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ORIGINAL ARTICLE

Dental acid etchant as a sensitizing agent in photodynamic therapy to reduce *S. mutans* in dentinal carious lesions

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Abstract

The study aims to assess the utility of dental acid etchant containing 37% phosphoric acid and methylene blue dye (DAE) as a sensitizing agent for photodynamic therapy (PDT) to reduce *Streptococci mutans* in dentinal caries. Forty-five permanent third molars were sectioned and the coronal dentin exposed. A cariogenic challenge was performed using brain-heart infusion (BHI) supplemented with 0.5% yeast extract, 1% glucose, 1% sucrose, and *S. mutans* ATCC 25175 standardized to 0.5 McFarland turbidity. Specimens were incubated in anaerobic jars at 37 °C for 15 days. During this period, BHI broth was renewed every 24 h. After 15 days, specimens were randomly divided into three groups ($n = 15$): DAE, application of dental acid etchant containing 37% phosphoric acid and methylene blue dye for 15 s; LLL, application of low-level laser (wavelength 660 nm, energy 4 J/cm^2, power 5 W) for 15 s; and PDT, application of DAE for 15 s followed by LLL irradiation (660 nm, 4 J/cm^2, 5 W). 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 DAE application (40.70%, $p < 0.0001$), LLL (12.35%, $p = 0.0036$), and PDT (55.22%, $p < 0.0001$). Dental acid etchant containing 37% phosphoric acid and methylene blue dye can be used as a photosensitizing agent for PDT to reduce *S. mutans* burden in dentinal caries.

Keywords Photodynamic therapy · Dental acid etchant · Laser irradiation · *S. mutans* · Cavity disinfection

Introduction

The cornerstone of minimally invasive treatment of dentinal caries involves partial removal of the carious tissue, with resection of infected dentin and preservation of affected dentin [1]. To Ferreira et al. [2], optimal sealing of the cavity enhances the longevity of dental restorations by cutting off the bacterial nutrient supply, thus halting progression of the carious lesion. However, recurrence of caries and postoperative sensitivity may occur due to the presence of viable microorganisms after cavity preparation. Thorough removal of carious tissue and optimal cavity sealing are paramount in restorative dentistry; however, reduction of microbial in the cavity before restoration also plays a relevant role in treatment success. Photodynamic therapy (PDT) is a promising alternative for this purpose [3].

The interaction between a light source and a pigment—known as the photosensitizing agent—that absorbs its energy causes the release of by-products toxic to microorganisms, such as reactive oxygen species, which damage the protein and lipid constituents of the bacterial cell membrane and affect nucleic acids [4–7]. Low-level laser (LLL) has photodynamic activity on specific dyes [8]. Furthermore, its application is painless, does not promote bacterial resistance, and is devoid of systemic effects [9, 10]. Garcez et al. [6], Hakimiha et al. [11], and Azizi et al. [12] demonstrated that PDT is capable of reducing *Streptococci mutans*, the causative organism of dental caries [13].

The synthetic, cationic phenothiazine dyes toluidine blue and methylene blue are already used as photosensitizing agents in dentistry [7]. These pigments are effective antimicrobials when exposed to red light, approximately in the 630–

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660-nm wavelength [7, 12, 14]. The dental acid etchants used to condition enamel and dentin prior to application of adhesive systems consist of a 37% solution of phosphoric acid with added methylene blue dye. When irradiated by LLL at the 660-nm wavelength, methylene blue may act as an effective alternative photosensitizer. This suggests that irradiation of dental acid etchant with LLL during etching of the cavity might enhance disinfection of enamel and dentin before composite resin restoration. This procedure—application of LLL onto the etchant—is not part of dental practice and has not been reported in the literature. Within this context, the present study sought to evaluate the potential of dental acid etchant containing methylene blue dye as a photosensitizing agent to reduce *S. mutans* burden in dentinal caries.

The null hypothesis of the study was that irradiation of dental acid containing methylene blue dye would not promote a significant reduction in *S. mutans* counts in carious lesions compared to the reductions obtained with application of dental acid etchant or LLL alone.

Methods

The present study was approved by the PUC-Campinas Research Ethics Committee (protocol number 1.230.452). Forty-five unerupted permanent third molars, with no visible cracks or fractures under ×10 magnification (Carl Zeiss, São Paulo, Brazil), were selected from the PUC-Campinas Dental Clinic.

