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ORIGINAL ARTICLE

Effect of cetrimide 2% with and without photodynamic therapy to reduce *Streptococcus mutans* burden in dentinal carious lesions

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Abstract

To evaluate the use of cetrimide alone and combined with photodynamic therapy to reduce *S. mutans* burden in carious lesions. Sixty permanent third molars were sectioned and the coronal dentin exposed. A cariogenic challenge was performed using brain-heart infusion (BHI) medium supplemented and *S. mutans* ATCC 25175. Specimens were incubated in anaerobic jars at 37 °C for 15 days, with BHI renewed every 24 h. After 15 days, specimens were randomly divided into six groups ($n = 10$): C, control (no treatment); CHX, application of chlorhexidine 2%; CT, application of cetrimide 2%; CT+aPDT, application of cetrimide 2% followed by methylene blue dye and aPDT (antimicrobial photodynamic therapy: wavelength 660 nm, energy 4J, power 100 mW, spot size 0.0028 cm^2, energy density 142 J/cm^2 for 40 s); ES+aPDT, application of experimental solution (methylene blue dye with cetrimide) and aPDT; and aPDT alone. Carious tissue from each specimen was collected before and after the applications. Five decimal dilutions were performed, and the resulting solution was seeded in mitis-salivarius-bacitracin agar. Plates were incubated in anaerobic jars at 37 °C for 48 h. Analysis of variance (ANOVA) with post hoc Tukey's test was used to compare total *S. mutans* counts. Significant reductions in *S. mutans* were observed after application of CT+aPDT (0.30 (0.97), $p < 0.0001$) and ES+aPDT (0.52 (1.13), $p < 0.0001$). Cetrimide 2% with methylene blue dye, applied consecutively or as a mixture, can be used as a photosensitizing agent for aPDT to reduce *S. mutans* burden in dentinal caries.

Keywords Cavity disinfection · *S. mutans* · Laser irradiation · Photodynamic therapy · Cetrimide (cetrimonium)

Introduction

Minimally invasive restorative dentistry techniques involve selective removal of the carious lesion while preserving affected but still repairable dentin. Cavity disinfection plays a key role in eliminating residual bacteria in this substrate, preventing the formation of secondary caries and postoperative sensitivity. Several antimicrobials are used for this purpose, particularly chlorhexidine (CHX), the most popular agent for cavity cleaning [1–3].

CHX has been used to disinfect decayed dental tissue since the 1970s [4]. Due to its broad antimicrobial spectrum, it has been used in endodontics, periodontics, and treatment of carious lesions [5]. CHX is a cationic compound which interacts with anions in the bacterial cell wall (phosphate groups of teichoic acids in Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria) [6]. The bacteriostatic effects of CHX are exerted through the release of low-molecular-weight substances [7].

Cetrimide (CT) is an alternative to CHX with similar properties. CT is a cationic surfactant of the quaternary ammonium class with proven effectiveness against Gram-positive and Gram-negative bacteria, as well as antifungal activity [8, 9]. It is used topically and is entirely nontoxic at a concentration of up to 2% [10]. As a mixture of amphipathic molecules, CT reduces the surface tension of liquids [11]. This characteristic could facilitate its

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penetration in hard-to-reach areas such as the inner lumen of dentinal tubules [12].

Antimicrobial photodynamic therapy (aPDT) is another technique that has been used in the treatment of carious lesions [13]. A light source is used to activate a photosensitizer such as methylene blue, which, in the presence of oxygen, forms singlet oxygen and other free radicals that lead to the irreversible rupture of cellular components, alter signal transduction pathways, and disrupt metabolic functions, ultimately leading to cell death [14]. This technique has been shown to reduce bacterial and fungal contamination in vivo [15].

In the context of the ongoing COVID-19 pandemic, minimal intervention techniques that reduce bioaerosol generation during dental practice are effective and should be considered [16–18]. In addition, the main advantages of photodynamic treatment in inactivating microorganisms are its broad spectrum of action and the non-development of resistance mechanisms. Cost-effective photosensitizers, such as phenothiazines or porphyrins, might also help mitigate the spread of COVID-19, considering their effectiveness against SARS-CoV-2 [19, 20]. Therefore, aPDT could be an alternative for the minimally invasive treatment of deep carious lesions while increasing safety for patients and practitioners [16].

