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Could chlorhexidine be an adequate positive control for antimicrobial photodynamic therapy in- *in vitro* studies?

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ABSTRACT

Background: Chlorhexidine digluconate (CHX) is commonly applied as positive control of new antimicrobials, because it is considered the gold-standard for chemical plaque control. The aim of this study was to compare the effect of treatments with curcumin-mediated aPDT and CHX in relation to the viability of specific microorganism groups in two distinct times (immediately and 24 h later).

Methods: Dentin caries microcosms were grown on bovine dentin discs (37 °C, anaerobiosis) for 3 days in the Active Attachment Amsterdam Biofilm Model. The biofilms were treated with 300 μM curcumin and 75 J.cm^{-2} LED, or 0.06% and 0.12% CHX. Then, total microorganisms, total streptococci, mutans streptococci, and total lactobacilli counts were determined. The statistical analysis was conducted by Kruskal-Wallis and post-hoc Dunn's tests ($P < 0.05$).

Results: Curcumin-mediated aPDT (C + L+), 0.06% and 0.12% CHX reduced mutans streptococci counts (0.19, 0.10 and 0.07 log10 respectively) in the immediate analysis. After 24 h, it was observed a re-growth of microorganisms treated by curcumin-mediated aPDT, whereas both CHX concentrations demonstrated a decrease of the viable microorganisms.

Conclusion: This study confirmed the substantive effect of CHX and the immediate effect of aPDT. The use of a neutralizer solution was important to block the substantivity of CHX and permit its fair comparison with aPDT, allowing its use as a positive control in further studies.

1. Introduction

The use of Antimicrobial Photodynamic Therapy (aPDT) is emerging as a new therapeutic modality option to treat local diseases caused by microorganisms. This therapy can be understood as a system used to promote the inactivation and/or death of microorganisms by the association of three components, a chemical photosensitizing agent, a complementary light source and molecular oxygen [1]. Photosensitizing molecules previously incorporated to cellular structures of microorganisms are able to absorb photons exciting electrons. These excited electrons are transferred to a substrate or molecular oxygen, resulting in molecules of high cytotoxicity, such as singlet oxygen ($^1O_2$), superoxide ions, hydroxyls and other free radicals, which can damage or kill microbial cells [2]. Comparing to conventional antimicrobial agents, aPDT provides a rapid bacterial death, making unnecessary the use of high concentrations of chemicals and long treatments, and consequently, decreasing the resistance of microorganisms [12]. In this sense, curcumin-mediated aPDT was developed to control the viability of oral microorganisms [3]. The dye presents economic advantages due to its lower cost, exerting a powerful phototoxic effect on microorganisms, even when applied in low concentrations [4,5]. Its efficacy has already been demonstrated on planktonic cells and biofilms of several species [6–13].

On the other hand, chlorhexidine digluconate (CHX) is a chemical compound used as the gold-standard for treating local infections, which exhibits a broad spectrum of action on Gram-positive and Gram-negative bacteria [14]. Its action depends on the adsorption of its cationic molecules on negatively charged microbial cellular walls, causing osmotic imbalance and loss of intracellular components [15,16]. Other important properties of CHX are its substantivity and residual effect, since it is slowly displaced from oral tissues carried by calcium ions. The maintenance of high concentrations of CHX even after 24 h of its

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application provides a longer contact with its target microorganisms, leading to a prolonged bacteriostatic and bactericidal effects [17,18].

Although CHX and aPDT present distinct mechanisms of action and properties, it is desirable to find a fair way to compare both antimicrobial strategies for the employment of CHX as a positive control of further studies involving complex biofilm models. Hence, the aim of this *in vitro* study was to compare the viability of specific microorganism groups from microcosms of dentin caries lesions after immediate and late treatments with curcumin-mediated aPDT and CHX, regarding a neutralizer solution of CHX for immediate analyses.