Sample size calculation

The design of this study provided for teeth to be randomly divided into three groups. The number of specimens in each group ($n = 15$) was obtained by sample size calculation, which was performed by ANOVA, with a minimum difference between treatment means = 0.04, standard error = 0.033, number of treatments = 3, statistical power = 0.80, and alpha = 0.05. Thus, the final sample size was calculated as 45 teeth.

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, Brazil) in a low-speed handpiece (KaVo do Brasil Ind. Com. Ltda., Joinville, Santa Catarina, 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, Brazil). A 4 × 4-mm label (3M do Brasil Ltda., São Paulo, 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, Brazil) and nail polish (Colorama, São Paulo, Brazil), except on the region covered by the label. The label was then removed to enable generation of the carious lesion.

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, São Paulo, Brazil), 1% sucrose (Labcenter Materiais para Laboratórios, Campinas, São Paulo, Brazil), 0.5% yeast extract (Oxoid Ltd., Basingstoke, Hampshire, England), and *S. mutans*-type strain ATCC 25175 (Fundação André Tosello, Campinas, São Paulo, Brazil), standardized to 0.5 McFarland turbidity. Samples were incubated in anaerobic jars (Probac do Brasil, São Paulo, Brazil) at 37 °C and subsequently stored in a bacteriological incubator (Sterilifer Ind. e Com. Ltda., Diadema, São Paulo, Brazil) for 14 days. During this period, BHI broth was replaced every 24 h.

Study interventions

After the cariogenic challenge, the 45 teeth were randomly divided (www.random.org.br) into three groups of 15 each:

DA: A 37% solution of phosphoric acid containing methylene blue dye (Condac 37, mid blue; FGM Produtos Odontológicos Ltda., Joinville, Santa Catarina, Brazil; wavelength 653.1 nm, absorbance 0.195) was applied onto the carious dentin for 15 s. The acid solution was then removed by rinsing with sterile saline solution (Laboratório Farmacêutico Arboreto Ltda, Juiz de Fora, Minas Gerais, Brazil). Carious dentin samples were collected with a sterile #5 spoon excavator (Millennium, Golgran Indústria Comercial de Instrumentos Odontológicos Ltda, S. Caetano do Sul, São Paulo, Brazil), before and after application of DAE.

LLL: The carious dentin was irradiated with low-level laser (DML, São Carlos, São Paulo, Brazil) for 15 s. LLL was administered at a spot energy of 4 J/cm^2, wavelength = 660 nm, and power = 5 W. Again, carious dentin was collected with a sterile #5 spoon excavator (Millennium, Golgran Indústria Comercial de Instrumentos Odontológicos Ltda, S. Caetano do Sul, São Paulo, Brazil), before and after LLL irradiation.

PDT: The same DAE solution used in group 1 (Condac 37, mid blue; FGM Produtos Odontológicos Ltda., Joinville, Santa Catarina, Brazil) was applied onto the carious dentin for 15 s. During the acid-etching period (15 s), carious dentin was irradiated with low-level laser (DML, São Carlos, São Paulo, Brazil), directly over the DAE, for 15 s. LLL was administered at a spot energy of 4 J/cm^2, wavelength = 660 nm, and power = 5 W. DAE was then removed with sterile saline solution (Laboratório Farmacêutico Arboreto Ltda, Juiz de Fora, Minas Gerais, Brazil). Carious dentin samples were collected with a sterile #5 spoon excavator (Millennium, Golgran Indústria Comercial de Instrumentos Odontológicos Ltda, S. Caetano do Sul, São Paulo, Brazil), before and after application of DAE and LLL irradiation.