There is no evidence to prove the effectiveness of cetrimide as a disinfectant for cavities, whether alone or in combination with aPDT. Therefore, the objective of this study was to evaluate the use of cetrimide alone and combined with photodynamic therapy to reduce *S. mutans* counts in dentinal caries. The null hypothesis was that there would be no significant difference in the reduction of *S. mutans* burden in decayed dentin with the use of cetrimide alone versus combined with aPDT.

Materials and methods

The present study was approved by the institution Research Ethics Committee (protocol number 2.167.351 and 1.230.452). Sixty unerupted permanent third molars, with no visible cracks or fractures under ×10 magnification (Carl Zeiss, São Paulo, SP, Brazil), were selected from the institution dental clinic. The chemical compounds used in this study are described in Table 1.

Sample size calculation

The sample size was calculated using analysis of variance (ANOVA). With a minimum difference between treatment means of 0.22, standard error of 0.125, number of treatments = 6, statistical power = 0.80, and alpha = 0.05, the number of teeth per group was defined as 10.

Specimens

After sample selection, the occlusal third was removed from each specimen using a double-sided diamond disk (KG Sorensen Indústria e Comércio Ltda., São Paulo, SP, Brazil) in a low-speed handpiece (KaVo do Brasil Indústria e Comérciol Ltda., Joinville, SC, Brazil), under refrigeration, to expose the dentin surface. The dentin surfaces were polished with wet silicon carbide sandpaper sheets, P600 grit (Água T223 advance, Norton, São Paulo, SP, Brazil). A 4×4-mm label (3M do Brasil Ltda., São Paulo, SP, Brazil) was placed onto the dentin surface of each specimen to standardize the location of the carious lesion. The specimens were sealed using epoxy resin (Araldite, São Paulo, SP, Brazil) and nail polish (Colorama, São Paulo, SP, Brazil), except on the region covered by the label. The label was then removed to enable generation of the carious lesion. After waterproofing, the specimens were sterilized with ethylene oxide (Acecil, Campinas, SP, Brazil).

Cariogenic challenge

Teeth were then exposed to a cariogenic challenge in brain-heart infusion (BHI) broth (Acumedia, Neogen Corporation, Lansing, MI, USA), supplemented with 1% glucose (Labsynth Materiais para Laboratório Ltda., Diadema, SP, Brazil), 1% sucrose (Labcenter Materiais para Laboratórios, Campinas, SP, Brazil), 0.5% yeast extract (Oxoid Ltd., Basingstoke, HPH, UK), and *S. mutans* type strain ATCC 25175 (Fundação André Tosello, Campinas, SP, Brazil), standardized to 0.5 McFarland turbidity. Samples were incubated in anaerobic jars (Probac do Brasil, São Paulo, SP, Brazil) at 37 °C and subsequently stored in a bacteriological incubator (Sterilifer Indústria e Comércio Ltda., Diadema, SP, Brazil) for 15 days. During this period, BHI broth was replaced every 24 h (adapted from Carvalho et al. [21] and Lima et al. [22]).

Study interventions

After 15 days, the specimens were randomly distributed (www.random.org.br) according to the cavity disinfection procedure (n=10), as shown in Table 2.

Assessment of cariogenicity

The collected samples were immediately placed in BHI transport medium (Acumedia, Neogen Corporation, Lansing, MI, USA) and homogenized for 1 min in a tube shaker (Phoenix, Araraquara, SP, Brazil). Immediately after homogenization, five decimal dilutions were performed into test tubes (Uniglass Produtos para Laboratórios Ltda., Cascavel, PR, Brazil), and three 25-μL aliquots from each dilution were seeded with a micropipette (Uniscience do Brasil, São

Table 1 Chemical compounds used in the study, manufacturer, and city/country of manufacture

Cetrimide	Neofórmula, Campinas, SP, Brazil
Chlorhexidine	Dental Cremer Produtos Odontológicos S.A, Blumenau, SC, Brazil
Experimental solution	Cetrimide–Neofórmula, Campinas, SP, Brazil Methylene blue 0.01%–DMC Importação e Exportação de Equipamentos Ltda., Sao Carlos, SP, Brazil
Epoxy resin	Araldite, Sao Paulo, SP, Brazil
Nail polish	Colorama, Sao Paulo, SP, Brazil
Brain-heart infusion (BHI) broth	Acumedia, Neogen Corporation, Lansing, MI, USA
Glucose	Labsynth Materiais para Laboratório Ltda., Diadema, SP, Brazil
Sucrose	Labcenter Materiais para Laboratórios, Campinas, SP, Brazil
Yeast extract	Oxoid Ltd., Basingstoke, HPH, UK
S. mutans type strain ATCC 25175	Fundação André Tosello, Campinas, SP, Brazil
Mitis-salivarius-bacitracin (MSB) medium	Oxoid Ltd., Basingstoke, HPH, UK; Merck KGaA, Darmstadt, HE, Germany

