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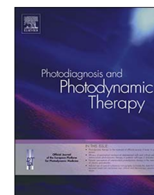

Effect of methylene blue-induced photodynamic therapy on a *Streptococcus mutans* biofilm model

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A R T I C L E I N F O

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A B S T R A C T

Background: Several studies have reported the use of antimicrobial photodynamic therapy (aPDT) to control biofilm but its efficacy depends on several factors, such as biofilm model used. This study aims to examine whether exposure to diode laser combined with methylene blue affects the bacterial viability and polysaccharide content in a *Streptococcus mutans* cariogenic biofilm model, which simulated 'feast-famine' episodes of exposure to sucrose that occur in the oral cavity.

Materials and methods: S. mutans biofilms were formed on acrylic resin discs and exposed to a 10% sucrose solution for 1 min, eight times/day. After growing for 48 h, the biofilms were submitted to the following treatments, twice daily (n = 4): (i) 0.9% NaCl (NaCl) as the negative control; (ii) 0.12% chlorhexidine digluconate (CHX) as the positive antibacterial control; (iii) diode laser combined with methylene blue, using an energy density of 320 J/cm^2 (aPDT). After 120 h of growth, the biofilm formed on each disc was collected to determine the viable bacterial counts and concentration of insoluble exopolysaccharides (IEPS) and intracellular polysaccharides (IPS).

Results: Bacterial counts in the biofilms formed differed among the treatments. Compared with NaCl, aPDT significantly destabilized biofilm ($p < 0.0001$). aPDT and CHX equally lowered the concentration of IEPS and IPS in biofilms.

Conclusion: Under the experimental conditions assessed, our findings indicate that a twice-daily treatment with diode laser combined with methylene blue effectively decreased bacterial viability and the intra- and extracellular polysaccharide concentration in biofilms of *S. mutans*, a cariogenic bacterium.

1. Introduction

It is well established that dental caries is a biofilm-dependent disease [1]. Biofilms are highly structured communities of microbial cells attached to a surface and embedded in a rich polymeric matrix [2], in which *Streptococcus mutans* is one of the most representative bacterial species. Once formed, biofilms are hard to disassemble and become more tolerant to antimicrobial agents than free microrganisms [3]. The polysaccharide matrix is the main factor that may hinder diffusion and access of antimicrobial agents [4]. In particular, insoluble exopolysaccharides (IEPS) favor the formation of dental plaque, biofilm stability and structural integrity, and are associated with caries prevalence [5,6]. In addition, intracellular polysaccharides (IPS) have been considered as an internal carbohydrate source for fermentation, contributing toward to the cariogenicity of *S. mutans* [7]. Considering that *S. mutans* is the most cariogenic bacterium [8], and that it can use dietary sucrose to synthesize polysaccharides [9], a *S. mutans* biofilm model that simulates different episodes of sugar exposure and pH-cycling that occur in the oral cavity represents a useful tool to study antimicrobial therapies, such as photodynamic therapy.

Several studies have reported the use of antimicrobial photodynamic therapy (aPDT) to control biofilm formation/growth [10–15]. As this therapy relies on the local delivery of visible light to living tissues, it suitable for dental applications, especially for irradiation of infected tissues previously exposed to a photosensitizing dye [16]. The lethal effect of aPDT is based on the principle that visible light activates a photosensitizer to generate reactive oxygen species that immediately induce phototoxicity. The efficacy of aPDT depends on several factors,

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such as the light type, laser setting parameters, interaction with the photosensitizer [17], and biofilm model used [11,12,15,18].

In the present study, we used a biofilm model that exhibits a concentration-dependent response to antimicrobials, with respect to biofilm formation and enamel demineralization [19]. This model presents a high frequency of exposure to sucrose and simulates "feast and famine" episodes of sugar consumption, with replacement of the culture medium during the incubation period to reproduce the oral cavity dynamics. In this sense, this study address whether exposure to diode laser affect the bacterial viability and polysaccharide content in *S. mutans* biofilms treated with methylene blue as the photosensitizer.

2. Material and methods

2.1. Experimental design

S. mutans biofilms were formed on acrylic resin discs suspended vertically in culture medium [20], using a validated model [19]. The formed biofilms were exposed to a 10% sucrose solution for 1 min, eight times/day. After growing for 48 h, the biofilms were treated twice daily (n = 4) with: (i) 0.9% NaCl (NaCl) as the negative control; (ii) 0.12% chlorhexidine digluconate (CHX) as the positive antibacterial control; or (iii) diode laser combined with methylene blue, using energy density of 320 J/cm^2 (aPDT). At 120 h of growth, the biofilm formed on each disc was collected to determine the viable bacterial counts and the concentration of IEPS and IPS.

2.2. Preparation of acrylic resin discs

Twelve discs of self-curing acrylic resin (JET – Artigos Odontológicos Clássico Ltda, SP, Brazil) were prepared according to the manufacturer's instructions (diameter, 3 mm; height, 2 mm). This dimension provided a uniform irradiation of the biofilm formed on the discs, considering the laser tip. The discs were randomly divided into three groups of four discs each, and further sterilized by X-ray radiation (100 Gy, average proton energy of 160 kV, 25 mA, 0.3 mm Cu filter) for 760 s (Rad Source's RS 2000 Biological Research Irradiator, Shangai Medicilon, Shangai, China) [21].

