

Changes in the Cell Surface Hydrophobicity of Oral *Candida albicans* from Smokers, Diabetics, Asthmatics, and Healthy Individuals following Limited Exposure to Chlorhexidine Gluconate

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Key Words

Candida albicans · Cell surface hydrophobicity · Chlorhexidine gluconate

Abstract

Objective: The objective of this study was to determine the cell surface hydrophobicity of 40 oral *Candida albicans* isolates obtained from smokers, diabetics, asthmatics using steroid inhalers, and healthy individuals, following brief exposure to subtherapeutic concentrations of chlorhexidine gluconate. **Materials and Methods:** Forty *C. albicans* oral isolates (10 isolates each from smokers, diabetics, asthmatics using steroid inhalers, and healthy individuals) were exposed to 3 subtherapeutic concentrations of chlorhexidine gluconate (0.00125, 0.0025, and 0.005%) for 30 min. Thereafter, the antiseptic was removed and the cell surface hydrophobicity was measured by a biphasic aqueous-hydrocarbon assay. **Results:** Compared to the unexposed controls, the cell surface hydrophobicity of *C. albicans* isolates was suppressed by 5.40% ($p > 0.05$), 21.17% ($p < 0.05$), and 44.67% ($p < 0.05$) following exposure to 0.00125, 0.0025, and 0.005% chlorhexidine gluconate, respectively. **Conclusions:** A brief period of transient exposure to subtherapeutic concentra-

tions of chlorhexidine gluconate may modulate the cell surface hydrophobicity of *C. albicans* isolates and thereby may reduce candidal pathogenicity.

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Introduction

Candida albicans is renowned as the foremost fungal pathogen implicated in oral candidosis and is considered the most common human fungal infection implicated in a variety of clinical manifestations [1]. Interestingly, more than 90% of human immunodeficiency virus (HIV)-infected individuals develop oral candidosis at some point in their disease, which is by far the commonest oral manifestation in these patients [2]. Further, *Candida* infections have been implicated in persistent apical periodontitis [3], and *Candida*-like organisms have been demonstrated in root canals and dentinal tubules in situ [4].

Adhesion of microorganisms to host mucosal surfaces is a fundamental prerequisite for successful microbial colonization and infection. The process of candidal adhesion is rather complex and involves both biological and nonbiological factors. Microbial cell surface hydropho-

bicity (CSH), which contributes to hydrophobic interactions between cells and surfaces, is thought to be an important nonbiological factor associated with the adherence of *Candida* to inert surfaces [5]. Studies have also shown that hydrophobic yeasts are more virulent than their hydrophilic counterparts [6, 7]. Significant correlations between CSH and candidal adhesion to buccal epithelial cells and denture acrylic surfaces has also been reported previously [8, 9].

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 [10]. The antifungal effect of CG has been demonstrated in several in vivo and in vitro trials, including some related to *Candida* infection [11]. It has also been demonstrated that exposure of either *Candida* isolates or buccal epithelial cells to 0.2% CG overwhelmingly suppresses the ability of the former to adhere to buccal epithelial cells in healthy [12] as well as in compromised patients, such as diabetics [13]. Likewise, pretreatment of acrylic dentures with 2% CG has also been shown to suppress the adhesion of the yeasts to denture acrylic surfaces [14]. In addition, a recent study showed that subtherapeutic levels of CG are also effective in suppressing germ tube formation of oral *C. albicans* isolates obtained from healthy individuals as well as diabetics, asthmatics using steroid inhalers, and smokers [15]. For these reasons, oral rinses containing CG may be an appropriate substitute for conventional antimycotics in the management of oral candidosis [16].

