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Antifungal activity of chlorhexidine on *Candida* spp. biofilm

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Resumo

Candidíase oral é a infecção oportunista mais comum em pacientes imunocomprometidos, sendo a clorexidina um importante antimicrobiano auxiliar no seu tratamento. O objetivo do presente estudo foi avaliar o efeito antifúngico de diferentes soluções de clorexidina (Periogard®, NoPlak Max®, Noplak®, Perioxidin®, Clorexidina 0.06%, Paradontax® e Clorexidina 1%) sobre biofilmes artificiais de *Candida* spp.: *C. albicans* (ATCC36801); *C. parapsilosis* (ATCC22019); *C. krusei* (ATCC6258); *C. glabrata* (ATCC2001) e *C. tropicalis* (ATCC750). As cepas foram cultivadas em meio de cultura BHI ágar sobre fragmentos de esmalte bovino por 72 horas a 37 °C. Após o crescimento, cada fragmento de esmalte bovino foi imerso nas diferentes soluções de Clorexidina por 3 minutos. Nistatina e solução salina foram utilizadas como controle negativo e positivo, respectivamente. Para remoção das células não aderidas, os fragmentos foram então imersos em solução salina por 10 minutos e agitados em vortex. Alíquotas de 100 µL foram inoculadas em placas contendo BHI ágar por 24 horas a 37 °C para contagem de unidades formadoras de colônias (UFC). Observamos que o número de UFC de *C. albicans* e *C. parapsilosis*, apresentou um percentual de redução variando de 79 a 99% quando do uso das diferentes soluções ($p < 0,001$), o mesmo não foi observado para o NoPlak Max® (2,94 e 1,3%, respectivamente); Para *C. krusei* e *C. glabrata,* a solução menos efetiva foi a Nistatina (23 e 3,4%, respectivamente) enquanto que para *C. tropicalis,* todas as soluções apresentaram um alto percentual de redução (99 a 100%). As soluções de clorexidina foram capazes de reduzir significativamente o número de UFC provenientes de biofilme de *Candida* spp. in vitro.

Palavras-chave: Clorexidina; candida; biofilmes.

Abstract

Oral candidiasis is the most common opportunistic infection in immunocompromised patients and chlorhexidine is an important antimicrobial for its treatment. The antifungal effect of different CHX solutions (Periogard®, NoPlak Max®, Noplak®, Perioxidin®, Chlorhexidine 0.06%, Paradontax® and Chlorhexidine 1%) was evaluated on artificial biofilms of *Candida spps*: *C. albicans* (ATCC36801), *C. parapsilosis* (ATCC22019), *C. krusei* (ATCC6258), *C. glabrata* (ATCC2001) and *Candida tropicalis* (ATCC750). The strains were grown, in a BHI agar medium on bovine teeth enamel for 72 hours at 37 °C. After growth, the fragments were immersed in the CHX solutions for 3 minutes. Nystatin and saline solutions were used as positive and negative controls respectively. To remove the non-adhered cells, the fragments were inoculated in saline solution for 10 minutes, transferred to Falcon tubes containing saline solution and mixed in a vortex. Aliquots of 100 µL were inoculated on BHI agar for 24 hours at 37 °C to count the number of colony forming units (CFU). We observed that the number of (CFU) of *C. albicans* and *C. parapsilosis*, showed a reduction rate ranging from 79 to 99% with the use of different solutions ($p < 0.001$), except for NoPlak Max® (2.94 and 1.3%, respectively). For *C. krusei* and *C. glabrata,* nystatin was the least effective solution (23 and 3.4%, respectively); and for *C. tropicalis,* all the substances presented a high reduction percentage (99-100%). The chlorhexidine solutions were able to reduce the colony forming units of *Candida* biofilm.

Keywords: Chlorhexidine; candida; biofilms.