2.3. S. mutans biofilm

S. mutans UA 159 samples were kindly donated by Dr. Jaime Aparecido Cury and Prof. Livia Andaló Tenuta from Piracicaba Dental School − UNICAMP (Piracicaba, SP, Brazil). Biofilms of *S. mutans* UA159 were formed on acrylic resin discs, during 5 days (37 °C, 5% CO_2), as reported by Ccahuana-Vásquez and Cury [19]. Briefly, *S. mutans* UA 159 was cultivated for 18 h in ultrafiltered culture medium containing ultrafiltered tryptone and yeast extract (UTYEB) supplemented with 1% glucose. Next, 0.1 mL of the culture were diluted in UTYEB with 0.1 mM glucose (the basal concentration of glucose in saliva); aliquots of this inoculum were transferred to 24-well culture plates containing individually suspended acrylic resin discs and incubated for 24 h (37 °C, 5% CO_2). Acrylic resin discs containing biofilms were transferred to fresh medium and treated with 10% sucrose for 1 min, eight times/day, at predetermined times (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00, and 18:30). After 48 h of growth, the biofilms were treated as described in the next section; during the whole treatment period, the eight daily exposures to sucrose were maintained, and the culture medium was replaced every 24 h (before 8:00 a.m.).

2.4. Treatments

Before treatment, the biofilms were maintained in the dark in plates containing 0.01% methylene blue (Chimiolux, DMC, São Carlos – SP) for 5 min (pre-irradiation time), both required conditions to kill bacteria according previous work [13]. The biofilms were submitted to the combined treatment with aPDT twice daily, before the first and the last exposure to sucrose. A preliminary study showed no difference between negative control group, methylene blue only or laser only, justifying the absence of the last two treatments in this work. A diode laser prototype (DMC Therapy XT, San Carlos − SP) (600-μm fiber diameter, 660 nm wavelength, spot size of 0.028 cm^2, 100 mW power output) was used as the laser source and device calibration has been checked before all the experiments (Fied-Top Max II laser power/energy meter, Coherent Radiation, Palo Alto, CA). The radiation originated from the equipment was delivered at energy density of 320 J/cm^2 (9 J), during 90 s to each disc side, at a 2-mm distance between the light source tip and the exposed disc. The irradiance was 1415 W/cm^2. An apparatus was used to hold and suspend the specimens during irradiation time and all area of the discs was treated, including its height. Control biofilms were treated for 1 min with sterile aqueous solution of 0.12% CHX (gold standard against biofilms at this concentration, positive control) (Sigma–Aldrich, St. Louis, MO, USA) or 0.9% NaCl (physiological saline solution, negative control) (Merck Millipore Corporation, Darmstadt, Germany). Although not equivalent chemically, the concentration used of methylene blue or chlorhexidine were based on its antimicrobial effects described by the literature. After each treatment, the biofilms were washed three times in 0.9% NaCl. The experiment was performed in quadruplicate (n = 4).

2.5. Biofilm collection and analysis

On the sixth day (t = 120 h of growth), the discs were washed three times in 0.9% NaCl to remove loosely adherent material and individually transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl; attached biofilms were removed by sonication [22]. Aliquots of 100 μL (biofilm suspension) of sonicated samples were diluted in 0.9% NaCl and serial decimal dilutions were inoculated in duplicate by the drop-counting technique [23] in Brain Heart Infusion agar. After incubation (48 h, 37 °C, 5% CO_2), the colony-forming units (CFU) were counted and the results expressed in CFU/mm^2 of specimen area. The concentrations of IEPS and IPS were determined as reported previously [22]. Briefly, the remaining biofilm suspension was centrifuged (10,000g for 10 min, 4 °C) and the precipitated was suspended in 1 M NaOH. After 15 min in agitation, the tube was centrifuged and the suspension containing IEPS was transferred to another microcentrifuge tube. For IPS extraction, 1 M NaOH was added to precipitated, the tube was vortexed and placed at 100 °C at 15 min, and the concentration of IPS determined in the supernatant. After ethanol precipitation, IEPS and IPS were determined by colorimetric method. The results of bacterial viability and polysaccharide concentration were expressed in CFU/mm^2 of specimen area and μg of polysaccharide/mm^2 of specimen area, respectively.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) model was used to test the effect of the treatments and the Tukey's test was applied to compare the means of significant variables. The assumption of adherence of the residuals to the Gaussian distribution was evaluated by Shapiro-Wilk test, by the coefficient of skewness, kurtosis, and graphical analysis. As the original CFU data showed no adherence to the Gaussian distribution, the statistical tests were applied to the rank transformed data, resulting in a normal distribution of the residuals. Statistical analyses were performed using SAS (SAS Institute Inc., version 9.3, Cary, NC, USA), at the significance level of 5%.

3. Results

Compared with NaCl, the combined treatment with aPDT markedly reduced the viable bacterial counts, but less strongly than CHX (Fig. 1).

The combined treatment with aPDT reduced the IEPS and IPS

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