Effects of Subtherapeutic Concentrations of Chlorhexidine Gluconate on Germ Tube Formation of Oral *Candida*

Arjuna N.B. Ellepola^a Bobby J. Joseph^b Z.U. Khan^c

Departments of ^aBioclinical Sciences and ^bDiagnostic Sciences, Faculty of Dentistry, and ^cDepartment of Microbiology, Faculty of Medicine, Health Sciences Center, Kuwait University, Jabriya, Kuwait

Key Words

Candida albicans · Germ tubes · Chlorhexidine gluconate

Abstract

Objective: The main objective of this study was to investigate the effect of brief exposure to subtherapeutic concentrations of chlorhexidine gluconate on germ tube formation of *Candida albicans* isolates obtained from smokers, diabetics, asthmatics using steroid inhalers and healthy individuals.

Materials and Methods: Forty isolates of *C. albicans* were used in this study. All these isolates were quantified for germ tube formation without exposure to the drug and were used as the control group for data analysis. Isolates were also exposed to three subtherapeutic concentrations of chlorhexidine gluconate (0.00125, 0.0025 and 0.005%) for 30 min (limited exposure); the antiseptic was then removed and germ tube formation of these isolates was quantified microscopically following incubation in a germ tube-inducing medium.

Results: Compared with the unexposed controls, brief exposure to all concentrations of chlorhexidine gluconate suppressed the ability of the *C. albicans* isolates to form germ tubes in increasing order by 13.72% ($p < 0.001$ to $p = 0.02$), 46.16% ($p < 0.001$) and 72.46% ($p < 0.001$). **Conclusions:** These findings show that brief exposure to subtherapeutic concentrations of chlorhexidine gluconate may modulate germ tube formation of *C. albicans* isolates, thereby sup-

pressing their pathogenicity, and further elucidate the pharmacodynamic mechanisms by which chlorhexidine gluconate may operate in vivo.

Copyright © 2011 S. Karger AG, Basel

Introduction

The frequency of life-threatening mycoses is rising worldwide, and oral candidiasis is considered the commonest human fungal infection, which displays a variety of clinical manifestations, including pseudomembranous and erythematous variants. *Candida* induces denture stomatitis and angular stomatitis, possibly of multifactorial origin [1]. *Candida* infections have also been implicated in persistent apical periodontitis [2], and *Candida*-like organisms have been demonstrated in root canals and dentinal tubules in situ [3].

Candida albicans is a pleomorphic opportunistic pathogen that takes different morphological forms under different environmental conditions, including budding yeast cells, germ tubes, and true hyphae and pseudohyphae [4]. Germ tubes, which mark the onset of hyphal growth, are a phenotypic characteristic of *Candida* that has been implicated in the pathogenesis of candidiasis [5] as these cylindrical extrusions are known to facilitate yeast adherence to epithelial cells and impart resistance

to phagocytic killing when compared with the blastospore form [6]. Furthermore, germ tubes tend to promote aggregation of yeast cells and bridging of adjacent hyphal elements, thereby bringing a large battery of organisms to intimate contact with the oral epithelium [7]. *Candida* hyphae have also been shown to penetrate dentinal tubules along cracks of tooth surfaces, enabling the organism to invade dental hard tissues [3].

Chlorhexidine gluconate at a concentration of 0.2% is widely prescribed in dentistry as an antiseptic mouth wash due to its broad-spectrum antimicrobial activity including *Candida* [8]. The antifungal effect of chlorhexidine gluconate has been demonstrated in several in vivo and in vitro trials, including some related to *Candida* infection [9]. Further, it has been demonstrated that exposure of either *Candida* isolates or buccal epithelial cells to 0.2% chlorhexidine gluconate profoundly suppressed their ability to adhere to buccal epithelial cells in healthy individuals [10] as well as in diabetics [11]. Moreover, pretreatment of acrylic dentures with 2% chlorhexidine gluconate has also been shown to suppress the adhesion of *Candida* to the denture's acrylic surfaces [12]. Exposure to chlorhexidine gluconate has also elicited the ability to reduce the cell surface hydrophobicity of these species [13]. Furthermore, a recent study [14] has revealed that surface coating of denture acrylic with chlorhexidine gluconate was capable of inhibiting biofilm growth of *C. albicans* isolates. For these reasons, oral rinses containing chlorhexidine gluconate may be an appropriate substitute to conventional antimycotics in the management of oral candidiasis [15].