INTRODUCTION

Candida is an opportunist pathogen which may cause acute or chronic infections[1], especially in immunocompromised patients, such as those infected with HIV. *Candida* species may be involved in various forms of oral diseases such as oral candidiasis, angular cheilitis, endodontic infections and periodontitis. *Candida albicans* is the most commonly found specie associated to oral lesions, however other species such as *C. glabrata, C. tropicalis, C. krusei and C. parapsilosis* may also be found in such lesions[2]. The presence of these non-albicans species may have important implications for the treatment, since some of them show different susceptibility to antifungal agents[3,4].

Several antifungal agents administered either topically or systemically, may be used for the management of candidosis. Chlorhexidine has been used as a therapeutic topical supplement due to its wide spectrum of antimicrobial activity against a wide variety of organisms, including *Candida*[5-7]. The mode of action of this substance is not entirely understood, but it is known that it acts as a fungicide and has a fungistatic function, leading to the coagulation of nucleoproteins and changes in cell walls allowing the possible escape of cytoplasmic components through the plasmalemma[6,8]. Also chlorhexidine is capable of inhibiting candidal adhesion to biological and inert surfaces[6].

Chlorhexidine is not the first choice of drug for the treatment of Candida infections. However, the increase in the number of opportunistic infections caused by fungus, mainly in HIV infected individuals, and the great number of strains that have become resistant to the common antifungals has encouraged new research in relation to alternative treatments of such infections, among which is the use of chlorhexidine. Consequently, the aim of this study is to evaluate, in vitro, the antifungal activity of seven chlorhexidine solutions on artificial biofilms of *Candida* spp.

MATERIAL AND METHOD

1. *Candida Species and Biofilm Formation*

Five different species of *Candida* were used in this study: *Candida albicans* (ATCC 36801), *Candida parapsilosis* (ATCC 22019), *Candida krusei* (ATCC 6258), *Candida glabrata* (ATCC 2001) and *Candida tropicalis* (ATCC 750).

A total of 45 healthy bovine teeth were selected. A careful, reflected-light microscopic examination was performed to exclude any teeth that were not intact or did not have a homogeneous enamel surface. The bovine teeth were cut in a standardized size of 8 × 9 mm (fragment) with a double face diamond disk mounted on a low rotation straight piece.

For the biofilm formation, the methodology was based on the study by Alviano et al.[9] (2003) with a few changes. The 45 bovine teeth fragments, previously sterilized (autoclaved at 121 °C for 15 minutes) were fixed in *Petri* plates with BHI (Brain Heart Infusion) Agar medium. One plate for each *Candida* specie (total of 5 plates) was prepared with 9 teeth fragments with the enamel surface face up and the other surfaces completely immersed in the culture medium agar. Cellular suspensions in BHI Broth of each species, with 10^5 cells.mL^{-1}, were inoculated into the *Petri* plates and biofilm formed on the specimens in 72 hours after incubation at 37 °C, without shaking.

2. *Application of the Solutions*

The following seven chlorhexidine solutions were tested: Periogard® (Colgate-Palmolive Company, São Paulo, SP, Brazil), NoPlak Max® (Laboratório Daudt Oliveira Ltda, Rio de Janeiro, RJ, Brazil), Noplak® (Laboratório Daudt Oliveira Ltda, Rio de Janeiro, RJ, Brazil), Perioxidin® (Lácer SA, Sardenya, Barcelona, Spain), Chlorhexidine 0.06% (Fórmula & Ação, São Paulo, SP, Brazil), Paradontax® (BYK Quím. e Farm. Ltda., São Paulo, SP, Brazil) and Chlorhexidine 1% (Fórmula & Ação, São Paulo, SP, Brazil). Nystatin (Bristol-Myers Squibb, São Paulo, Brazil) was used as the negative control and sterile saline as the positive control.

After the induction of artificial biofilm by the different species of *Candida*, one tooth fragment from each specie was immersed in 1 mL of one chlorhexidine solution for 3 minutes. Subsequently, the fragments were kept for 10 minutes in 1 mL of saline solution, in order to remove any non adhered cells. After, each fragment was transferred to a Falcon tube, containing 1 mL of saline solution and the same was shaken in a vortex mixer for 20 seconds to remove the cells pertaining to the biofilm[9]. Aliquots of 100 µL were inoculated on a *Petri* plate with BHI Agar and the counting of the colony forming units (cfu) was carried out after incubation for 24 hours at 37 °C.

