene surfaces.

2. Materials and methods

2.1. Synthesis of silver colloidal nanoparticles

In this research, the average size of SN used was approximately 5 nm. These nanoparticles were synthesized, stabilized and characterized as described previously [5].

2.2. Human epithelial cells

A monolayer of epithelial cells from a HeLa cell line with origin in human cervical carcinoma (obtained from Gulbenkian Institute of Science, Lisbon, Portugal) was grown (at 37 °C in 5% CO_2) in Dulbecco's modified Eagle's medium (D-MEM; Gibco, Carlsbad,

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USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA). When the cultures reached 80% of confluence, the cells were detached using a 25% trypsin-EDTA (Gibco, USA) solution. The cell concentration was adjusted to 10^6 cells/mL with fresh D-MEM without P/S and then transferred to a 24-well microtiter plate. Prior to the adhesion tests, the wells were washed twice with phosphate buffered saline (PBS; pH 7, 0.1 M).

2.3. Candida strains and growth conditions

Two *Candida* strains were evaluated. *C. albicans* 324LA/94 and *C. glabrata* D1, which are oral clinical isolates obtained from the culture collection of Cardiff Dental School, Cardiff, UK, and from the Biofilm Group of the Centre of Biological Engineering, University of Minho, Braga, Portugal, respectively. They were grown overnight in Sabouraud dextrose broth (Liofilchem, Roseto degli Abruzzi, Italy) medium at 37 °C and 120 rpm, harvested by centrifugation (8000 rpm for 5 min at 15 °C), and washed twice in PBS. Then, fungal cells were counted using a Neubauer chamber and resuspended in mucin-containing artificial saliva medium [5] to achieve 10^7 cells/mL.

2.4. Biofilm formation and treatment with SN

Candida biofilms were produced in 6-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). Aliquots of 4 mL of standardized cell suspensions, as prepared above, were transferred to each well of a microtiter plate. The plates were incubated for 48 h at 37 °C in an orbital shaker at 120 rpm, and the artificial saliva medium was renewed every 24 h. After the incubation stage, the medium was aspirated and the biofilms were washed with 4 mL of PBS in order to remove loosely adherent cells prior to SN treatment. SN suspensions were diluted in RPMI 1640 medium (Sigma–Aldrich, St Louis, USA) and the biofilms were then treated with SN at 13.5 and 54 μg/mL for 24 h at 37 °C (at 120 rpm). Appropriate untreated controls were also included.

After treatment with SN, the resulting biofilms were washed as described above and then 1 mL of PBS was added to each well and the biofilms were carefully scraped off the well walls using sterilized cell scrapers (Orange Scientific, Braine-l'Alleud, Belgium). The biofilm suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W and gently vortexed for 2 min to disrupt the aggregates. Following, for enumeration of viable cells, 0.5 mL of each biofilm suspension were mixed with 0.1 mL of 0.4% trypan blue stain (Sigma–Aldrich, USA). After 5 min, the number of viable cells was adjusted to a concentration of 10^7 cells/mL in RPMI 1640 medium, using a Neubauer chamber. These *Candida* cell suspensions were used in the succeeding assays.

2.5. Candida biofilm cells adhesion assay

A volume of 0.5 mL of viable yeast suspension prepared as above was added to the wells of the 24-well plate covered with a monolayer of human epithelial cells or to empty wells of 24-well plates (to study adhesion to polystyrene). In both conditions the plates were incubated at 37 °C for 2 h. Next, the wells were washed once with PBS to remove non- adherent yeasts.

The quantification of adhered yeasts to epithelial cells and to the polystyrene surfaces was carried out by the crystal violet staining method, as described by Negri et al. [7] and Monteiro et al. [5], respectively. For adhesion assays, the mean absorbance of yeasts was standardized by number of adhered yeasts per area of the well using *C. albicans* 324LA/94 and *C. glabrata* D1 standard curves [7].

2.6. Statistical analysis

In all cases, the assays were performed in triplicate on three separate occasions. The results were analyzed using ANOVA with Bonferroni test. A p value < .05 was taken to be statistically significant. The statistical analysis was obtained in the SPSS software (SPSS - Statistical Package for the Social Sciences, Inc., Chicago).