Assessment of cariogenicity

The collected samples were immediately placed in BHI transport medium (Acumedia, Neogen Corporation, Lansing, MI, USA) and homogenized for 3 min in a tube shaker (Phoenix, Araraquara, São Paulo, Brazil). Immediately after homogenization, five decimal dilutions were performed into test tubes (Uniglass Produtos para Laboratórios Ltda., Cascavel, Paraná, Brazil), and three 25-μL aliquots from each dilution were seeded with a micropipette (Uniscience do Brasil, São Paulo, Brazil) onto the surface of *mitis-salivarius*-bacitracin (MSB) medium (Oxoid Ltd., Basingstoke, Hampshire, England and Merck KGaA, Darmstadt, Germany). All plates (Olen, China) were incubated in anaerobic jars (Probac do Brasil, São Paulo, 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 Biostat 4.0. Results obtained as CFU/mL were $\log_{10}$-transformed. The Shapiro–Wilk test of normality was applied. The sample was normally distributed. Comparisons of reduction in microbial counts before and after application of DAE and/or LLL were performed by analysis of variance (ANOVA) with Tukey's post hoc test at the 1% significance level.

Results

Significant reductions in *S. mutans* counts were observed after DAE application ($p < 0.0001$), LLL ($p = 0.0036$), and PDT ($p < 0.0001$). The greatest relative reduction in *S. mutans* counts was obtained with PDT (55.22%). Still significant reductions, though to a lesser extent, were obtained with DAE application alone (40.70%) and LLL alone (12.35%) (Table 1 and Fig. 1).

Discussion

Dental acid etchants containing methylene blue dye are widely used in dentistry. This substance is able to demineralize the dental structure, allowing establishment of a hybrid layer and creation of resin tags, thus contributing to the bonding of resin-based materials. Its characteristic blue color, imparted by methylene blue dye, gives the dentist greater control and precision during application. The results of this study rejected the null hypothesis, as application of DAE followed by low-level laser irradiation (wavelength = 660 nm, spot energy = 4 J/cm^2, and power = 5 W) for 15 s over carious dentin, significantly reduced viable *S. mutans* counts. This suggests that acid etchant containing methylene blue dye can be used as a photosensitizing agent for PDT with antimicrobial intent. The methylene blue present in the etchant absorbs laser energy at the same wavelength (approximately 630–660 nm).

According to Araújo et al. [15], Araújo et al. [14], Hakimiha et al. [11], Paschoal et al. [16], and Diniz et al. [17], free radicals and singlet oxygen are highly reactive molecules that cause cell-membrane and DNA damage, leading to irreversible metabolic and structural changes and, ultimately, bacterial cell death.

In the present study, acid etchant containing methylene blue dye was applied to the carious lesions for 15 s, which is the standard time used for etching of dentin prior to the application of adhesive systems. Thus, the present study confirmed that dentists can perform both procedures simultaneously; that is, PDT can be performed while acid etchant containing methylene blue dye is being applied in the cavity, thus reducing operative time and enhancing cavity disinfection.

Factors such as the type of microorganism, its organization (suspension or biofilm), the characteristics of the light source, and the concentration of the dye (photosensitizer) play an essential role in the success or failure of PDT [3]. Guglielmi et al. [3] applied 0.01% methylene blue and LLL (wavelength = 660 nm, power = 100 mW, duration = 90 s) to deep caries and obtained a significant reduction in *S. mutans* counts, which is consistent with the results of the present study.

Melo [18] evaluated the size and shape of *S. mutans* after PDT in artificially demineralized dentin using atomic force microscopy. PDT was performed using methylene blue as the photosensitizer and a 638.8-nm LED as the light source. After PDT, no morphological alterations were observed in the bacterial cells; however, their dimensions were reduced enough to lead to cell death, which is consistent with the results obtained herein.

In the present study, we also assessed the activity of LLL alone as a light source when applied directly to dentinal caries. Microbial counts were performed before and after application of LLL (660 nm, energy 4 J/cm^2, power 5 W) for 15 s to carious dentin, without prior application of an exogenous photosensitizer. The results of this group demonstrated a statistically significant 12.35% reduction in *S. mutans* counts after application of laser alone ($p = 0.0036$). One possible explanation for this microbial reduction is the presence of endogenous dyes produced by bacterial metabolism in the carious dentin [19]. *S. mutans* exhibits active metabolism in acidic pH conditions, which causes progressive demineralization of tooth structure [20]. Carious lesions are known to contain

Table 1 Arithmetic means (M), standard deviations (SD), and ANOVA (Tukey) statistics for comparison of reductions in microbial count before and after application of low-level laser (LLL); before and after application of dental acid etchant containing 37% phosphoric acid and methylene blue dye (DAE); and before and after photodynamic therapy (PDT) with LLL, using DAE as the photosensitizing agent