It has been reported that 30% of the total chlorhexidine gluconate dose may be retained in the mouth for 24 h after a 1-min rinse but is removed from the oral cavity during the first hour due to the diluent effect of saliva and the cleansing effect of the oral musculature, thus compromising its therapeutic efficacy [16]. Hence, intra-orally, pathogenic yeasts undergo brief exposure to high concentrations of chlorhexidine gluconate following an oral rinse during therapy while thereafter the drug concentration may be subtherapeutic. Yet the behavior of yeasts under the later conditions has been little studied [17]. For instance, only one study has quantitatively compared germ tube formation of oral *C. albicans* isolates. This study used 10 oral *C. albicans* isolates obtained from HIV-infected ($n = 5$) as well as HIV-free healthy ($n = 5$) individuals in Hong Kong to investigate germ tube formation following a single limited exposure (30 min) to subtherapeutic concentrations of chlorhexidine gluconate [17]. In addition to HIV infection, *C. albicans* has

also been implicated in oral candidiasis in other patient groups, such as diabetics, asthmatics using inhalation steroids and smokers [18–20]. However, the effect on germ tube formation of oral *C. albicans* isolates obtained from these patients following brief exposure to subtherapeutic concentrations of chlorhexidine gluconate has not been studied. Hence the main aim of this study was to investigate germ tube formation of oral *C. albicans* isolates after brief exposure to three subtherapeutic concentrations of chlorhexidine gluconate.

Materials and Methods

Organisms

A total of 40 oral isolates of *C. albicans* stock cultures recovered from oral rinse samples from patients attending the Kuwait University Dental Clinic (KUDC) for dental treatment were included in the study. Ten isolates each were from smokers, diabetics, asthmatics using steroid inhalation and healthy individuals. The KUDC provides a full range of dental treatments for those who have dental treatment needs that correspond to the teaching needs of dental students. The diabetic patients were under oral hypoglycemic drugs and the asthmatic patients were under steroid inhalation therapy, and were otherwise healthy at the time of attending the KUDC. Patients who smoked more than 25 cigarettes per day were considered as smokers. None of the patients from whom the isolates were recovered had oral candidiasis. Initially, all the yeast isolates were tested for germ tube formation. Thereafter, the colony characteristics were observed using CHROMagar *Candida* medium (Becton Dickinson, Sparks, Md., USA) and identified by Vitek 2 Yeast ID System (BioMérieux, France) as well as API 20C AUX Yeast ID System (BioMérieux, Inc., Hazelwood, Mo., USA).

Antifungal Agents and Media

Chlorhexidine gluconate 0.2% (Corsodyl, GlaxoSmithKline, Brentford, UK) was dissolved in sterile phosphate-buffered saline (PBS) at pH 7.2 and was prepared as 0.005, 0.0025 and 0.00125% solutions immediately prior to each experiment as previously described [17].

Preparation of Cell Suspension for the Germ Tube Assay

A previously described method was used for this purpose [17]. Briefly, yeast cells maintained on Sabouraud's dextrose agar were inoculated onto fresh plates and incubated overnight at 37°C for 24 h prior to use. The organisms were harvested and a cell suspension was prepared in sterile PBS at 520 nm to an optical density of 1.5. From this cell suspension, 0.5 ml was added to tubes containing only 2 ml of PBS without the drug (control) and 2 ml of three different concentrations of chlorhexidine gluconate (three test groups) dissolved in PBS. This gave a cell suspension of 10^6 ml⁻¹ in each assay tube. The tubes were then incubated at 37°C for a period of 30 min. Following this limited exposure, the drugs were removed by two cycles of dilution with sterile PBS and centrifuged for 10 min at 3,000 g. The supernatant was completely decanted and the pellets were resuspended in 2.5 ml of sterile PBS. This

Table 1. Mean $\pm$ SEM percentage of germ tube-positive cells in 40 oral *C. albicans* isolates obtained from healthy individuals, diabetic patients, asthmatic patients and smokers