3. *Data Analysis*

All the experiments were performed in triplicate and the results were expressed as means and standard deviation. The perceptual reduction of the viable cells after the treatment with each solution was determined and was compared with the positive control (saline solution). ANOVA was carried out with Tukey's test ($p < 0.05$) for comparison of the effectiveness of the evaluated substances.

RESULT

Table 1 presents the perceptual reduction after the use of each of the chlorhexidine solutions on the biofilm of each species of *Candida.*

In relation to *C. albicans* and *C. parapsilosis* species, the solution NoPlak Max® presented a smaller percentage reduction compared to the other solutions and this result was statistically different. For the *C. krusei* and *C. glabrata* species, all the chlorhexidine solutions were able to reduce viable cells on the biofilm; however Nystatin was the least effective, but this result was statistically significant only for the last species. In relation to *C. krusei*, the significance occurred only when Nystatin was compared to the 1% chlorhexidine solution and Paradontax® (Table 1).

The results for *C. tropicalis* show a similar effectiveness for all the tested solutions.

Table 1. The percentage reduction and standard deviation averages of the 5 species of *Candida*, in relation to the evaluated substances. Aa; Bb; Cc and Dd mean a significant statistical difference between the substances ($p < 0.05$) (Tukey's test)

Solutions	Perceptual reduction of the biofilm after using the solution (% ± sd)				
	C. albicans	*C. parapsilosis*	*C. krusei*	*C. glabrata*	*C. tropicalis*
Nystatin	92.0 ± 12.2^{a}	98.7 ± 0.0^{b}	23.6 ± 33.4^{C}	3.4 ± 0.6^{D}	97.7 ± 2.1
Periogard® CHX 0.12%	89.8 ± 14.3^{a}	95.9 ± 6.4^{b}	74.5 ± 9.7^{c}	84.7 ± 9.8^{d}	95.4 ± 6.4
NoPlack Max® CHX 0.12%	2.4 ± 3.4^{A}	1.3 ± 1.9^{B}	57.0 ± 39.9^{c}	62.9 ± 38.0^{d}	90.2 ± 12.9
NoPlack® CHX 0.12%	79.9 ± 27.9^{a}	96.0 ± 6.5^{b}	96.0 ± 4.3^{c}	94.1 ± 5.3^{d}	99.9 ± 0.1
CHX 0.06%	92.8 ± 0.7^{a}	98.9 ± 1.8^{b}	92.7 ± 9.6^{c}	85.5 ± 20.5^{d}	99.7 ± 0.1
Paradontax® CHX 0.2%	99.8 ± 0.2^{a}	99.3 ± 0.7^{b}	99.8 ± 0.1^{c}	99.0 ± 1.4^{d}	99.1 ± 1.6
CHX 1%	92.0 ± 13.9^{a}	92.8 ± 12.5^{b}	99.6 ± 0.5^{c}	91.3 ± 12.7^{d}	100 ± 0.0
Perioxidin® CHX 0.12%	80.9 ± 4.8^{a}	95.1 ± 6.9^{b}	95.5 ± 6.2^{c}	82.0 ± 25.0^{d}	98.1 ± 2.7

DISCUSSION

In addition to the adhesion phenomenon, *Candida* species may form biofilms in which the microorganisms express a new and sometimes more virulent phenotype, making such microorganisms less susceptible to antifungals[3,4]. In the literature microorganisms pertaining to the biofilm are reported to be 500 times more resistant to antimicrobial agents than planktonic cell microorganisms[14,15]. So, it is very important to test new treatment options for candidiasis, such as chlorhexidine, which is the drug of choice for this study.

