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Effect of oral antiseptic agents on phospholipase and proteinase enzymes of *Candida albicans*

Banu Uygun-Can[a,*], Tanju Kadir[a], Birsay Gumru[b]

[a] Department of Microbiology, Faculty of Dentistry, Marmara University, Istanbul, Turkey
[b] Department of Oral Diagnosis and Radiology, Faculty of Dentistry, Marmara University, Istanbul, Turkey

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ABSTRACT

Objective: *Candida*-associated denture stomatitis is the most prevalent form of oral candida infections among the denture wearers. Generally, antiseptic oral rinses used in the treatment of these infections are considered as an adjunct or alternative antifungal treatment. Studies have suggested that the intraoral concentrations of antiseptics decrease substantially to the sub-therapeutic levels on account of the dynamics of the oral cavity. This condition yields the question about the minimum antiseptic concentration that effect the character or pathogenesis of *Candida* during treatment. The extracellular phospholipase and proteinase enzymes of *Candida albicans* are regarded to have a crucial role in the pathogenesis of human fungal infections. Therefore, the aim of this study was to investigate the effect of different sub-therapeutic concentrations of chlorhexidine gluconate, hexetidine and triclosan on the production of these enzymes by *C. albicans* strains isolated from 20 patients with denture stomatitis.
Methods: Phospholipase test was done by using Sabouraud dextrose agar with egg yolk, proteinase test was done by using bovine serum albumin agar.
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Results: Exoenzyme production of 20 strains which were brief exposured to sub-therapeutic concentrations of three antiseptic agents decreased significantly compared with the strains that were not exposured with antiseptic values ($p < 0.05$). There was significant difference between the sub-therapeutic concentrations of each of three antiseptics ($p < 0.05$). When the same concentrations of each antiseptic was compared, there were no significant differences between enzymatic activities ($p > 0.05$).
Conclusions: The results of this study show that sub-therapeutic levels of each antiseptic may modulate candidal exoenzyme production, consequently suppressing pathogenicity of *C. albicans*.

1. Introduction

Candida albicans is an opportunistic pathogen that can colonize the oral mucosa (Arendorf and Walker, 1979). Almost 60% of healthy adults and 26–65% of healthy children are reported to harbor candidal microorganisms in absence of signs or symptoms of candidiasis (Berdicevsky, Ben-Aryeh, Szargel, & Gutman, 1984; Fotos, Vincent, & Hellstein, 1992; Kadir, Uygun, & Akyüz, 2005). Some local and systemic factors lead to an increase in the number of *Candida albicans* strains causing various forms of oral candidiasis particularly *Candida*-associated denture stomatitis which is frequently observed in individuals wearing dentures (Dreizen, 1984; Kadir et al., 2002). As most pathogens, *C. albicans* have developed effective virulence factors and specific strategies to facilitate colonization in host tissues and causing disease. Although many factors have been mentioned among the virulence attributes for *C. albicans*, adhesion, germ tube formation, cell surface hydrophobicity, and exoenzyme production have been extensively studied in recent years (Calderone and Fonzi, 2001; Mayer, Wilsona, & Hubeabc, 2013). The exoenzymes, including the secreted aspartyl proteinase and phospholipase, have been shown to directly contribute to virulence of *C. albicans* (Calderone and Fonzi, 2001). These enzymes assist in adherence and tissue penetration and consequently invasion of the host (Datta, Ganesan, & Natarajan, 1989; Ibrahim et al., 1995). So the amount of these enzymes is important as well as their presence (Budtz-Jörgensen, 2000).

* Corresponding author at: Department of Microbiology, Faculty of Dentistry, Marmara University, Basıbuyuk Mah. No:9/1, 34854 Maltepe/Istanbul, Turkey. Fax: +90 212 2465247.
E-mail address: drbanucan@gmail.com (B. Uygun-Can).

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Mouthrinses containing chlorhexidine, hexetidine and triclosan have been used as convenient adjuncts and/or alternatives to conventional antimycotics in the treatment of oral candida infections. In the treatment of *Candida*-associated denture stomatitis 0.2% chlorhexidine gluconate, 0.1% hexetidine and 0.045% triclosan antiseptic mouthrinses have been successfully used (Budtz-Jörgensen, 1990; Ellepola and Samaranayake, 2000a; Giuliana, Pizzo, Milici, Musotto, & Giangreco, 1997; Jeganathan, Lin, Jacobsen, Bryhni, & Gjermo, 1979; Salim, Moore, Silikas, Satterthwaite, & Rautemaa, 2013; Sharma et al., 2003). Furthermore, chlorhexidine can be incorporated into denture lining materials or denture acrylic resin to reduce candidal biofilm formation (Bertolini et al., 2014; Salim, Moore, Silikas, Satterthwaite, & Rautemaa, 2012). But intraoral concentrations of antifungal agents have been reported to reduce substantially as a result of the diluent effect of saliva and the cleansing effect of the oral musculature, consequently the drug concentration is likely to be sub-therapeutic decreasing the therapeutic efficacy (Bonesvoll, Lokken, Rolla, & Paus, 1974; Bonesvoll, Lokken, & Rolla, 1974; Ellepola and Samaranayake, 2000b; Martin, 1990). In the literature, there is some information about the behaviors and virulence factors of yeasts under the mentioned conditions. In addition to the antifungal effect of oral antiseptics, the candidal adhesion (Darwazeh, Lamey, MacFarlane, & McCuish, 1994; McCourtie, MacFarlane, & Samaranayake, 1985; McCourtie, MacFarlane, & Samaranayake, 1986; Pizzo, Giuliana, & Angelo, 2001), germ tube formation (Ellepola and Samaranayake, 2000b; Ellepola, Joseph, & Khan, 2012), and cell surface hydrophobicity (Ellepola, Joseph, & Khan, 2013) following brief exposure of *C. albicans* to these agents was investigated in several studies. Further, some studies investigated the effects of some antimycotic and oral antiseptic agents on phospholipase production by *C. albicans*. For instance, Anil and Samaranayake (2003) investigated the effects of sub-minimal inhibition concentrations (S-MICs) of nystatin and amphotericin B on the phospholipase production of *C. albicans* and *C. tropicalis* isolates following brief exposure (60 min). In a previous study (Kadir, Gümrü, Uygun-Can, 2007), we examined the effects of three different sub-therapeutic concentrations of chlorhexidine gluconate on phospholipase production of *C. albicans* strains isolated from patients with denture stomatitis. However, proteinase enzyme production, the other important virulence factor, was not investigated. In addition, the effect of other oral antiseptic agents on the enzyme production was not included in none of these studies.