2. Materials and methods

2.1. Study design

The present study was approved by the Committee for Ethics in Human Research of the Bauru School of Dentistry (CAAE: 34559314.6.0000.5417). This experimental protocol was based on Mendéz et al. [19]. Three 7–11 years-old children with at least one dental caries lesion were selected for collection of infected dentin. The action of aPDT on the viability of microcosm biofilms was tested in two stages (immediate and late), including six different groups, as follows: C-L- (no treatment, control), C + L-, C-L+, C + L+, 0.06%CHX, and 0.12%CHX. The experiment was performed in duplicate with one replicate (n = 4/group). The viability of microorganisms was evaluated immediately and 24 h after treatments, by counting colony forming units (CFU) of viable total microorganisms, total streptococci, mutans streptococci, and total lactobacilli.

2.2. Collection of infected dentin

Three authorized children with 7–11 years-old and at least one dentin caries lesion in the first molars were referred to the Clinics of Pediatric Dentistry, Bauru School of Dentistry, where samples of infected dentin were collected using a sterile curette. Children with lesions with pulp exposure, diagnosis of syndromes and/or systemic diseases, and use of systemic antibiotics in the last three months before procedures were excluded. First, children were anesthetized and the teeth were isolated with a rubber dam. Collected samples were transferred to microcentrifuge tubes containing Brain Heart infusion [37 g of BHI, deionized water per liter, pH 7.2] with 20% glycerol, and then stored at −80 °C until the moment of use. Following, the teeth were restored appropriately with Glass Ionomer Cement (Ketac Fil Plus® 3MEspe, Maplewood, USA). Infected dentin samples were collected from children due to the greater application of minimal intervention methods for restorative procedures in pediatric dentistry.

2.3. Preparation of inoculum

Microtubes with infected dentin were thawed at room temperature and then sonicated vigorously for 2 min A poll of microorganisms was prepared by mixing 400 µL of samples of each child, and then stored in at −80 °C until the moment of use.

2.4. Preparation of dentin discs

The specimens were obtained from roots of bovine inferior incisors. The roots were sectioned longitudinally by a diamond disc (Diaflex-F, Wilcos do Brasil, Petrópolis, Brazil), obtaining two halves (mesial and distal). From each half, dentin discs of 5 mm of diameter were made using a trephine drill #5 (Alpha Instrumentos, Ribeirão Preto, Brazil) adapted into a dental handpiece (Kavo Kerr, Joinville, Brazil). Sequentially, the discs were ground flat with water-cooled silicon carbide discs (600, 800, and 1200 grades of Al_2O_3 papers, Buehler Ltd., Lake Bluff, USA) for 10 s and polished with felt paper wet by diamond spray (1 µm; Buehler Ltd.). After each sandpaper, the discs were washed in the T7 Thornton ultrasound (Unique Ind. And Com of Electronic Products Ltda., São Paulo, Brazil) for 20 min and stored in deionized water at 4 °C until the moment of use [19].

2.5. Biofilm model

A modified version of the active attachment biofilm model (ACTA, Amsterdam, The Netherlands) was used for biofilm growth. The model consists of a stainless steel lid with 24 polystyrene clamps attached to a 24-well tissue culture plate (Greiner Cellstar®, Greiner Bio-one, Kremsmünster, Germany) [19]. The dentin discs were fixed to the polystyrene clamps with condensation silicone impression material (Zetaplus®, Zhermack, Badia Polesine, Italy). After 24 h, the stainless steel lids together with the assembled dentin discs were autoclaved at 121 °C for 30 min.

2.6. Microcosm biofilm growth

A volume of 400 µL of the microbial pool was added in 10 mL of the McBain medium plus 0.2% sucrose and incubated in anaerobic conditions at 37 °C overnight [20]. Then, suspensions were uniformly distributed in each well of a 24-well microtiter plate and the biofilm model was positioned on the plate. The set was incubated under anaerobic conditions at 37 °C. The culture media were refreshed each 24 h for 3 days.