Paulo, SP, Brazil) onto the surface of mitis-salivarius-bacitracin (MSB) medium (Oxoid Ltd., Basingstoke, HPH, UK; Merck KGaA, Darmstadt, HE, Germany). All plates (Olen, China) were incubated in anaerobic jars (Probac do Brasil, São Paulo, SP, Brazil) at 37 °C (candle-flame method) for 48 h. After incubation, the viable bacterial count was determined in CFU/mL.

Statistical analyses

Statistical analyses were carried out in BioEstat 4.0. The Shapiro-Wilk test of normality was applied. The sample was normally distributed. Comparisons of reduction in microbial counts were performed by ANOVA with Tukey's post hoc test at the 1% significance level.

Results

The arithmetic means and standard deviations of the *S. mutans* count of each sample group (CFU/mL-log10) are described in Table 2. After cetrimide application combined with aPDT (CT + aPDT), *S. mutans* counts decreased significantly (0.30 [0.97], $p<0.0001$). The experimental solution plus aPDT

Table 2 Description of study groups by disinfection procedure

Groups	Procedure
C	*S. mutans* count after curettage of carious lesion with a sterile #5 spoon excavator (Millenium, Golgran Indústria Comercial de Instrumentos Odontológicos Ltda., São Caetano do Sul, SP, Brazil); no further disinfection
CT	*S. mutans* count after application of 2% cetrimide (Neofórmula, Campinas, SP, Brazil) for 1 min with a microbrush (KG Sorensen Medical Burs Indústria e Comércio de Produtos Abrasivos Ltda., Cotia, SP, Brazil)
CHX	*S. mutans* count after application of 2% chlorhexidine (Dental Cremer Produtos Odontológicos S.A, Blumenau, SC, Brazil) for 1 min with a microbrush (KG Sorensen Medical Burs Indústria e Comércio de Produtos Abrasivos Ltda., Cotia, SP, Brazil)
CT + aPDT	*S. mutans* count after application of 2% cetrimide (Neofórmula, Campinas, SP, Brazil) for 1 min, followed by antimicrobial photodynamic therapy with 0.01% methylene blue photosensitizer (DMC Importação e Exportação de Equipamentos Ltda., São Carlos, SP, Brazil), applied for 3 min with a microbrush (KG Sorensen Medical Burs Indústria e Comércio de Produtos Abrasivos Ltda., Cotia, SP, Brazil). Subsequently, low-level laser (DMC Importação e Exportação de Equipamentos Ltda., São Paulo, SP, Brazil; wavelength = 660 nm, energy = 4J, power = 100mW, spot size = 0.0028 cm^2, energy density =142 J/cm^2) was applied for 40 s
ES + aPDT	*S. mutans* count after application of an extemporaneously compounded mixture of 2% cetrimide (Neofórmula, Campinas, SP, Brazil) and 0.01% methylene blue (DMC Importação e Exportação de Equipamentos Ltda., São Carlos, SP, Brazil) with a microbrush (KG Sorensen Medical Burs Indústria e Comércio de Produtos Abrasivos Ltda., Cotia, SP, Brazil) for 4 min. Subsequently, low-level laser (DMC Importação e Exportação de Equipamentos Ltda., São Paulo, SP, Brazil; wavelength = 660 nm, energy = 4J, power = 100 mW, spot size = 0.0028cm^2, energy density =142 J/cm^2) was applied for 40 s
aPDT	*S. mutans* count after photodynamic therapy with 0.01% methylene blue (DMC Importação e Exportação de Equipamentos Ltda., São Carlos, SP, Brazil), applied for 3 min with a microbrush (KG Sorensen Medical Burs Indústria e Comércio de Produtos Abrasivos Ltda., Cotia, SP, Brazil). Subsequently, low-level laser (DMC Importação e Exportação de Equipamentos Ltda., São Paulo, SP, Brazil; wavelength = 660 nm, energy = 4 J, power = 100 mW, spot size = 0.0028 cm^2, energy density =142 J/cm^2) was applied for 40 s

aPDT, antimicrobial photodynamic therapy; *C*, control; *CHX*, chlorhexidine; *CT*, cetrimide; *ES*, experimental solution

group (ES + aPDT), which did not differ significantly from the CT + aPDT group, also presented a low *S. mutans* count (0.52 [1.13]).