Source of <i>C. albicans</i> isolates (n = 40)	Unexposed controls	0.00125% chlorhexidine	0.0025% chlorhexidine	0.005% chlorhexidine
Healthy (n = 10)	26.92 $\pm$ 1.16	23.57 $\pm$ 0.75**	13.03 $\pm$ 0.73*	6.21 $\pm$ 0.60*
Diabetic (n = 10)	28.08 $\pm$ 0.88	23.98 $\pm$ 0.34*	15.24 $\pm$ 0.52*	7.25 $\pm$ 0.55*
Asthmatic (n = 10)	28.76 $\pm$ 1.03	25.12 $\pm$ 0.79***	16.63 $\pm$ 0.89*	9.67 $\pm$ 0.34*
Smokers (n = 10)	29.67 $\pm$ 0.67	25.16 $\pm$ 0.52*	16.27 $\pm$ 0.55*	8.19 $\pm$ 0.42*

* p < 0.001; ** p = 0.02; *** p = 0.008.

Table 2. Mean percentage reduction of germ tube formation in 40 oral *C. albicans* isolates obtained from healthy individuals, diabetic patients, asthmatic patients and smokers

Source of <i>C. albicans</i> isolates (n = 40)	0.00125% chlorhexidine	0.0025% chlorhexidine	0.005% chlorhexidine
Healthy (n = 10)	12.44	51.59	76.93
Diabetic (n = 10)	14.60	45.73	74.18
Asthmatic (n = 10)	12.65	42.17	66.37
Smokers (n = 10)	15.20	45.16	72.39
Mean	13.72	46.16	72.46
SEM	0.69	1.97	2.24

technique has been shown to reduce the drug concentration as much as 10,000-fold [21], thereby minimizing any carryover effect of the drug following its removal. Viable counts of the control and the test were performed after drug removal. As the procedure of drug removal effectively eliminated any carryover effect, there was virtually no difference in the viable counts of the controls and the tests following exposure to already diluted subtherapeutic concentrations of the drug as observed in previous studies [13, 17].

Microscopic Quantification of Germ Tube-Forming Cells

Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine (Sigma, St. Louis, Mo., USA) was chosen for the assay because it induces germ tube formation effectively as described previously [22]. For germ tube induction, 250 μ l of yeast suspension, obtained after drug removal, was added to 1 ml RPMI-1640 medium with L-glutamine and incubated at 37°C for 90 min. The tube was vortexed for 10 s and a drop of each cell suspension was placed on a Neubauer's hemocytometer chamber and covered with a cover slip for quantification of germ tubes. Thereafter, 300 yeast cells in contiguous fields were counted (under $\times 40$ magnification) and the percentage of germ tube-forming cells calculated as previously described [17]. The counting criterion was only yeast cells with a germ tube, without constriction at the junction between the cell and the elongation; clumped cells with germ tubes and pseudohypha-forming yeast cells were excluded.

All experiments were repeated on three times with duplicate determinations on each occasion.

Statistical Analysis

The effect of chlorhexidine gluconate on each isolate was statistically analyzed. The data obtained from all three germ tube assays in duplicate were analyzed using ANOVA Dunnett's t tests, which treated one group as a control (unexposed to chlorhexidine gluconate) and compared all other groups (exposed to chlorhexidine gluconate) against it. A p value <0.05 was considered statistically significant.

Results

The mean percentage of germ-positive cells of the 40 *C. albicans* isolates following limited exposure to three different subtherapeutic concentrations of chlorhexidine gluconate, drug removal and subsequent incubation in RPMI-1640 medium is shown in table 1. Compared to the controls and other concentrations of the drug, most suppression of germ tube induction in all the isolates was seen following exposure to 0.005% chlorhexidine gluconate, indicating close dose dependence. The mean percentage reduction was, in increasing order, 13.72 $\pm$ 0.69% at 0.00125% to 72.46 $\pm$ 2.24 at 0.005% chlorhexidine gluconate (table 2).