3. Results and discussion

As it is possible to observe in Fig. 1a and b, compared to the control groups (biofilm cells without SN treatment), *C. albicans* 324LA/94 and *C. glabrata* D1 biofilm cells showed reductions in the capacity to adhere to HeLa cells by around 53% ($p = .001$) and 34% ($p < .001$) when pretreated with silver at 13.5 μg/mL and around 40% ($p = .003$) and 86% ($p < .001$) at a silver concentration of 54 μg/mL, respectively.

The number of yeast cells adhered to polystyrene surfaces is presented in Fig. 1c and d. *C. albicans* 324LA/94 and *C. glabrata* D1 biofilm cells exhibited reductions in adhesion to polystyrene by around 43% ($p < .001$) and 34% ($p = .013$) when pretreated with silver at 13.5 μg/mL and around 70% ($p < .001$) and 46% ($p = .006$) at a silver concentration of 54 μg/mL, respectively, compared to controls. While for *C. albicans* 324LA/94 the adhesion inhibition is dose dependent, for *C. glabrata* D1 the pretreatment with the higher concentration of silver showed no significant difference in the adhesion capacity when compared to the group pretreated with the lower concentration ($p > .05$). It is worth noting that the silver concentrations used herein were unable to completely eradicate *C. albicans* 324LA/94 and *C. glabrata* D1 biofilms [5,6].

The adhesion capacity of yeasts is related to several factors, including non-specific ones, such as cell surface hydrophobicity and electrostatic interactions (zeta potential) between microbial cells and substrate surfaces [8], or specific factors, such as connection of cell wall adhesins to specific binders [9]. The phenomenon of adhesion to inert surfaces (polystyrene) is commanded by physicochemical properties of yeast cell surfaces [10]. Thus, the reduction in adhesion to polystyrene could have occurred due to alterations on electrostatic and electrodynamic properties of live yeast cell surfaces generated by the action of SN. Probably, these nanoparticles reduced the relative cell surface hydrophobicity.

Among the factors affecting *Candida* adhesion to epithelial cells are the peripheral proteins called adhesins. Several *Candida* adhesins have been identified and the adhesin Eap1p plays an important role in adherence to both mammalian cells (HeLa cells) and polystyrene [11]. During the adhesion of *Candida* cells to epithelial cells, the yeast cells produce an extracellular polymeric material containing a mannoprotein adhesion [12]. Miyauchi et al. [12] demonstrated that the adhesion of *Candida* to HeLa cells was regulated by the protein parts of the mannoprotein and no involvement by the polysaccharide parts was evidenced. On the other hand, although the mechanism of action of SN is multifactorial, these nanoparticles preferably bind to sulphur-containing proteins and deactivate their enzymatic functions [13]. In this context, as silver has an affinity for proteins, one reason for the reduction of adhesion to HeLa cells obtained may be the binding of SN to adhesins present in the yeast cells. Moreover, we also believe that, probably, SN interfered with the expression or synthesis of adhesins on the viable yeasts [14,15]. Interspecies differences were also found in the extension of adhesion to HeLa cells. It is highly likely that these results are due to the inherent physiological differences among the species tested. Accordingly, literature data show that *C. glabrata* does not adhere well to oral keratinocytes, but adheres better to denture surfaces, when compared with *C. albicans* [16]. Thus, these features may hinder or facilitate the action of SN in reducing adhesion of *Candida* strains to HeLa cells.