It has been shown that 30% of the total CG 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 affecting its therapeutic efficacy [17]. Hence, intraorally, the pathogenic yeasts undergo a brief exposure to high concentrations of CG following an oral rinse during therapy, while thereafter the drug concentration is likely to be subtherapeutic. However, the conduct of yeasts under the latter conditions has not been adequately studied. For instance, there has only been one study [18] which has quantitatively compared the CSH of oral *C. albicans* isolates following brief exposure to CG. In addition to HIV infection, *C. albicans* has also been implicated in oral candidosis in other patients such as diabetics, asthmatics using inhalation steroids, and smokers [19–21]. However, the CSH of oral *C. albicans* isolates obtained from these patients following brief exposure to subtherapeutic concentrations of CG has not

been studied heretofore. Hence, the main aim of this study was to investigate the CSH of 40 oral *C. albicans* isolates obtained from diabetics, asthmatics using inhalation steroids, smokers, and healthy individuals after their brief (30 min) exposure to 3 subtherapeutic concentrations of CG.

Materials and Methods

Organisms

A total of 40 oral isolates of *C. albicans* recovered from oral rinse samples from patients attending the Kuwait University Dental Clinic (KUDC) for dental treatment were included in the study (10 isolates each were from smokers, diabetics, asthmatics using steroid inhalation, and healthy individuals). These isolates were from a total of 370 patients screened at the KUDC in a previous prevalence study [22]. Though non-*albicans* species of *Candida* were also isolated in the previous study [22], only *C. albicans* isolates were used for the current study. The diabetic patients were on oral hypoglycemic drugs, and the asthmatic patients were on steroid inhalation therapy but were otherwise healthy at the time of attending the KUDC. The patients who smoked more than 25 cigarettes per day were considered as smokers. None of the patients from which the isolates were recovered had oral candidosis. Initially, all of the yeast isolates were tested for germ tube formation. Thereafter, the colony characteristics were observed using CHROMagar *Candida* medium (Becton Dickinson and Company, Sparks, Md., USA) and identified using the VITEK 2 yeast ID system (BioMérieux, France) as well as the API 20C AUX yeast ID system (BioMérieux, Inc., Hazelwood, Mo., USA).

Antifungal Agents and Media

The CG 0.2% (Corsodyl; GlaxoSmithKline, Brentford, UK) was dissolved in sterile phosphate-buffered saline (PBS) at pH 7.2 and was prepared as 0.00125, 0.0025, and 0.005% solutions immediately prior to each experiment as previously described [16, 18].

Preparation of the Cell Suspension for the Hydrophobicity Assay

A previously described method was used for this purpose [16, 18]. 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 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 2 cycles of dilution with sterile PBS and centrifugation for 10 min at 3,000 g. Afterwards, the supernatant was completely decanted and the pellets were re-suspended in 5 ml of sterile PBS. This procedure, as previously used for drug removal [16, 18], has been shown to reduce the concentration of the drug by as much as 10,000-fold, thereby minimizing any carryover effect of the drug following its removal. Viable counts of the control and the test were performed after drug

Table 1. Mean (\pm SEM) CSH of 40 oral *C. albicans* isolates

Source of <i>C. albicans</i> isolates (n = 40)	Unexposed controls	Chlorhexidine, %		
		0.00125	0.0025	0.005
Healthy (n = 10)	21.62 \pm 1.24	20.32 \pm 0.92	16.34 \pm 0.78	11.43 \pm 0.84
Diabetic (n = 10)	23.73 \pm 1.08	22.92 \pm 1.04	18.65 \pm 1.02	12.68 \pm 0.76
Asthmatic (n = 10)	21.46 \pm 0.92	19.82 \pm 0.62	16.93 \pm 0.88	12.17 \pm 1.22
Smokers (n = 10)	24.22 \pm 0.87	23.12 \pm 0.64	19.92 \pm 1.05	14.12 \pm 0.83
Mean \pm SEM	22.76 \pm 0.71	21.55 \pm 0.86	17.96 \pm 0.82	12.60 \pm 0.57
p value		>0.05	<0.005	<0.005

removal. As the procedure of drug removal effectively eliminated any carryover effect, there was virtually no difference in the viable counts of the control and the tests following exposure to already diluted subtherapeutic concentrations of the drug as observed in previous studies [16, 18].