Discussion

The present study showed that limited exposure of oral *C. albicans* isolates from diabetics, asthmatics using inhalation steroids, smokers and healthy individuals to different concentrations of chlorhexidine gluconate suppressed the ability of isolates to form germ tubes. This observation is similar to that reported previously for 10 oral *C. albicans* isolates from HIV-infected as well as HIV-free healthy patients [17] subjected to the same treatment. Though the later study failed to demonstrate an overall significant suppressive effect on germ tube formation following exposure to 0.00125% chlorhexidine gluconate (9.13%), the suppressive effect on 30% of the isolates was statistically significant following exposure to this concentration of the drug whereas in the current study the suppressive effect was significant on all isolates tested following exposure to 0.00125% of chlorhexidine gluconate. Exposure to 0.0025 and 0.005% chlorhexidine gluconate elicited a suppressive effect of 42.74 and 81.23%, respectively, at these two concentrations [17]; the effect was statistically significant for all isolates tested similar to that of the current study. Though significant in the current study, the reduction in germ tube formation of yeasts exposed to the same concentrations of chlorhexidine gluconate was not uniform on all the isolates tested. For instance, the mean percentage reduction in germ tube formation ranged from 12.44 to 15.20%, 42.17 to 51.59% and 66.37 to 76.93% following exposure to 0.00125, 0.0025 and 0.005% chlorhexidine gluconate, respectively. As a wide range of isolates from different patient groups was used, it can be speculated that this difference in sensitivity to a specific chemical at the same concentration seen in this study may be due to factors such as origin, previous exposure to drugs, underlying disease entities or inherent genomic profile. However, a study with a larger battery of organisms is warranted to verify this speculation.

The observed suppression of germ tube formation by chlorhexidine gluconate may be related to the pharmacodynamic action of the antiseptic on the cell wall of *Candida*. Scanning and transmission electron-micrographic studies have demonstrated that the antifungal effect of this antiseptic is most likely due to the induction of a loss of cytoplasmic components and coagulation of nucleoproteins, and associated morphological changes in the cell wall structure [23]. Further, a decrease of budding *Candida* cells was also observed in the aforementioned study. Others have demonstrated complex ultrastructural dynamics of the cell wall during the transition from

blastospore to hyphal phase, and to the fact that the cell wall of the germ tube possesses stratification comparable to that of the blastospore wall [24]. Therefore it is reasonable to speculate that chlorhexidine gluconate affects the cell wall structure as well as other cellular events leading to germ tube formation as shown in the current study.

Oral candidiasis has been observed in populations of diabetics, asthmatics using inhalation steroids and smokers [18–20]. Therefore, the information provided herein contributes to broadening the understanding of the effectiveness of chlorhexidine gluconate against a vital function (i.e. germ tube formation), incriminated in the pathogenesis of *C. albicans*, which frequently colonizes compromised patients such as diabetics, asthmatics using steroid inhalers and smokers. Even subtherapeutic concentrations such as 0.00125% chlorhexidine gluconate, which is far below the normal therapeutic concentration of this drug (0.2%), has the ability of suppressing germ tube formation of all *Candida* isolates tested in different patient groups. As isolates from all these patient groups were used in the current study, this information may further advocate the use of chlorhexidine gluconate as an adjunct in the management of oral candidiasis. However, further studies on the impact of this antiseptic on other pathogenic properties of *Candida*, such as adhesion to oral epithelial cells, adhesion to denture acrylic and cell surface hydrophobicity, including a larger battery of organisms are warranted.

Conclusion

These findings showed that brief exposure to subtherapeutic concentrations of chlorhexidine gluconate may modulate germ tube formation of *C. albicans* isolates, thereby suppressing its pathogenicity and elucidates further the pharmacodynamic mechanisms by which chlorhexidine gluconate may operate in vivo.

Acknowledgements

The work was partially supported by Kuwait University Research Grant No. DB 01/07. The technical support from Ms. Leeba Philip (Faculty of Dentistry) and Ms. Rachel Chandy (Faculty of Medicine), Health Sciences Center, Kuwait University is appreciated and thankfully acknowledged.

