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# Stability and efficacy of combined nystatin and chlorhexidine against suspensions and biofilms of *Candida albicans*

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ABSTRACT

*Objective:* Nystatin and chlorhexidine are extensively used in oral medicine; however, there is some controversy about the possibility of these drugs showing antagonism. To clarify this issue, this study investigated the efficacy and stability of nystatin and chlorhexidine in combination.
*Design:* An *in vitro* study was conducted to analyze the effect of nystatin and chlorhexidine combined on *Candida albicans* ATCC 18804, using the drugs mixed as a single formulation and as independent formulations used sequentially with different time intervals between them. The minimum inhibitory concentration (MIC) and effects on *C. albicans* suspensions and biofilms were evaluated. Also, the stability of nystatin and chlorhexidine in a mixture was tested by high performance liquid chromatography (HPLC).
*Results:* When nystatin and chlorhexidine were mixed in a single formulation, there was no significant difference in MIC compared to that of the drugs used alone (as the only treatment). However, when these drugs were used as independent formulations, sequentially with time intervals in between, their MICs were higher than the respective MIC of the drug used alone, except for the MIC of chlorhexidine with a 10-min interval. Nystatin/chlorhexidine combinations showed lower activity against *C. albicans* biofilms, except for that with a 30-min interval. The drugs when combined showed high percentages of degradation at all the times evaluated.
*Conclusions:* The combination of nystatin and chlorhexidine seems to interfere with the efficacy of the drugs and to increase their rate of degradation.

## 1. Introduction

Candidiasis is the most prevalent fungal infection in the oral cavity of humans. Even though most cases are associated with *Candida albicans,* other non-albicans species such as *C. glabrata*, *C. tropicalis, C. parapsilosis* and *C. krusei* have also been found to be pathogenic (Barkvoll & Attramadal, 1989; Ellepola, 2005). This disease is an opportunistic infection, which has predisposing factors such as hyposalivation, alcohol and tobacco use and ill-fitting and poorly cleaned dentures (Garcia-Cuesta, Sarrion-Pérez, & Bagán, 2014), and heredity, severe nutritional deficiencies, immunosuppressive conditions and endocrine disturbances including diabetes mellitus (Akpan & Morgan, 2002; Barkvoll & Attramadal, 1989; Ellepola, 2005; Garcia-Cuesta et al., 2014; Olczak-Kowalczyk et al., 2015). The treatment of oral candidiasis requires the avoidance or control of predisposing factors and administration of antifungal drugs, either topical or systemic. These drugs are classified into polyenes and azole group antimycotics, where the latter include imidazoles and triazoles (Ellepola, 2005).

Nystatin is an antimicrobial agent with both fungicidal and fungistatic properties, which since its discovery in 1951, has been used in topical formulations to treat oral candidiasis. The drug is obtained by fermentation using cultures of *Streptomyces noursei* (Sklenár, Scigel, Horácková, & Slanar, 2013). Since it is a polyene, it binds to ergosterol, a component of the fungal cytoplasmic membrane, and causes changes in cell permeability (Akpan & Morgan, 2002; Brescansin, Portilho, Teixeira, & Benedito, 2013). Nystatin has a local effect with low risk of hepatotoxicity (Garcia-Cuesta et al., 2014), since it is poorly absorbed in the gastrointestinal tract. Moreover, drug interactions and adverse effects have not been reported (Sklenár et al., 2013).

