

The postantifungal effect and phospholipase production of oral *Candida albicans* from smokers, diabetics, asthmatics, denture wearers and healthy individuals following brief exposure to subtherapeutic concentrations of chlorhexidine gluconate

Arjuna N. B. Ellepola,¹ Bobby K. Joseph² and Z. U. Khan³

¹Department of Bioclinical Sciences, Faculty of Dentistry, Health Sciences Center, Kuwait University, Safat, Kuwait, ²Department of Diagnostic Sciences, Faculty of Dentistry, Health Sciences Center, Kuwait University, Safat, Kuwait and ³Department of Microbiology, Faculty of Medicine, Health Sciences Center, Kuwait University, Safat, Kuwait

Summary

Candida albicans is the major aetiological agent of oral candidosis and one of its important virulent factors is the production of extracellular phospholipases, which can be modulated by subtherapeutic concentrations of antifungal agents thus decreasing their pathogenicity. Hence, considering that chlorhexidine gluconate (CG) is a common antimicrobial mouthwash used in dentistry and that its concentration in the mouth reaches subtherapeutic levels during dosage intervals due to the diluent effect of saliva and cleansing effect of the oral musculature, the postantifungal effect (PAFE) and the phospholipase production of oral *C. albicans* following brief exposure to subtherapeutic concentrations of CG was studied. Fifty *C. albicans* planktonic oral isolates obtained from smokers, diabetics, asthmatics using steroid inhalers, partial denture wearers and healthy individuals were exposed to three subtherapeutic concentrations of CG (0.005%, 0.0025% and 0.00125%) for 1 h. Isolates unexposed to CG was the control group. Thereafter the antiseptic was removed and the PAFE and phospholipase production was determined by a turbidometric method and a plate assay using an egg yolk agar medium respectively. Mean PAFE (hours) of 50 oral isolates of *C. albicans* following 1-h exposure to 0.005%, 0.0025% and 0.00125% CG was 6.97, 1.85 and 0.62 respectively. The phospholipase production of these isolates was significantly suppressed with a percentage reduction of 21.68, 18.20 and 14.04% following exposure to 0.005%, 0.0025% and 0.00125% CG respectively. Brief exposure of *C. albicans* isolates to subtherapeutic concentrations of CG would yield an antifungal effect by suppressing growth and phospholipase production, thereby quelling its pathogenicity.

Key words: *Candida albicans*, postantifungal effect, phospholipase, chlorhexidine gluconate.

Correspondence: Dr A. N. B. Ellepola, BDS, PhD, FIBiol, Department of Bioclinical Sciences, Faculty of Dentistry, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait.
Tel.: 00965 24636714. Fax: 00965 25326049.
E-mail: arjuna@hsc.edu.kw

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Introduction

Candida albicans is renowned as the foremost fungal pathogen implicated in oral candidosis which is considered the commonest human fungal infection which reveals in variety of clinical manifestations.¹ Interestingly, more than 90% of human immunodeficiency virus (HIV)-infected individuals develop oral candidosis during some point of their disease which is by far the

commonest oral manifestation in these patients. In addition to HIV infection, *C. albicans* has also been implicated in oral candidosis in other patients such as diabetics, asthmatics using inhalation steroids, smokers and denture wearers.¹⁻⁴

Candida albicans isolates develop specific virulence mechanisms that confer the ability to colonise epithelial cells, adhere to denture material, to invade deeper cells, or to influence host defences. One of the most important virulence factors of *C. albicans* is extracellular phospholipases, which degrade phospholipid constituents of host cell membrane leading to disruption of host cells or alteration of surface characteristics that facilitate adherence and subsequent infection.⁵ In fact, a positive correlation between phospholipase activity and two major pathogenic attributes of *Candida* has been documented, where it was shown that *C. albicans* with the highest phospholipase activity had the highest adherence to oral epithelial cells and a greater ability to kill mice after intravenous inoculation compared with yeast with a low degree of phospholipase activity.⁵