The chlorhexidine molecule is a highly cationic biguanide and binds avidly to negatively charged surfaces, including epithelial cells[16] and can be used in various concentrations, as shown in this study. When a low chlorhexidine dose is used, the cellular transport of bacterial cells is damaged with the creation of pores in the cellular membrane. At higher concentrations, the solution penetrates the bacterial cells and leads to microorganism destruction[17]. Also, studies with *Candida* exposed to chlorhexidine have shown the coagulation of nucleoproteins and alterations of the cell wall allowing the possible escape of cytoplasmic components to the plasmalemma[8]. Our results show a large reduction of viable cells in the *Candida* spp. biofilms after the use of different chlorhexidine solutions, suggesting that such cells were destroyed. According to MacNeill et al.[18] (1997), deep effects in the structural viability and integrity of the *Candida* species occur after exposure to chlorhexidine, both at macroscopic levels, where adhesion to the surface of the substrate is disrupted and at cellular levels[18]. The effects of chlorhexidine on the adhesion abilities of *Candida*, one of the main contributing factors to its virulence[6].

Chandra et al.[3] (2001) showed that *C. albicans* biofilms, when grown in vitro, are highly resistant to antifungal agents, including nystatin and chlorhexidine. However, in this study, there was a high percentage reduction of *C. albicans* biofilm after the use of nystatin and 6 of the 7 chlorhexidine solutions studied, showing that these solutions were effective against this species. Confirming such findings, other authors have also observed an effective performance of chlorhexidine against *C. albicans* biofilms[4,19]. Different chlorhexidine-based solutions also showed an effective result against the biofilms of the other Candida species used in this study. The only exception was NoPlak Max® against *C. albicans*. Giuliana et al.[20] (1997) reported that different chlorhexidine solutions show similar antifungal properties, suggesting that differences in ingredients incorporated in the solutions have not affected the chlorhexidine antifungal activity. This study showed a different result, considering that for both *C. albicans* and *C. parapsilosis* the NoPlak Max® solution presented a smaller perceptual reduction than the other substances and the difference was statistically significant. NoPlak Max® is the only one with *propolis* in its composition, which could be a cause of interference in the way chlorhexidine acts. However additional studies are necessary to confirm this hypothesis. It is important to emphasize that the comparison between the different chlorhexidine solutions was not the objective of this study. These 7 solutions were chosen because they are the most commercialized in Brazil and studies of the performance of these solutions on the artificial biofilm of Candida are scarce in the literature.

One limitation of our study is that the artificial biofilm was formed on bovine enamel teeth modifying the methodology on which this experiment was based[9]. This was due to the difficulty of acquiring permanent or deciduous human teeth[10] and the use of bovine teeth is very common in dentistry researches[11-13], so the authors do not believe that this influenced the results. Also, the study was an in vitro study and the biofilms were formed by only one microorganism, which does not reflect an oral biofilm

environment. Therefore, the results must be analyzed with caution.

In HIV-infected patients, the development of anti-retroviral therapy has caused a change in the opportunistic oral infection patterns and causing a reduction in their emergence[21]; however, in some cases serious lesions resistant to conventional antifungal therapy may occur. This is due to a response failure from the host to the anti-retroviral treatment, leading to a need for prophylactic therapies with antibacterials and antifungals[22]. However in our study we observed that *C. glabrata* and *C. krusei species* were resistant to nystatin, which is considered the topical antifungal choice for treating fungal lesions[2,23]. The literature also reports that there is a resistance of such species to azole antifungals (clotrimazole, ketoconazole, fluconazole and itraconazole), generally used in a systemic manner[24,25].

In general studies of alternative drugs for this infection will be a scientific contribution to dentistry. Our results suggest that the chlorhexidine-based solutions commercialized in Brazil may be an alternative or adjunct treatment for candidiasis, mainly in immunocompromised patients.

CONCLUSION

The chlorhexidine-based solutions were satisfactory in reducing the viable cells of Candida spp. biofilm, except for NoPlak Max®. Consequently chlorhexidine can be considered an alternative to conventional antimycotic therapy in the management of oral *Candida* infection.

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