2.7. Antimicrobial agents

The photosensitizing agent employed was curcumin (Sigma-Aldrich, St. Louis, USA). The solution of curcumin was thinned in sterile deionized water with 0.2% DMSO to the final concentration of 300 µM. Biotable® RGB (Institute of Physics of São Carlos, São Carlos, Brazil) was used as a light source. It consists of an acrylic box containing 24 light-emitting diodes which permits the simultaneous irradiation of 24 specimens. An optical power meter (1916-C Optical Power Meter, Newport, Irvine, USA) was used to control the optical power output of Biotable® RGB. When required, the irradiation time was corrected by the ratio between energy and power densities. Chlorhexidine digluconate (Maquira Indústria de Produtos Odontológicos S.A, Maringá, Brasil) was diluted to final concentrations of 0.06% and 0.12%, using deionized water (late analyses) or a neutralizer solution (immediate analyses), containing 3 g of lecithin, 0.34 g of K2HPO4, 30 g of polysorbate, and 500 mL of demi water (per liter, pH 7.2) [21].

2.8. Antimicrobial photodynamic therapy

To remove unbound cells, biofilms were washed three times in the cysteine peptone water (CPW) medium prior to treatments. Then, the models were positioned on the 24-well plate containing 300 µM curcumin, 0.06% CHX, 0.12% CHX or deionized water, and they were incubated for 2 min. Subsequently, samples of groups C-L + and C + L + were irradiated with blue LEDs [wavelength 420 nm, size of 1 cm^2, distance of ≈ 25 mm and irradiance of 40 $mW.cm^2$]. The energy density (75 $J.cm^2$) was determined by the meantime variation of 11 min. After treatment, the models were washed in CPW medium. Biofilms of immediate analyses were removed from polystyrene clamps and transferred to universal tubes containing 2 mL of CPW medium. Dentin discs of late analyses were re-incubated in McBain medium supplemented with 0.2% sucrose for additional 24 h, and then were collected as aforementioned.

2.9. Determination of CFU counts

The biofilms adhered to the substrate were dispersed by sonication on ice at 40 mW, 1 $pulse.s^{-1}$ for 1 min (Single Ultra-Sonic Cell Disruptor, Merse, Campinas, Brazil). Serial dilutions (1:10, 1: 100, 1:

1000, 1: 10,000, and 1: 100,000) were performed using the CPW medium. Aliquots of 50 μL of each dilution were inoculated into Petri dishes containing Tryptone Soy Blood Agar Mitis for selective growth of total microorganisms, Mitis Salivarius for total streptococci, Agar Mitis Salivarius containing 0.2% bacitracin and 20% sucrose for mutans streptococci, and Rogosa agar with 0.13% glacial acetic acid for total lactobacilli. After the incubation of 48–72 h under anaerobic or microaerophilic conditions at 37 °C, CFUs per milliliter of each microorganism group were determined by a trained operator using a stereoscopic microscope.

2.10. Statistical analysis

The data were analyzed using SPSS 21.0 software (IBM® SPSS® Statistics, New York, USA). The hypothesis of normal distribution was rejected by the Shapiro-Wilk test. Then, Kruskal-Wallis and post-hoc Dunn's tests were used to indicate differences between groups. Values of $P < 0.05$ were adopted for significant differences.

3. Results

The viability of intact biofilms is shown in Table 1. Curcumin-mediated aPDT (C + L+) reduced total microorganisms (1.32 log10), total streptococci (1.2 log10), total lactobacilli (0.5 log10) and mutans streptococci counts (0.19 log10). On the other hand, the viability of total lactobacilli and total streptococci increased after treatments with 0.06% CHX. Also, treatments with 0.12% CHX increased the vitality of all microorganism groups, except mutans streptococci that showed a slight reduction (0.07 log10) (Fig. 1).