Hydrophobicity Assay

A biphasic aqueous-hydrocarbon assay previously used for the assessment CSH on oral *Candida* species [8, 9, 18, 23] was used in the current study. In brief, 5 ml of yeast suspension was vortex mixed and the absorbance was measured at 520 nm. For each organism tested, 2.5-ml volumes of suspension were added to 2 sterile glass test tubes, representing 1 test and 1 control. In addition, a test and a control were prepared from the suspending medium alone as spectrophotometer blanks. Xylene (0.5 ml) was added to each test suspension. The test and the controls were placed in an incubator at 37°C for 10 min to equilibrate, and then taken in turn and vortex mixed for 30 s and returned to the incubator for a further 30 min to allow the immiscible xylene and aqueous phases to separate. The lower aqueous phase of the sample was carefully removed using a pipette and transferred to a clean test tube. Any traces of contaminating xylene that may have been carried over in the pipette or bound to the yeast was removed by bubbling air through the suspension at a rate of 180 ml per min, for 2 min. The absorbance was measured as before at 520 nm following vortex mixing for 5 s to disrupt and resuspend any aggregates that may have formed. The hydrophobicity was expressed as the percentage reduction in optical density of the test suspension compared with the control. Thus, the greater the change in absorbance, the greater the shift in yeasts from the bulk medium to the interface, i.e. the more hydrophobic the yeast strain. Suspensions without xylene were used as the negative controls.

All experiments were repeated on 3 separate occasions with duplicate determinations on each occasion.

Statistical Analysis

The effect of CG on each isolate was statistically analyzed. The data obtained from all 3 CSH assays in duplicate were analyzed using ANOVA Dunnett's t tests, which treated one group as the control (unexposed to CG) and compared all other groups (exposed to CG) to it. The difference between the suppressive effect on CSH elicited by the 3 different concentrations (0.00125 vs. 0.0025 vs. 0.005%) was also compared by Tukey's multiple comparison test.

Table 2. Summary of the mean percentage suppression of CSH in 40 oral *C. albicans* isolates

Source of <i>C. albicans</i> isolates (n = 40)	Chlorhexidine, %		
	0.00125	0.0025	0.005
Healthy subjects (n = 10)	6.01	24.42	47.13
Diabetics (n = 10)	3.41	21.41	46.57
Asthmatics (n = 10)	7.64	21.11	43.29
Smokers (n = 10)	4.54	17.75	41.70
Mean \pm SEM	5.40 \pm 0.92	21.17 \pm 1.36	44.67 \pm 1.30

Results

The mean CSH of the 40 *C. albicans* isolates following limited exposure to 3 different subtherapeutic concentrations of CG, drug removal, and subsequent biphasic aqueous-hydrocarbon assay is shown in table 1. The mean CSH of the unexposed isolates was 22.76 \pm 0.71, whereas this value following exposure to 0.00125, 0.0025, and 0.005% CG was 21.55, 17.96, and 12.6, respectively. The mean percentage reduction in CSH of these isolates compared to the unexposed controls is shown in table 2. Compared to the controls, a marked reduction in the CSH of all of the isolates was seen following exposure to 0.005% CG, with a mean percentage reduction of 44.67% ($p < 0.005$). The overall suppressive effect on CSH following exposure to 0.0025% dilution of the antiseptic was also significant in comparison to that of the unexposed controls, although it was considerably lower than for the higher concentration (21.17 vs. 44.67%). Overall, though there was a reduction in the CSH of all the yeasts exposed to 0.00125% of CG (5.4%), the suppressive effect was statistically not significant on the isolates tested.

Analysis of the suppressive effect on CSH elicited by the 3 different concentrations revealed a statistically significant difference in the mean percentage reduction for each concentration tested.

