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The *in vitro* effect of Antimicrobial Photodynamic Therapy on dental microcosm biofilms from partially erupted permanent molars: A pilot study

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ABSTRACT

Background: Antimicrobial Photodynamic Therapy (aPDT) could enhance the prevention of dental caries lesions in pits and fissures of partially erupted molars, by killing microorganisms from complex dental biofilms. This pilot study aimed to evaluate the effect of Antimicrobial Photodynamic Therapy (aPDT) on the viability of specific microorganism groups of dental microcosm biofilms from occlusal surfaces of first permanent molars in eruption.

Methods: Dental microcosm biofilms grown on bovine enamel blocks, from dental plaque collected on occlusal surfaces of a partially erupted lower right first permanent molar, with McBain medium plus 1% sucrose in anaerobic condition at 37 °C for 72 h. The experiments were performed in eight groups: L-P- = no treatment (control), L18.75P- = 18.75 J/cm^2 LED, L37.5P- = 37.5 J/cm^2 LED, L75P- = 75 J/cm^2 LED, L-P+ = 200 mM TBO, L18.75P+ = 200 mM TBO + 18.75 J/cm^2 LED, L37.5P+ = 200 mM TBO + 37.5 J/cm^2 LED, and L75P+ = 200 mM TBO + 75 J/cm^2 LED. The counts of total microorganisms, total streptococci and mutans streptococci were determined on selective media agar plates by colony-forming units per mL. The log-transformed counts were analyzed by Kruskal-Wallis and post-hoc Dunn's test ($P < 0.05$).

Results: The counts of all microorganisms treated in the group L75P+ were statistically lower than those treated in L-P-. The aPDT promoted a significant reduction of microorganisms, with a trend of dose-dependent effect.

Conclusion: TBO-mediated aPDT was effective in reducing the viability of specific microbial groups in dental microcosm biofilms originated from occlusal of permanent molars in eruption.

1. Introduction

The susceptibility to dental caries varies according to the age of person, tooth and dental surface [1]. The first permanent molars are considered the most vulnerable teeth for the development and progression of lesions, especially during the eruption [2], because the accumulation and retention of dental plaque by complex occlusal morphology and limited effectiveness of toothbrushing [3,4].

Antimicrobial Photodynamic Therapy (aPDT) has been proposed as an alternative method for the prevention and treatment of biofilm-dependent oral diseases, such as dental caries [5,6]. It consists of the interaction of a photosensitizing agent with a complementary light source in the presence of endogenous oxygen, leading to the production of free radicals, such as superoxydes and synglet oxygen, which promote oxidative stress and death of microbial cells [5–7].

The effectiveness of aPDT against cariogenic microorganisms using toluidine blue O (TBO) was predominantly demonstrated by the reduction of the viability of planktonic cells [8–14] and mono/dual-species biofilms [15–19]. Few studies considered testing TBO-mediated aPDT on multi-species biofilms [20–23]. Taking into account that cariogenic microorganisms are found exclusively in complex biofilms on dental surfaces, in addition to the fact that the antimicrobial effect is progressively reduced from planktonic cells through polymicrobial biofilms [24], it would be desirable to test aPDT on more pragmatic *in vitro* models as a contribution for the development of clinical protocols based on more accurate backgrounds.

A recent systematic review [25] showed that there is no agreement on the efficacy of aPDT on dental plaque, by the disparities of results

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and the use of distinct methodologies. It suggested the design of studies with standardized experimental protocols containing human dental biofilm. However, the application of this relatively novel therapeutic procedure on humans, considering only the adjusting of its settings for better performance instead of exploring its maximum efficacy, could be considered precipitate and inadequate.

Based on the higher dental caries risk of occlusal surfaces of permanent molars in eruption, and the limitation of results of preventive strategies in this life stage, the aim of this pilot study was to evaluate the effect of aPDT on the viability of specific microorganism groups in microcosm biofilms grown from the occlusal surface of a partially erupted first permanent molar. The null hypothesis (*H0*) was that the aPDT would have no effect on the viability of different microorganism groups in dental microcosm biofilms.

2. Materials and methods

2.1. Ethics

This research protocol was submitted and approved by the Human Research Ethics Committee of *(track omitted for the peer-review process)* (#CAAE *track omitted for the peer-review process*), in accordance with the ethical standards of the Declaration of Helsinki.

The parents of a 7-year-old girl, with partially erupted first molars (DMFT = 0), were invited to authorize their child's participation in this study, through the donation of dental biofilm samples. They received information concerning the aims of the study and signed a statement of informed consent to the inclusion of material pertaining to their children, with the maintenance of anonymous information and no identification of their acknowledge via the paper.