After 24 h of incubation, biofilms treated with curcumin-mediated aPDT presented a tendency of increasing CFUs, with a non-significant decrease of lactobacilli counts (Table 1). Also, there was a decrease of viable microorganisms treated with both CHX concentrations, except for total microorganisms and total lactobacilli treated with 0.06% CHX (Fig. 1). The reduction of total streptococci counts (1.45 log10) provided by 0.06% CHX and total lactobacilli counts (1.91 log10) provided by 0.12% CHX were statistically significant (Table 1).

4. Discussion

Traditionally, total caries removal can cause damage to dental pulp, leading to pain and reduction of tooth structure resistance. With this in mind, it has been recommended the selective caries removal with the maintenance of the affected dentin, capable of remineralization [30]. In this sense, aPDT may be a promising adjunct for dentine disinfection, increasing the chances of successful treatment [22]. Some studies demonstrated the efficacy of curcumin-mediated aPDT on planktonic cells and specific biofilms of *Candida* spp., *Candida albicans, Enterococcus faecalis*, salivary microorganisms and cariogenic bacteria [6,12,13,23]. Its effectiveness on multispecies biofilms composed of *C. albicans, C. glabrata, and S. mutans* was also demonstrated [31].

Araújo et al. [24] evaluated the susceptibility of multi-species biofilms of *S. mutans* (ATCC25175) and *L. acidophilus* (ATCC#ITAL-523) grown in dentin caries lesions to curcumin-mediated aPDT. The biofilms were exposure to different concentrations of curcumin (750, 1500, 3000, 4000 and 5000 mg/L) combined with 5.7 $J.cm^{-2}$ blue LED, resulting in reductions of viable cells from 97.5 through 100%. In this study, the reduction of the viability of mutans streptococci was 4% for biofilms treated with curcumin and LED (C + L+). These differences can be justified by some factors, such as the lower concentration of curcumin and the more complexity of biofilms employed here. The concentration of photosensitizer significantly affects its diffusivity, especially when microorganisms are in a complex culture media. The photodynamic effect can be reduced by the organic structure of carious dentin, decreasing the binding efficiency of photosensitizer to bacterial colonies and attenuating the penetration of irradiation necessary to photoactivate the dye [25]. Moreover, microcosms maintain the natural complexity found in oral biofilms, regarding their biodiversity and heterogeneous structure, which can reduce the effectiveness of antimicrobial therapies on different groups of microorganisms [19]. Finally, CFU increased for all microorganisms groups after 24 h of treatment with aPDT, which demonstrates the absence of a substantivity effect of curcumin-mediated aPDT. Same results were demonstrated by Garcez et al. [33], who analyzed the effect of the association of aPDT and endodontic treatment on microbiological samples of root canals, with a mean reduction of 0.74 log10 in the microbial burden; however, after 1 week the root canals showed 40% of bacterial recolonization.

Several *in vivo* and *in vitro* studies have demonstrated the efficacy of 0.2% chlorhexidine as an antiplaque agent [26,27]. Netto et al. [28] reported significant differences in mutans streptococci levels of saliva samples collected 7 and 14 days after 0.12% CHX mouthrinses, although they were not able to observe the same differences for lactobacilli counts. However, Albertsson et al. [32] showed a significant reduction of mutans streptococci and lactobacilli after a period of 16 days with 0.12% CHX mouthrinses. In this study, we observed that there was a significant reduction (30%) in the viability of total lactobacilli after 24 h of application of 0.12% CHX, without changes observed in mutans streptococci counts. Borges et al. [29] studied the susceptibility of biofilms of *Streptococcus mutans* to 0.2% CHX. The treatment of biofilms with the antimicrobial agent led to reductions of 3.71 log10, a more accentuated variation than that observed in this study (0.22 log10). These differences can also be justified by the lower

Table 1
Immediate and late effect of aPDT and CHX on CFU counts (log10) of distinct microorganisms. Distinct letters represent significant statistical differences between groups.