Chlorhexidine, in turn, is an antiseptic compound that can also be used as an adjuvant to specific antifungal agents (Barkvoll & Attramadal, 1989; Calamari, Bojanich, Barembaum, Berdicevski, &

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Azcurra, 2011; Fathilah, Himratul-Aznita, Fatheen, & Suriani, 2012; Nittayananta et al., 2008). Chlorhexidine was developed in 1940 in England; however, its potential for inhibiting dental biofilm was not investigated until 1969 by Schroeder (Balagopal & Arjunkumar, 2013). It is a cationic compound that forms low solubility salts with anions such as phosphate, sulfate, and chloride (Barkvoll, Rölla, & Bellagamba, 1988). Chlorhexidine digluconate is an antiseptic agent with a broad antibacterial spectrum, which acts against Gram-positive and Gram-negative bacteria and some fungi (Varoni, Tarce, Lodi, & Carrassi, 2012). Its formulations have been widely used to chemically control dental biofilm (Adams & Mouthrinses, 1994) and to treat oral cavity infections, including those affecting patients in intensive care units. Patients with hyposalivation caused either by head and neck radiation therapy or other factors, and those receiving chemotherapy as well have been treated with chlorhexidine, especially when showing high risk of periodontal disease, dental caries, and mucositis. Furthermore, chlorhexidine is recommended as an antiseptic in oral surgery procedures and as a cleaning agent for dental prostheses (Lambert, Morris, & Ochi, 1997; Pusateri, Monaco, & Edgerton, 2009; Varoni et al., 2012).

There are reports contraindicating the simultaneous use of nystatin and chlorhexidine. According to these reports, their combination would result in the formation of a salt, which would lead to the loss of desired pharmacological effects (Barkvoll & Attramadal, 1989; Epstein, Vickars, Spinelli, & Reece, 1992; Gaibi, 2006). Barkvoll and Attramadal (1989) tested, *in vitro,* the efficacy of the combined use of these compounds against cultures of *C. albicans*. The authors observed a higher minimum inhibitory concentration (MIC) for the combined drugs than the drugs alone, suggesting that chlorhexidine digluconate and nystatin are both inhibitors of *C. albicans* but act antagonistically. Nevertheless, these authors used the combined drugs in equal volumes in a single formulation, without testing them alone according to an alternate schedule of use at different times, like in routine clinical practice. Moreover, the effects of the combined drugs on biofilms of *C. albicans* have not been tested and the inference regarding salt formation as being responsible for the decrease in drug efficacy was based on the chemical structure of the drugs and the appearance of precipitate in the combined formulation. No chemical analysis was preformed.

Nystatin and chlorhexidine are extensively used in oral medicine, and may be administered simultaneously to hospitalized immunosuppressed patients. Even though there are some recommendations about the need of respecting a 30-min interval between the use of chlorhexidine and toothpaste (De Paola & Spolarich, 2007), the literature does not report recommendations concerning a safe time interval between chlorhexidine and nystatin. Moreover, there is a lack of studies investigating the efficacy of their combination in a systematic and standardized way. Therefore, the aim of the present study was to investigate, *in vitro,* the efficacy of nystatin and chlorhexidine in combination against *C. albicans* suspensions and biofilms, and also to evaluate the chemical interaction between these two compounds.

## 2. Material and methods

### *2.1. Study design*

An *in vitro* study was conducted to evaluate the effect of nystatin and chlorhexidine combined on *C.albicans.* MIC was determined by the microdilution broth method, and fungal growth inhibition was evaluated in either fungal planktonic colonies or in biofilms. Also, the drugs were analyzed by HPLC to investigate their stability in combination.