aPDT alone also reduced *S. mutans* counts (1.97 [0.32]), however with no difference to the CHX group (2.58 [0.92]), which, in turn, did not differ significantly from the CT group (3.03 [0.45]). Individually, all groups differed significantly from the control group (4.53 [0.43]), which presented the highest *S. mutans* counts (p<0.01), as described in Tables 3 and 4.

Table 4 Arithmetic means and SD of the *S. mutans* counts of each sample group (CFU/mL-$\log_{10}$). In columns, different capital letters denote significant differences among groups ($p < 0.0001$). Lowercase letters denote no differences between groups ($p < 0.0001$)

Groups	Mean (SD)	
C	4.53 (0.43)	B
CT	3.03 (0.45)	A
CHX	2.58 (0.92)	Aa
aPDT	1.97 (0.32)	Ca
ES + aPDT	0.52 (1.13)	D
CT + aPDT	0.30 (0.97)	D

aPDT, antimicrobial photodynamic therapy; *C*, control; *CFU*, colony formation unit; *CHX*, chlorhexidine; *CT*, cetrimide; *ES*, experimental solution; *SD*, statistical deviation

Discussion

The present study used the microbiologic caries challenge methodology. Briefly, with the aid of orthodontic wires, teeth were secured in glass jars containing BHI medium supplemented with 1% glucose, 1% sucrose, 0.5% yeast extract, and a type strain of *S. mutans* (ATCC 25175) standardized to 0.5 McFarland turbidity. To induce the carious lesion, the specimens were incubated in an oven at 37 °C for 15 days in anaerobic jars, using the candle flame method. During this period, the BHI medium was renewed every 24 h and the pH was measured to ensure an acidic medium conducive to the presence of bacterial activity (around pH 4.5) [14, 15]. In vitro models are commonly used to simulate the cariogenic challenge and to analyze the cariostatic effects of different materials; however, it is noteworthy that in vitro studies provide only simplified insight into the in vivo oral environment, as they do not provide for the properties of saliva, erosive tooth wear, or even oral hygiene habits. In addition, the duration of in vitro experiments is often shorter than in vivo studies [16–18]. There are other models for the production of artificial caries; the chemical model uses acid gel or solutions that stimulate the tooth demineralization process. Studies have compared the depth of caries produced in dentin by microbiological and chemical models and have shown that the microbiological model achieves greater caries depth. In addition, it produces decayed tissue with more demineralization and well-defined tubules, while the chemical model produces a more dense, compact structure, without well-defined dentinal tubules [19, 20]. These factors are relevant to the result of the present study, and justify the method used.

Surface-associated bacterial biofilms are complex, three-dimensional structures in which bacteria are incorporated into a matrix composed of extracellular polymeric substances, which provide mechanical stability to the biofilm and provide several functions that allow its organization [23]. Once formed, biofilms are difficult to remove completely. Several chemical agents capable of acting on the biofilm are often employed for this purpose, including surfactants. As a cationic surfactant, cetrimide has cytotoxic bactericidal action and can act on biofilms [18, 19]. It is mostly non-irritating to host tissues and has the capacity to reduce the surface tension of liquids, facilitating their entry into hard-to-reach areas, such as the dentinal tubules [20]. Accordingly, a previous study found that application of cetrimide 0.2% for 1 min achieved eradication of *S. mutans* in most specimens, as well as an increase in the rate of biofilm removal [24]. Another study showed that cetrimide 0.2% has longer-lasting substantivity compared to chlorhexidine 0.2% and almost as long as that of chlorhexidine 2% in a dentine model [21]. This may be related to the cationic nature of cetrimide, which is able to interact with dentin. These studies point to the potential use of cetrimide and are in line with the results of the present work, in which cetrimide application reduced *S. mutans* burden in dentinal caries.