Groups	Total microorganisms	Total streptococci	Mutans streptococci	Total lactobacilli
Immediate effect				
C-L-	6.93 ± 0.48^{BC}	5.82 ± 1.07^{AC}	4.90 ± 0.46^{A}	4.69 ± 0.06^{BC}
C + L-	6.03 ± 1.16^{BD}	5.00 ± 1.26^{A}	4.73 ± 0.05^{A}	4.86 ± 0.35^{ABC}
C-L+	6.42 ± 0.34^{BD}	5.13 ± 1.22^{A}	5.01 ± 0.90^{A}	4.44 ± 1.15^{BC}
C + L+	5.61 ± 0.49^{B}	4.61 ± 1.01^{A}	4.71 ± 1.01^{A}	4.19 ± 0.21^{B}
0.06% CHX	6.89 ± 0.20^{BC}	6.06 ± 0.46^{AC}	4.80 ± 0.39^{A}	6.13 ± 0.33^{AD}
0.12% CHX	7.08 ± 0.05^{AB}	6.47 ± 0.51^{AD}	4.83 ± 0.28^{A}	5.47 ± 1.07^{ABC}
Late effect				
C-L-	6.66 ± 1.62^{ACD}	7.24 ± 0.16^{BD}	5.31 ± 0.74^{A}	6.23 ± 1.04^{AD}
C + L-	8.07 ± 0.46^{A}	7.54 ± 0.98^{BD}	5.58 ± 0.44^{A}	6.21 ± 0.79^{AD}
C-L+	7.56 ± 0.38^{AC}	7.27 ± 0.22^{BD}	6.28 ± 0.66^{A}	5.41 ± 0.25^{ACE}
C + L+	7.40 ± 0.22^{AC}	6.99 ± 0.27^{CD}	5.66 ± 1.53^{A}	5.68 ± 0.61^{CD}
0.06% CHX	6.61 ± 1.23^{BC}	5.79 ± 1.14^{AC}	5.46 ± 0.75^{A}	5.21 ± 0.90^{ABC}
0.12% CHX	6.87 ± 0.19^{AB}	6.46 ± 0.46^{AD}	5.09 ± 0.32^{A}	4.32 ± 0.67^{BE}