### *2.2. Microbiological assays*

A 2 mg/mL stock solution of nystatin (Fagron, Barsbüttel, Germany) in 1% dimethylsulfoxide (DMSO, Dinâmica Química Contemporânea Ltda, São Paulo, SP, Brazil) (Carrillo-Muñoz et al., 1999), and a 20% aqueous solution of chlorhexidine digluconate (All Chemistry, São Paulo, SP, Brazil) were prepared. The experimental groups were allocated, according to treatment: (1) negative control (n = 10): *C. albicans* treated with saline; (2) nystatin (n = 10): *C. albicans* treated with nystatin; (3) chlorhexidine (n = 10): *C. albicans* treated with chlorhexidine; (4) nystatin/chlorhexidine single (n = 10): *C. albicans* treated with nystatin and chlorhexidine mixed in a single formulation; (5) nystatin/2 min/chlorhexidine: *C. albicans* treated with nystatin and, after 2 min, with chlorhexidine (n = 10); (6) chlorhexidine/1 min/nystatin: *C. albicans* treated with chlorhexidine and, after 1 min, with nystatin (n = 10); (7) nystatin/10 min/chlorhexidine: *C. albicans* treated with nystatin and, after 10 min, with chlorhexidine (n = 10); (8) chlorhexidine/10 min/nystatin: *C. albicans* treated with chlorhexidine and, after 10 min, with nystatin (n = 10); (9) nystatin/30 min/chlorhexidine: *C. albicans* treated with nystatin and, after 30 min, with chlorhexidine (n = 10); (10) chlorhexidine/30 min/nystatin: *C. albicans* treated with chlorhexidine and, after 30 min, with nystatin (n = 10).

The time intervals of 2 min and 1 min were based on the recommended time of the mouthrinse respectively for nystatin and chlorhexidine. That is, there was no interval between the drugs except for the time that the patient would spend with the mouthrinse. The time of 30 min, in turn, was chosen considering that this is the time to be respected for the use of chlorhexidine before or after brushing (De Paola & Spolarich, 2007), while the interval of 10 min was chosen as an intermediate period.

#### *2.2.1. Preparation of Candida albicans inoculum*

Cultures of *C. albicans* strain ATCC 18804 were prepared in Sabouraud dextrose agar (SDA), consisting of 2% D-glucose (Oxoid Ltd, Basingstoke, Hampshire, UK), 1% peptone (Merck, Darmstadt, Germany) and 1.5% bacteriological agar (Himedia, Mumbai, India). The inoculum was prepared by selecting 5 colonies each greater than 1 mm in diameter, which were suspended in sterile saline, and mixed for 15 s to obtain a turbidity of 0.5 on the McFarland scale. The suspensions were also quantified with a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 530 nm and adjusted to an optical density (OD) of 0.11–0.14.

#### *2.2.2. Broth microdilution/MIC*

MIC assays were performed by the broth microdilution method, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). An initial inoculum of 1 to $5 \times 10^6$ CFU/mL was diluted 1:1000 in RPMI 1640 medium with glutamine and no bicarbonate (Gibco, Grand Island, NY, USA), adjusted to 1 to $5 \times 10^3$ CFU/mL and transferred to 96-well microplates. The drugs were added to the microplates using twofold serial dilutions with concentrations varying from 0.25 to 32 μg/mL for nystatin and 0.5 to 64 μg/mL for chlorhexidine (CLSI, 2008). The same process was repeated performing the different treatments according to the group distribution. Afterwards, the OD of *C. albicans* suspensions was determined with a spectrophotometer (SpectraMax 190) at 530 nm. MIC was defined as the lowest concentration of the drug that was capable of reducing fungal growth by 50% at 48 h.

#### *2.2.3. C. albicans biofilm formation and treatment*

*C. albicans* was inoculated in 2% YPD broth (2% D-glucose, 2% peptone, 1% yeast extract), in 8 tubes (5 mL each), and incubated on a shaker at 125 rpm for 18 h overnight at 30 °C. The OD of the inoculum was then read at 570 nm, and the OD of the samples was adjusted to 0.800–1. Cultures were distributed in 10 microtubes (1.5 mL each) and the samples centrifuged. The supernatant was removed, and the cell pellet was resuspended in 1 mL of sterile PBS and vortexed. The cells were centrifuged and resuspended 3 times, where the last suspension was made in 8% YPD. A 100-μL aliquot of cell suspension was added to each well of a 96-well flat-bottom polystyrene microplate, and incubated at 37 °C for 2 h. Next, the culture medium was removed, and 100 μL of YPD broth with 2% glucose were added to the wells; the

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