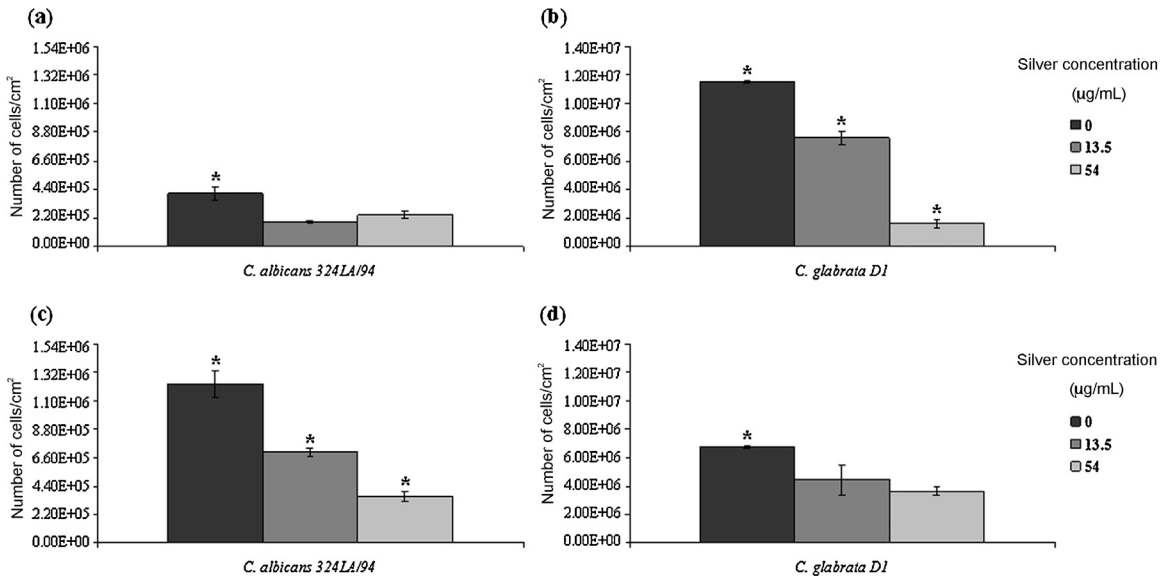

Fig. 1. Adhesion capacity (2 h) of viable cells recovered from *Candida* biofilms pretreated with two different silver nanoparticles concentrations (13.5 and 54 μg/mL) to HeLa cells (a and b) and polystyrene surfaces (c and d), measured by crystal violet staining assay and standardized as cells/cm^2 ($n = 9$). Error bars symbolize the standard deviations of the means. $^*p < .05$ between the different pretreatments for the same species.

Furthermore, this study demonstrated that the amount of adhered yeasts varies according to the type of substrate (epithelial cells and polystyrene surfaces). For *C. albicans* 324LA/94, the untreated and pretreated with SN at 13.5 μg/mL groups showed a significantly higher capacity to adhere to polystyrene than to HeLa cells ($p < .001$). At a SN concentration of 54 μg/mL there was no significant difference in the amount of adhered yeasts to HeLa cells and to polystyrene ($p = .211$). Interestingly, for *C. glabrata* D1, it was possible to verify that the control group and the group pretreated with SN at 13.5 μg/mL demonstrated a significantly higher capacity to adhere to HeLa cells than to polystyrene ($p < .001$). However, when biofilm cells were pretreated with SN at 54 μg/mL, the extent of adhesion to polystyrene was significantly higher ($p = .005$). These results may indicate differences between the isolates tested in terms of adhesion mechanisms, hydrophobic nature after treatment with SN and number and type of adhesins involved in the adhesion. All these factors may also be related to different infective abilities of *Candida* isolates.

Importantly, this work revealed the ability of SN to affect an important virulence factor of *Candida* species – surface colonization. Thus, the findings of the present study taken together with our previous results [5,6] demonstrate an obvious dual function of SN: anti-adhesion and antifungal effects against *C. albicans* 324LA/94 and *C. glabrata* D1.

4. Conclusion

In conclusion, our results sustain the hypothesis that the pretreatment of *Candida* biofilm cells with SN significantly reduces the subsequent adhesion capacity of *C. albicans* 324LA/94 and *C. glabrata* D1 viable cells to human epithelial cells and polystyrene surfaces. These new findings highlight the potential use of SN in controlling the dissemination of *Candida* infections, especially in susceptible patients. However, additional studies with a wide number of strains, and directed to investigate the accurate mechanisms by which adhesion capacity of *Candida* cells is decreased after treatment with SN, are needed.

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