The suppression of candidal growth that occur following limited exposure to antifungal agents, as in the oral environment, has been described as the postantifungal effect (PAFE). It has been documented that the knowledge of PAFE would be clinically useful in evaluating new dosage regimens of a drug and such transient exposure to antifungal agents may be a determinant of candidal virulence itself and also influence other virulent factors of *Candida* pertaining to their adhesion.⁶⁻⁸

Chlorhexidine gluconate (CG) in a concentration of 0.2% is widely prescribed as an antiseptic mouthwash in routine dental practice due to its broad-spectrum antimicrobial activity which includes *Candida* species.⁹ The antifungal effect of CG has been demonstrated in several *in vivo* and *in vitro* trials including some related to *Candida* infection.¹⁰ It has also been demonstrated that exposure of either *Candida* isolates or the buccal epithelial cells (BEC) to 0.2% CG overwhelmingly suppresses the ability of the former to adhere to BEC in healthy,¹¹ as well as in compromised patients such as diabetics.¹² Likewise, pretreatment of acrylic dentures with 2% CG has also been shown to suppress the adhesion of the yeast to denture acrylic surfaces.¹³ In addition, a recent study has shown that subtherapeutic levels of CG are also effective in suppressing germ tube (GT) formation of oral *C. albicans* isolates obtained from healthy as well as diabetics, asthmatics using steroid inhalers and smokers.¹⁴ Furthermore, it has been shown that CG alone or in combination with other antimycotic agents were capable of inducing a

PAFE as well as modulating virulent attributes of *Candida* including phospholipase production.¹⁵ For these reasons, oral rinses containing CG may be an appropriate substitute to conventional antimycotics in the management of oral candidosis.¹⁶

It has been recounted that 30% of the total CG dose may be retained in the mouth for 24 h after a 1-min rinse but 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 conceding its therapeutic efficacy.¹⁷ Hence, the pathogenic *Candida* may undergo a brief exposure to topically applied antifungal drugs, while thereafter, the drug concentration is likely to be subtherapeutic,¹⁸ a scenario all too familiar in the niches of the oral cavity, which is similar to the phenomena as in PAFE. Yet, the impact of PAFE on the phospholipase production of oral *C. albicans* isolates obtained from different predisposing patient groups (i.e. diabetics, asthmatics using inhalation steroids, smokers and partial denture wearers) following brief exposure to subtherapeutic concentrations of CG has not been extensively studied hitherto.

Hence, taken together the foregoing information, the main aim of this study was to determine the CG-induced PAFE and to observe the phospholipase production of 50 oral *C. albicans* isolates obtained from diabetics, asthmatics using inhalation steroids, smokers, partial acrylic denture wearers and healthy individuals following their brief exposure to three subtherapeutic concentrations of CG (i.e. 0.005%, 0.0025% and 0.00125%).

Materials and methods

Organisms

A total of 50 oral isolates of *C. albicans* recovered from oral rinse samples from patients attending the Kuwait University Dental Clinic for dental treatment were included in the study (10 isolates each were from smokers, diabetics, asthmatics using steroid inhalation, patients wearing partial acrylic dentures and healthy individuals). The diabetic patients were under oral hypoglycaemic drugs and the asthmatic patients were under steroid inhalation therapy, and were otherwise healthy at the time of attending the dental clinics. The patients who smoked more than 25 cigarettes per day were considered as smokers. Denture wearers were using partial removable acrylic dentures for a period of more than 6 months. None of the patients from which the isolates were recovered had oral candidosis. Initially, all the yeast isolates were tested for GT

formation. Thereafter the colony characteristics were observed using CHROMagar Candida medium (Becton Dickinson and Company, Sparks, NV, USA) and identified by VITEK 2 yeast ID system (BioMérieux, Craponne, France) as well as API 20C AUX yeast ID system (BioMérieux, Inc, Hazelwood, MO, USA).