The production of proteinase enzyme also serves as another virulence factor of *C. albicans* because of its central effect in invasiveness (Calderone and Fonzi, 2001; Odds, 1988). There are two studies evaluating the interaction between proteinase production and some antifungal substances. The first one (Wu, Samaranayake, Cao, & Wang, 1996) investigated the effect of S-MICs of nistatin, amphotericin B, clotrimazole and miconazole on in vitro proteinase production by 14*C. albicans* isolates from HIV positive and negative patients. The other study (Lyon, dos Santos, de Moraes, & Moreira, 2011) evaluated the effect generated by the surfactants on proteinase production by *Candida*. Despite great interest focussing on virulence factors, few studies have evaluated the effects of oral antiseptics on exoenzyme production.

The aim of the present study was to evaluate the phospholipase and proteinase production of 20 oral *C. albicans* isolates following 30 min exposure to 4 different sub-therapeutic concentrations (1/150, 1/200, 1/250, 1/300) of 3 different oral antiseptic agents including chlorhexidine gluconate, hexetidine and triclosan in vitro. The hypothesis was that exposure to four sub-therapeutic concentrations of these three oral antiseptic agents could decrease the phospholipase and proteinase production of *C. albicans*.

2. Materials and methods

2.1. Isolates

A total of 20 phospholipase and proteinase positive *C. albicans* isolates collected from patients with denture stomatitis between 2003 and 2004 and preserved in our laboratory stock collection were included in the study. *Candida glabrata* strain (from our laboratory stock collection) was used as negative control for phospholipase and proteinase assays. All isolates were revived from stock cultures maintained on Sabouraud's dextrose agar (SDA) stored at 4 °C. Cultures from the maintained medium were inoculated onto fresh SDA plates and incubated at 37 °C for 48 h. The obtained fresh cultures were used for the enzyme assays.

The study protocol was approved by the local ethics committee of the Marmara University, Istanbul, Turkey.

2.2. Antifungal agent

In order to prepare the prime chlorhexidine solution (0.2%), 0.2 ml of chlorhexidine gluconate 4% (Merkez Lab Inco, Istanbul, Turkey) was added to 3.8 ml sterile phosphate buffered saline (PBS). Hexetidine (0.1%) was prepared by adding 0.01 ml of hexetidine 1% (Sigma) to 0.09 ml PBS and triclosan (0.045%) by completing 0.045 g of stock triclosan (Merkez Lab Inco, Istanbul, Turkey) to 100 ml with alcohol. The pH of each stock solution was adjusted to be 7.2.

Sub-therapeutic concentrations (S-TCs) of antiseptic agents were prepared by adding 10 μl therapeutic concentrations of each antiseptic agent to tubes containing respectively 1.49, 1.99, 2.49 and 2.99 ml PBS. The solutions were prepared shortly before each experiment.

2.3. Preparation of the yeast suspension

Following a 48 h incubation period of *C. albicans* isolates on fresh SDA, yeasts were suspended equalling to the 0.5 McFarland turbidity (i.e., optical density = 0.12 – 0.15 at 530 nm, corresponding to $1-5\times10^6$ colony forming units (CFU)/mL) in sterile PBS.

2.4. Exposure of albicans isolates to antiseptic agent

A cell suspension (10^6 yeast/ml) of each 20 *C. albicans* isolates was prepared in 1 ml of PBS for two tubes. The tubes were then centrifuged for 10 min at 3000 × *g*. The supernatant was completely decanted and each of yeast pellets was resuspended in 1 ml of sterile PBS (control) and 1 ml of PBS/antiseptic. This process was applied to each sub-therapeutic concentration of different antiseptic solutions. The tubes were incubated for 30 min at 37 °C on shaker and then the supernatant which contains antiseptic was completely removed by two cycles of suspension with sterile PBS and centrifugation in 4 °C for 10 min at 3000 × *g*. For elimination of any carry-over effect of the antiseptics following its removal, the complete decanting of the supernatant with two washings were repeated and pellets were resuspended in 1 ml of sterile PBS (Samaranayake, Raeside, & MacFarlane, 1984). Following removal of the antiseptic, viable counts of the control and tests were performed. From each pellet suspension samples of 0.1 ml were removed and serially diluted 10-folds with PBS. 0.1 ml from each dilution was plated onto SDA in triplicate, incubated at 37 °C for 48 h and colonies were counted (Kadir et al., 2007). When any reduction in the cell concentration of test was observed, the final cell concentration of the control was adjusted to be equal to test concentration.