aPDT is an antimicrobial technique used in the treatment of oral infections, such as tooth decay. It consists of the application of light to activate a photosensitive agent in the presence of oxygen, generating reactive oxygen species (such as singlet oxygen) in situ and resulting in bacterial lysis [22]. When used on dentin, aPDT has been found to decrease *S. mutans* counts in deep carious lesions [25]. In addition, another study

Table 3 Arithmetic means and standard deviations of the *S. mutans* count of each sample group (CFU/mL-$\log_{10}$)

C	CHX 2%	CT 2%	CT + aPDT	ES + aPDT	aPDT	(p-ANOVA)
4.53 (0.43)	2.58 (0.92)	3.03 (0.45)	0.30 (0.97)	0.52 (1.13)	1.97 (0.32)	< 0.0001

aPDT, antimicrobial photodynamic therapy; *C*, control; *CFU*, colony formation unit; *CHX*, chlorhexidine; *CT*, cetrimide; *ES*, experimental solution

evaluated the use of aPDT (application of toluidine blue for 3 min) in periodontal pockets and found in a reduction in the number of bacteria [26]. Gong et al. demonstrated a significant reduction in *S. mutans*, *L. casei*, and *Candida albicans* counts after aPDT, using the photosensitizer erythrosine for 3 min and a blue light-emitting diode (LED), resulting in a significant antimicrobial effect against oral biofilms [27]. The results of the present study are consistent with the literature; a reduction in *S. mutans* counts was obtained with aPDT, with the same duration of photosensitizer application, and no difference in the reduction of *S. mutans* counts with the addition of cetrimide, whether as a separate step or in a mixed experimental solution, both followed by low-level laser irradiation. Therefore, the null hypothesis was confirmed.

Ornellas et al. demonstrated the effectiveness of methylene blue (0.01% applied for 5 min) followed by red laser irradiation in reducing the total number of *Streptococcus* spp., *S. mutans*, and *Lactobacillus* spp. [28]. The advantage of aPDT is that it reduces the microbial load of dentin immediately. Ricatto et al. further demonstrated the effectiveness of methylene-blue PDT, with a laser or LED light source, in reducing *S. mutans* and *L. casei* burden [29]. Guglielmi et al. [24] observed a reduction in total microorganism counts (*S. mutans*, *Lactobacillus* spp.) during an in vivo study evaluating the effect of methylene-blue PDT on contaminated dentin of permanent teeth. Again, the results are in line with the present study, as PDT was able to significantly reduce *S. mutans* burden in carious lesions.

An additional advantage of aPDT concerns the ongoing pandemic of coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2 virus originating in China [25]. Because this virus is transmitted via respiratory droplets and bioaerosols or contact with contaminated surfaces [30], the pandemic has had an impact on aerosol-generating dental procedures [26]. As these aerosols can spread for considerable distances and remain suspended in the air for several hours, the dental clinic environment is thus a high-risk area for nosocomial spread [23]. Use of atraumatic, noninvasive, or minimally invasive treatment techniques that promote minimal or near-zero generation of bioaerosols would be safer, especially considering the high success rate that these treatments have in dentistry [16].

In short, this study found that combining cetrimide—a cationic surfactant capable of penetrating the deeper layers of the dentinal tubules—with aPDT was able to promote deeper penetration of photosensitizer (methylene blue 0.01%) into the tubules, thus allowing better disinfection of the carious lesion and consequent eradication of *S. mutans*. This is a minimally invasive technique with reduced aerosol generation, which makes it particularly suitable for the current context of COVID-19. Nevertheless, additional studies are needed to evaluate the potential of cetrimide and photosensitizer combinations, particularly in a single experimental solution, as a means of streamlining the procedure.

Conclusion

Based on the results of this in vitro study, aPDT with a combination of cetrimide 2% plus a photosensitizing agent reduced *S. mutans* CFU counts in dentinal caries. This protocol is a promising, minimally invasive alternative to conventional techniques which involves minimal bioaerosol generation, thus making it safer for clinical practice during the COVID-19 pandemic.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Gabriele Giorgi Moro, Natalia Cunha Massat, Diana Roberta Pereira Grandizoli, Augusto Etchegaray Junior, Giovanna Rosa Degasperi, Carlos Eduardo Fontana, and Sérgio Luiz Pinheiro. The first draft of the manuscript was written by Sérgio Luiz Pinheiro, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the local Research Ethics Committee (protocol numbers 2.167.351 and 1.230.452).

Consent to participate Written informed consent was obtained from the donors of all teeth used in this study.

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