Discussion

The present results indicate that, limited exposure of isolates of *C. albicans* from 0.0025 to 0.005% and CG could suppress the CSH of these isolates to a significant level, with a mean percentage reduction of 21.17 and 44.67% ($p < 0.005$), respectively, which confirmed our previous results [18]. However, exposure to 0.00125% CG did not generate a significant mean percentage reduction of CSH, which was only 5.4% ($p > 0.05$). Interestingly, in a recent study [24] with *C. dubliniensis* isolates, it was shown that 0.00125, 0.0025, and 0.005% CG were capable of reducing the CSH of these isolates by 7.01% ($p > 0.05$), 21.82% ($p < 0.005$), and 44.49% ($p < 0.005$), respectively, which is almost similar to the suppressive effect elicited in the current study. Though the number of *C. dubliniensis* isolates used in this study [24] was much smaller ($n = 12$) compared to the current study ($n = 40$), the similarity is not surprising as these two *Candida* species are closely related in evolutionary terms, sharing each other's phenotypic properties of commensalism and opportunistic infection.

The relative CSH of *Candida* is considered a nonbiological factor of critical importance relating to candidal adhesion as reported by Hazen and Hazen [25] that hydrophobic yeasts are more virulent than their hydrophilic counterparts. Others have shown that the reduction in CSH following limited exposure to antibiotics promoted increased ingestion of bacteria by polymorphonuclear leukocytes, thus increasing the susceptibility of bacteria to the killing effect of polymorphonuclear leukocytes [26, 27]. Hydrophobic cells also exhibited greater adherence to epithelial cells and extracellular matrix proteins and decreased susceptibility to phagocytic killing [28]. In addition, the superior virulence of hydrophobic cells over hydrophilic cells may be due, in part, to the potential of hydrophobic cells to bind throughout various organs following clearance from the bloodstream [28]. Microbial structures that contribute to the CSH include outer membrane protein, lipoprotein, phospholipid, lipopolysaccharide, and fimbriae [27]. Thus, drugs that alter these structural features have been shown to reduce the CSH of bacteria. In the case of *C. albicans* it has been shown that CSH correlates with

the concentration of fibrils in the exterior layer of the cell wall [28]. Hence, the aforementioned suppression of CSH elicited by CG on phenotypically similar *Candida* species (i.e. *C. albicans* and *C. dubliniensis*) may be related to the pharmacodynamics 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 the result of a loss of cytoplasmic components and coagulation of nucleo-proteins and associated morphological changes in the cell wall structure [29]. Therefore, it is rational to surmise that by affecting the cell wall structure CG may be capable of subduing the CSH of *Candida* species.

Analysis of the effect among the 3 different concentrations of CG (0.00125 vs. 0.0025 vs. 0.005%) revealed that there was a significant difference between all 3 concentrations tested on the suppressive effect elicited on the CSH of the *Candida* species tested. 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, it can be speculated that the aforementioned significant concentration-dependent difference in suppressing the CSH of oral *Candida* species may have some implication in designing appropriate dosage regimens of topical antiseptic agents used in the oral cavity.

Oral candidosis has been implicated in populations of diabetics, asthmatics using inhalation steroids, and smokers [19–21]. Therefore, the information provided herein is noteworthy as it contributes to broadening the understanding of the pharmacodynamics of CG against a vital attribute (i.e. CSH) incriminated in the pathogenesis of *C. albicans*, which frequently colonizes in the compromised patients (diabetics, asthmatics using steroid inhalers, and smokers). As isolates from all of these patient groups were used in the study, this information may further promote the use of CG as an adjunct in the management of oral candidosis. In addition, antimicrobial resistance has become increasingly important in antifungal therapy. Resistance to nearly all antifungal agents has been reported in clinical isolates of *Candida* [30]. 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 candidiasis. In this context, this study clearly revealed that exposure to CG reduced candidal CSH to varying degrees, which appear to be an unrecognized yet salutary feature potentiating action of this

antiseptic, and therefore the information provided lends further credence to the use of CG as an adjunct in the management of oral candidiasis.

Conclusions

Brief and transient exposure to subtherapeutic concentrations of CG may modulate the CSH of *C. albicans* isolates, thereby quelling its pathogenicity, and exemplifies additional pharmacodynamics of CG. Further stud-

ies on the impact of CG on other pathogenic attributes related to *Candida* adhesion with a larger battery of organisms are warranted.

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