Antifungal agents and media

Chlorhexidine gluconate 0.2% (Glaxo Smith Kline, 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.^{15,18}

Preparation of cell suspension for the PAFE and phospholipase production assay

A previously described method was used for this purpose.^{7-9,15,18} Briefly, yeast cells maintained on Sabouraud's dextrose agar (SDA) 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 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 2 ml of PBS (control) and 2 ml of PBS/CG (test). This gave a cell suspension of 10^6 cells 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 centrifugation for 10 min at 3000 *g* (gravity). Afterwards the supernatant was completely decanted and the pellets were resuspended in 2.5 ml of sterile PBS. This procedure has been used previously for drug removal^{7-9,15,18} and has shown to reduce the concentration of the drug as much as 10 000-fold,¹⁹ thereby minimising any carry-over 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 carry-over effect there was virtually no difference on the viable counts of the control and the tests following exposure to already diluted subtherapeutic concentrations of the drug as observed in previous studies.^{7-9,15,18}

PAFE assay

Following drug removal, to determine the growth suppression and subsequent recovery of fungal growth, namely, the PAFE, the growth was

determined by a previously described optical density method with slight modification using the equation $PAFE = T - C$.⁷⁻⁹ *T* is the time required for optical density of the drug-exposed cell suspension to reach the selected relative optical density of 0.05 value at 520 nm. *C* is the time required for optical density of the drug-free control cell suspension to reach the selected relative optical density value at 520 nm. Thus, *T - C* expresses the time in which the antifungal agent was capable of causing growth suppression of the organism following limited exposure to the drug (i.e. PAFE). As done in previous studies,²⁰ for this purpose 1600 µl of cell suspension was incubated with 2.4 ml of RPMI (Roswell Park Memorial Institute) 1640 at 37 °C and the relative optical density of the suspension was measured at frequent intervals (i.e. 15 min) for 8, 3 and 1 h for 0.05%, 0.025% and 0.0125% CG concentrations, respectively, by which time, both the controls and tests reached the selected relative optical density value, enabling to calculate the PAFE.

Phospholipase production assay (egg yolk agar method)

Candida albicans isolates were screened for phospholipase activity by growing the isolates on egg yolk medium and measuring the size of the zone of precipitation (i.e. production of phospholipase) as done in previous studies.^{21,22} The egg yolk medium consisted of SDA, 13.0 g; NaCl, 11.7 g; CaCl₂, 0.11 g; and 10% sterile egg yolk emulsion in 184 ml of distilled water. The egg yolk emulsion was centrifuged at 500 *g* for 10 min at room temperature, and 20 ml of the supernatant was added to the cooled sterilised medium. A 10 µl suspension of 10^7 yeast cells (drug-exposed and control) per ml in PBS was plated on the surface of egg yolk medium in a 90-mm-diameter petri dish and left to dry at room temperature. For the CG assay, one isolate was tested on two plates. Plates were divided into two equal sections (i.e. to have four sections in two plates). First section was used for the control, second, third and fourth sections for the test isolate exposed to three different concentrations of CG. The plates were then incubated at 37 °C for 72 h. The phospholipase activity of the isolates was interpreted positive when a precipitation zone was visible around the growth and the value of phospholipase production (Pz) was determined by the ratio of the diameter of the colony plus the precipitation zone to the diameter of the colony as done previously.^{21,22}

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

Statistical analysis

For the suppression effect on phospholipase production, the raw data did not follow normal distribution. Therefore, as per statisticians advice non-parametric, Mann–Whitney *U*-test was used to compare the control group with each of the three groups exposed to three different concentrations of CG. The difference among three groups exposed to three different concentrations of CG was also compared using Kruskal–Wallis test in relation to CG-induced PAFE and suppression of phospholipase production. The relationship between the CG-induced PAFE and suppression of phospholipase production was analysed using non-parametric correlation (Spearman's rho). A *P* value of less than 0.05 was considered statistically significant.