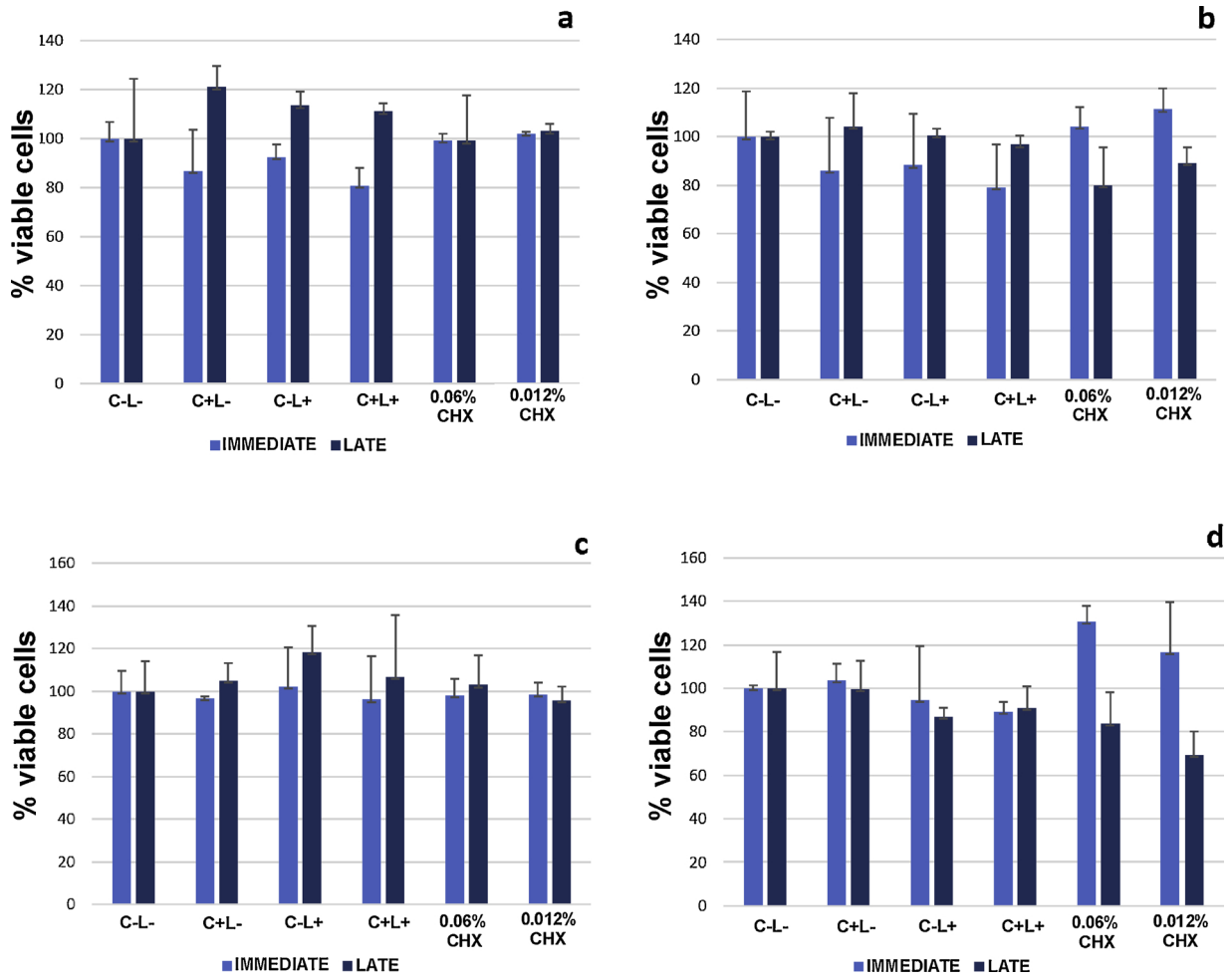

Fig. 1. Percentage of viable cells of total microorganisms (a), total streptococci (b), mutans streptococci (c), and total lactobacilli (d) after different treatments in relation to no treatment group (C-L-).

effectiveness of antimicrobial therapies against microcosms, especially considering a lower concentration of chlorhexidine. Distinctly of aPDT, chlorhexidine diluted in deionized water reduced the viability of microorganisms after 24 h of treatment, confirming its substantivity. On the other hand, the use of a neutralizer solution seemed to suppress the antimicrobial effect of CHX, avoiding additional reductions of viable cells during the bacterial growth on Petri dishes. Jenkins et al. [34] demonstrated that CHX inhibits plaque by an immediate bactericidal action during the time of application, followed by a prolonged bacteriostatic action. Maybe because the combination of CHX with a neutralizer, the immediate antimicrobial effects were not observed.

This study presents some limitations. Although the use of a neutralizer solution decreased the substantivity of CHX, which permitted the comparison of its immediate antimicrobial effects against aPDT, this measure should not be indicated therapeutically, under the risk of hampering its clinical benefits for dental patients. Also, the efficacy of aPDT observed in these experiments might be limited by the employment of lower concentration of curcumin in relation to previous studies [12,13], especially because the concerns about dental pigmentation. Finally, the cost-effectiveness of aPDT as an adjuvant for restorative procedures needs to be critically analyzed, because it demands new technologies, training and additional clinical time.

In conclusion, these results indicate an immediate effect of curcumin-mediated aPDT, by the observation of re-growth of microorganisms after 24 h of treatment, and a substantive effect of CHX, confirmed by the reduction of viable cells of specific microorganisms. Also, the use of a neutralizer solution was effective in blocking the substantivity of CHX, which can permit its fair comparison with aPDT, allowing its use as a positive control in further studies.

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Ethical approval

This research protocol was approved by the Committee for Ethics in Human Research of the Bauru School of Dentistry (CAAE: 34559314.6.0000.5417), following ethical standards of the Declaration of Helsinki.

Conflict of interest

The authors declare that they have no conflict of interest.

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