	Before DAE	After DAE	Before LLL	After LLL	Before PDT	After PDT
Mean (SD)	4.57 (1.23) a	2.71 (1.75) b	4.21 (1.09) a	3.69 (1.08) b	3.73 (0.81) a	1.67 (1.22) b
Percent reduction	40.70%		12.35%		55.22	
p	< 0.0001		0.0036		< 0.0001	

Reductions expressed as percentage. Different letters denote $p = 0.0036$, $p < 0.0001$, $p < 0.0001$

DAE dental acid etchant containing 37% phosphoric acid and methylene blue, LLL low-level laser, PDT photodynamic therapy

endogenous dyes, such as porphyrins. These endogenous porphyrins are pigments found in blood, urine, and also in bacteria that, when irradiated, can act as photosensitizers [19] and absorb light from LLL, releasing singlet oxygen and free radicals that cause microbial death, especially in Gram-positive species such as *S. mutans* [21].

Cieplik et al. [22] and Komerik et al. [21] state that porphyrins are part of a class of photosensitizing chemical compounds effective against Gram-positive bacteria such as *S. mutans*. According to de Melo et al. [8], the high photosensitivity of Gram-positive bacteria may be explained by the porosity of their peptidoglycan layer and associated lipoprotein acid.

In the present study, application of acid etchant containing methylene blue dye onto carious lesions caused a reduction in viable *S. mutans* counts. Phosphoric acid (H_3PO_4) is an acidic functional monomer with extremely low pH. Its application to dentin exposes the collagen layer, enabling bonding of an adhesive system [23, 24]. Settembrini et al. [25] notes that acid etchants such as phosphoric acid have antimicrobial effects. The extremely low pH of H_3PO_4 (approximately 1) has bacteriostatic and bactericidal effects on *S. mutans*.

Both direct LLL irradiation and application of acid etchant alone are able to reduce *S. mutans* counts in carious tissue. However, the effectiveness of LLL increases when it is combined with a dental acid containing methylene blue dye for PDT. Thus, acid etchants containing methylene blue can be used as photosensitizers for LLL irradiation to promote a photodynamic effect, reducing *S. mutans* counts and providing disinfection of dentinal caries, and may act an important clinical resource prior to application of adhesive systems and composite resins.

Compliance with ethical standards

Financial disclosure The authors have no financial relationships relevant to this article to disclose.

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The present study was approved by the local Research Ethics Committee (protocol number 1.230.452).

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.

Informed consent Forty-five permanent third molars were selected at the Dental Clinic of PUC-Campinas. All donor patients signed an informed consent form.

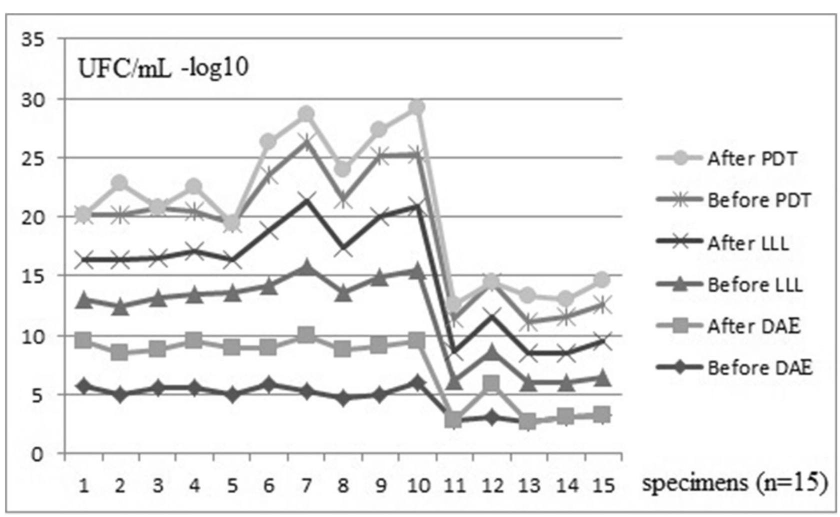

Fig. 1 Colony-forming unit counts in each specimen before and after application of DAE, LLL, and PDT

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