Results

Based on the equation $\text{PAFE} = T - C$, the mean *in vitro* PAFE (hours) on 50 oral isolates of *C. albicans* following 1-h exposure and subsequent removal of 0.005%, 0.0025% and 0.00125% CG was 6.97 ± 0.77 , 1.85 ± 0.58 and 0.62 ± 0.01 respectively (Table 1). Hence, in summary brief exposure to 0.005%, 0.0025% and 0.00125% CG was able to suppress the growth of *C. albicans* isolates giving a PAFE of approximately 7, 2 and ½ h respectively.

Table 1 The PAFE (in hours) of 50 oral *Candida albicans* isolates following 1-h exposure and subsequent removal of three different concentrations of chlorhexidine gluconate. Each value indicates the mean ($\pm$ SEM) of 10 oral *C. albicans* obtained either from healthy individuals, diabetic patients, asthmatic patients, smokers or denture wearers. In relation to PAFE, 0.25 = 15 min; 0.50 = 30 min; 0.75 = 45 min; 1.0 = 60 min.

Source of <i>C. albicans</i> isolates (<i>n</i> = 50)	0.005% Chlorhexidine	0.0025% Chlorhexidine	0.00125% Chlorhexidine
Healthy (<i>n</i> = 10)	6.96 (0.17)	1.75 (0.14)	0.60 (0.04)
Diabetic (<i>n</i> = 10)	6.93 (0.17)	2.01 (0.10)	0.61 (0.03)
Asthmatic (<i>n</i> = 10)	6.85 (0.17)	1.93 (0.12)	0.66 (0.03)
Smokers (<i>n</i> = 10)	7.27 (0.16)	1.87 (0.13)	0.62 (0.03)
Denture wearers (<i>n</i> = 10)	6.84 (0.18)	1.71 (0.14)	0.63 (0.03)
Mean ($\pm$ SEM)	6.97 (0.77)	1.85 (0.58)	0.62 (0.01)
Standard deviation	0.54	0.41	0.09

PAFE, postantifungal effect; SEM, standard error of mean.

Mean phospholipase production of the 50 *C. albicans* isolates, following limited exposure to three different subtherapeutic concentrations of CG, drug removal and subsequent production of phospholipase activity is shown in Table 2. Mean phospholipase production of the unexposed isolates was 1.72, whereas this figure following exposure to 0.005%, 0.0025% and 0.00125% CG was 1.35, 1.41 and 1.48 respectively. Hence, as shown in Table 3, a significant reduction of phospholipase activity was seen following exposure to 0.005% CG, with a mean percentage reduction of 21.68% ($P < 0.001$). Overall suppressive effect on the phospholipase production following exposure to 0.0025% and 0.00125% dilutions of CG was also significant in comparison to the unexposed control, although lower than for the higher concentration (18.20% and 14.04% vs. 21.68%).

Analysis of the difference on PAFE and the suppressive effect on phospholipase production elicited by the three different concentrations of CG revealed a statistically significant difference between each concentration tested.

When the relationship between CG-induced PAFE and phospholipase production was analysed, there was no positive co-relation between these two parameters for all three concentrations tested (i.e. $r = 0.108$ and $P = 0.455$ for 0.05% CG; $r = 0.084$ and $P = 0.564$ for 0.0025% CG; $r = 0.00$ and $P = 0.999$ for 0.00125% CG).

Discussion

Candida albicans is by far the major aetiological agent associated with oral candidosis.¹ The ability of *C. albicans* to produce extracellular enzymes such as phospholipases has been associated with its capacity to colonise host tissue and cause tissue invasion. In fact *C. albicans* has consistently shown superior phospholipase activity than other non-*albicans* *Candida* species, making it one of the most virulent of all *Candida* species.^{2,2} In addition, to phospholipase production, another form of measuring *Candida* virulence is the PAFE, which measures the growth recovery capacity after a limited exposure to antifungal agents, where more virulent and resistant organisms will have low PAFE, whereas a susceptible and less virulent organisms will have higher PAFEs.^{7–9,20,23}

The present results indicate that brief exposure to subtherapeutic concentrations of CG elicited PAFEs ranging from approximately 0.5 to 7 h on the *C. albicans* isolates tested based on the concentration used. In addition, such exposure of *C. albicans* isolates to CG

Table 2 Mean ($\pm$ SEM) Pz activity of 50 oral *Candida albicans* isolates following 1-h exposure and subsequent removal of three different concentrations of chlorhexidine gluconate. Each value indicates the mean ($\pm$ SEM) CSH of 10 oral *C. albicans* obtained either from healthy individuals, diabetic patients, asthmatic patients, smokers or denture wearers.