References

- 1 Ellepola AN, Samaranayake LP: Antimycotic agents in oral candidosis: an overview: 1. Clinical variants. Dent Update 2000;27: 111–112, 114–116.
- 2 Waltimo TM, Sirén EK, Torkko HL, Olsen I, Haapasalo MP: Fungi in therapy-resistant apical periodontitis. Int Endod J 1997;30:96–101.
- 3 Sen BH, Safavi KE, Spångberg LS: Colonization of *Candida albicans* on cleaned human dental hard tissues. Arch Oral Biol 1997;42: 513–520.
- 4 Shepherd MG: Biology of *Candida* species; in Samaranayake LP, MacFarlane TW (eds): Oral Candidosis. London, Wright, Butterworth, 1990, pp 10–20.
- 5 Richardson MD, Smith H: Production of germ tubes by virulent and attenuated strains of *Candida albicans*. J Infect Dis 1981; 144:565–569.
- 6 Tronchin G, Bouchara JP, Robert R, Senet JM: Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. Infect Immun 1988;56:1987–1993.
- 7 Sobel JD, Muller G, Buckley HR: Critical role of germ tube formation in the pathogenesis of candidal vaginitis. Infect Immun 1984;44: 576–580.
- 8 MacNeill S, Rindler E, Walker A, Brown AR, Cobb CM: Effects of tetracycline hydrochloride and chlorhexidine gluconate on *Candida albicans*. An in vitro study. J Clin Periodontol 1997;24:753–760.
- 9 Budtz-Jorgensen E: *Candida*-associated denture stomatitis and angular cheilitis; in Samaranayake LP, MacFarlane TW (eds): Oral Candidosis. London, Wright, Butterworth, 1990, pp 156–183.
- 10 Tobgi RS, Samaranayake LP, Macfarlane TW: Adhesion of *Candida albicans* to buccal epithelial cells exposed to chlorhexidine gluconate. J Med Vet Mycol 1987;25:335–338.
- 11 Darwazeh AM, Lamey PJ, MacFarlane TW, McCuish AC: The effect of exposure to chlorhexidine gluconate in vitro and in vivo on in vitro adhesion of *Candida albicans* to buccal epithelial cells from diabetic and non-diabetic subjects. J Oral Pathol Med 1994;23: 130–132.
- 12 McCourtie J, MacFarlane TW, Samaranayake LP: A comparison of the effects of chlorhexidine gluconate, amphotericin B and nystatin on the adherence of *Candida* species to denture acrylic. J Antimicrob Chemother 1986;17:575–583.
- 13 Anil S, Ellepola AN, Samaranayake LP: The impact of chlorhexidine gluconate on the relative cell surface hydrophobicity of oral *Candida albicans*. Oral Dis 2001;7:119–122.
- 14 Pusateri CR, Monaco EA, Edgerton M: Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. Arch Oral Biol 2009;54:588–594.
- 15 Ellepola AN, Samaranayake LP: Adjunctive use of chlorhexidine in oral candidoses: a review. Oral Dis 2001;7:11–17.
- 16 Bonesvoll P, Lökken P, Rölla G, Paus PN: Retention of chlorhexidine in the human oral cavity after mouth rinses. Arch Oral Biol 1974;19:209–212.
- 17 Ellepola AN, Samaranayake LP: The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine gluconate on the germ tube formation of oral *Candida albicans* and its relationship to post-antifungal effect. Oral Dis 2000;6:166–171.
- 18 Ellepola AN, Samaranayake LP: Inhalation and topical steroids, and oral candidosis: a mini review. Oral Dis 2001;7:211–216.
- 19 Soysa NS, Ellepola AN: The impact of cigarette/tobacco smoking on oral candidosis: an overview. Oral Dis 2005;11:268–273.
- 20 Soysa NS, Samaranayake LP, Ellepola AN: Diabetes mellitus as a contributory factor in oral candidosis. Diab Med 2005;23:455–459.
- 21 Craig WA, Gudmundsson S: The postantibiotic effect; in Lorian V (ed): Antibiotics in Laboratory Medicine. Baltimore, Williams & Wilkins, 1996, pp 296–329.
- 22 Brayman TG, Wilks JW: Sensitive assay for antifungal activity of glucan synthase inhibitors that uses germ tube formation in *Candida albicans* as an end point. Antimicrob Agents Chemother 2003;47:3305–3310.
- 23 Bobichon H, Bouchet P: Action of chlorhexidine on budding *Candida albicans*: scanning and transmission electron microscopic study. Mycopathologia 1987;100:27–35.
- 24 Cassone A, Simonetti N, Strippoli V: Ultrastructural changes in the wall during germ-tube formation from blastospores of *Candida albicans*. J Gen Microbiol 1973;77:417–426.