Source of <i>C. albicans</i> isolates (n = 50)	Unexposed controls	0.005% Chlorhexidine	0.0025% Chlorhexidine	0.00125% Chlorhexidine
Healthy (n = 10)	1.68 (0.02)	1.36 (0.03)	1.43 (0.02)	1.47 (0.01)
Diabetic (n = 10)	1.71 (0.02)	1.35 (0.03)	1.41 (0.02)	1.48 (0.02)
Asthmatic (n = 10)	1.74 (0.03)	1.35 (0.04)	1.41 (0.03)	1.47 (0.02)
Smokers (n = 10)	1.72 (0.03)	1.37 (0.04)	1.41 (0.03)	1.48 (0.02)
Denture wearers (n = 10)	1.76 (0.02)	1.31 (0.03)	1.38 (0.02)	1.50 (0.01)
Mean ($\pm$ SEM)	1.72 (0.01)	1.35 (0.02)	1.41 (0.01)	1.48 (0.01)
Standard deviation	0.07	0.09	0.07	0.05
P value ¹		P < 0.001	P < 0.001	P < 0.001

SEM, standard error of mean; CSH, cell surface hydrophobicity.

¹Compared to the controls the P value was also <0.001 for each patient group tested.

Table 3 Summary of the mean percentage suppression of phospholipase production in 50 oral *Candida albicans* isolates following 1-h exposure and subsequent removal of three different concentrations of chlorhexidine gluconate. Each value indicates the mean percentage reduction of phospholipase production in 10 oral *C. albicans* obtained from either healthy individuals, diabetic patients, asthmatic patients, smokers or denture wearers.

Source of <i>C. albicans</i> isolates (n = 50)	0.005% Chlorhexidine	0.0025% Chlorhexidine	0.00125% Chlorhexidine
Healthy (n = 10)	19.04	14.04	12.50
Diabetic (n = 10)	21.05	17.54	13.45
Asthmatic (n = 10)	22.41	18.97	15.52
Smokers (n = 10)	20.35	18.02	13.95
Denture wearers (n = 10)	25.57	21.59	14.77
Mean ($\pm$ SEM)	21.68 (1.11)	18.20 (1.08)	14.04 (0.52)
Standard deviation	2.49	2.42	1.16
P value ¹	P < 0.001	P < 0.001	P < 0.001

SEM, standard error of mean.

¹The P value was also <0.001 for each patient group tested.

was also capable of suppressing the phospholipase production to a significant level with a percentage reduction ranging from approximately 14% to 22%. The observed CG-induced PAFE and suppression of phospholipase production by limited exposure to CG may be related to the pharmacodynamics of this antiseptic on *Candida*. For instance, scanning and transmission electron microscopic studies have demonstrated loss of cytoplasmic components and coagulation of nucleoproteins and associated morphological changes in the *Candida* cell wall structure.²⁴ In addition, a decreased percentage of budding *Candida* cells was also observed in the aforementioned study.²⁴ Interestingly, other chlorhexidine derivatives such as chlorhexidine

diacetate have also produced cytological changes in other yeast species such as *Saccharomyces cerevisiae*, involving dense and granular cytoplasmic constituents, withdrawal of the interior constituents from the cell wall and a general loss of the typical cellular organisation.²⁵ Moreover, chlorhexidine-induced leakage of K⁺ and pentose material from *S. cerevisiae* and protoplast lysis has also been documented.²⁶ Taken together these possible effects of CG and other chlorhexidine derivatives on yeast species, in addition to the fact that CG may be removed from the oral cavity during the first hour due to the diluent effect of saliva and the cleansing effect of the oral musculature following an oral rinse (i.e. similar to the exposure time in our study),¹⁷ it is rational to surmise that brief exposure to CG as seen in this study is capable of eliciting a substantial PAFE and suppresses the phospholipase production of *C. albicans* planktonic oral isolates.

Analysis of the effect among the three concentrations of CG revealed that there was a significant difference among all three concentrations tested on the PAFE and suppressive effect elicited on the phospholipase production of the *Candida* species tested. A significant difference between similar concentrations of CG on the suppressive effect elicited by this antiseptic on the cell surface hydrophobicity (CSH) and germ tube (GT) formation of *C. albicans* isolates has been reported.^{27,28} In the oral cavity the diluent effect of saliva and the cleansing effect of the oral musculature results in changeability of drug concentrations resulting in alterations in the initial therapeutic concentration, usually making it subtherapeutic. Hence, the aforementioned difference between concentration gradients in inducing a PAFE and suppressing the phospholipase production, CSH and GT formation on *C. albicans* planktonic isolates may have possible

implications in designing future dosage regimens of topical antiseptics for usage *in vivo* in the oral cavity. However, in this study there was no positive relationship between the CG-induced PAFE and phospholipase production with all three concentrations tested. This may be due to the fact that PAFE is a measurement of growth suppression, whereas phospholipase production is a measurement of extracellular enzyme production, which are two divergent attributes from each other and determined by two distinct methodologies.

Taken together, the aforementioned information contributes to broadening of the understanding of pharmacodynamics of CG against two virulent determinants (i.e. PAFE and phospholipase production) incriminated in the pathogenesis of *C. albicans*, which frequently colonises in diabetics, asthmatics using steroid inhalers, smokers and denture wearers. Although only planktonic *Candida* isolates from all these patient groups were used in the study this information may possibly accentuate the use of CG *in vivo* as an adjunct in the management of oral candidosis. In addition, antimicrobial resistance to nearly all antifungal agents has been reported in clinical isolates of *Candida*.²⁹ Development of such resistance may have important implications for antifungal therapy and indicate the need for possible alternative therapies, which may facilitate the management of oral candidosis. In this context, the current study reveals that exposure to CG induces a PAFE and reduces candidal phospholipase production to varying degrees, which appear to be an unrecognised, yet a salutary feature potentiating action of this antiseptic on planktonic *Candida* cells *in vitro* thereby possibly providing further credence to the use of CG *in vivo* as an adjunct in the management of oral candidosis.

Although there have been previous studies on CG as well as other antimycotic induced PAFE's and its impact on various pathogenic attributes of *Candida*,^{7–9,15,20,23,28,30} the methodological differences between researchers, in addition to variations in the concentrations of the drugs used, number and the types of *Candida* species engaged, exposure time of the drug and pathogenic attribute tested make comparisons extremely arduous between this study with previously studies. In a nutshell, these studies^{6–8,14,15,20,23,28,30} have revealed that sub-cidal concentrations of nystatin, amphotericin B, 5-fluorocytosine, ketoconazole and fluconazole as well as CG in concentrations used in the current study are capable of producing PAFE's of varying durations and modulating pathogenic candidal attributes such as adhesion to BEC and denture acrylic, GT formation and CSH to varying degrees. However, to our knowledge the current study is the

first to document the CG-induced PAFE and the suppression of phospholipase production during the PAFE period, covering the largest number of oral *C. albicans* isolates obtained from a single geographic location in the Middle-East Asia. Testing with a larger number of sessile isolates encased in a *Candida* biofilm scenario instead of using only planktonic isolates as used in this study is warranted to further magnify the current findings.

Conclusions

Brief and transient exposure to subtherapeutic concentrations of CG may induce a PAFE and modulate phospholipase production of *C. albicans* isolates, thereby quelling its pathogenicity.

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