2.2. Collection of dental biofilm

After relative isolation with cotton rolls and drying with air jets, using a spoon excavator, samples of dental biofilm were collected from the central fossa of the occlusal surface of the lower right first permanent molar. These procedures were repeated eight times, in weekly intervals. The samples were immediately transferred to cryotubes (Corning Incorporated, Corning, USA) containing 2.0 mL of Brain Heart Infusion medium [BHI, 37 g of brain heart infusion, and deionized water/L, pH 7.2] and 20% glycerol. These stocks were subsequently stored at −80 °C until the moment of use.

2.3. Preparation of substrata and biofilm models

The bovine enamel blocks (4 × 4 × 2 mm) were prepared as described by Fushida and Cury [26], and maintained in deionized water at 4 °C until the moment of their use. The Active Attachment Model Amsterdam (ACTA, Amsterdam, The Netherlands) was used to grow biofilms as previously described by Deng et al. [27] and Silva et al. [28]. This model consists in two parts, a stainless-steel lid on which 24 polystyrene clamps were fixed to support substrata, and a 24-well tissue culture plate (Greiner Cellstar®, Greiner Bio-one, Kremsmünster, Germany) that holds biofilm medium. The model was assembled with dentinal faces of specimens fixed to the clamps with condensation silicone impression material (Zetaplus®, Zhermack, Badia Polesine, Italy), maintaining the enamel faces parallel to the bottom of well. The stainless-steel lids together with the assembled enamel blocks were sterilized by autoclaving before usage.

2.4. Biofilm growth

Initially, a stock solution of biofilm diluted in BHI medium was thawed at room temperature for 15 min and vortexed for 10 s. Then, 400 µL of the suspension was mixed with 10 mL of modified McBain medium [2.5 g/L mucin, 2.0 g/L peptone, 2.0 g/L casein peptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L CaCl2, 1.0 mg/L hemin, 0.2 mg/L vitamin K1, 50 mmol/L PIPES, 0,4% sucrose, and deionized water, pH 7.0] [29], and subsequently stored in anaerobic conditions at 37 °C overnight.

After that, a volume of microorganism suspension was mixed with modified McBain medium plus 1% sucrose in the proportion 1:1. Aliquots of 1.5 mL of final microbial suspension were distributed in each well of a 24-well microtiter plate (Greiner Cellstar®, Greiner Bio-One, Kremsmünster, Austria). The biofilm models covered the sterile plate, with the enamel blocks being entirely dipped in the modified McBain medium. Then, the models were incubated under anaerobic conditions at 37 °C for 24 h. The culture media without microbial inoculum were refreshed each 24 h until 3 days completed.

2.5. Photosensitizing agent and light source

The toluidine blue O (TBO, Pharmácia Específica, Bauru, Brazil) and the Biotable® RGB (Institute of Physics of São Carlos, São Carlos, Brazil) were used as a photosensitizing agent and a light source, respectively. The TBO was diluted in deionized water until the final concentration of 200 mM, and stocked in the dark at 4 °C. Biotable® RGB is an equipment that permits the simultaneous irradiation of until 24 specimens. It consists of an acrylic box with a similar size of a microtiter plate, containing 24 light emitting diodes (LEDs) uniformly distributed by the extension of its bottom. Each LED RGB is able to irradiate an individual sample through the application of three different wavelengths [blue (455 ± 30 nm), green (520 ± 30 nm), and visible red (625 ± 30 nm)], and four distinct power densities (10, 20, 30, and 40 mW/cm^2).

The optical power output of Biotable® RGB was controlled by an optical power meter (1916-C Optical Power Meter, Newport, Irvine, USA) throughout the study. When necessary, the time of irradiation was corrected according to the following formula:

$$time(s) = \frac{energydensity\left(\frac{J}{cm^2}\right)}{powerdensity\left(\frac{W}{cm^2}\right)}$$

2.6. Experimental groups

The biofilms were allocated in eight experimental groups, one control and seven treatments, as follows: L-P- = no treatment (control), L18.75P- = 18.75 J/cm^2 LED, L37.5P- = 37.5 J/cm^2 LED, L75P- = 75 J/cm^2 LED, L-P+ = 200 mM TBO, L18.75P+ = 200 mM TBO + 18.75 J/cm^2 LED, L37.5P+ = 200 mM TBO + 37.5 J/cm^2 LED, and L75P+ = 200 mM TBO + 75 J/cm^2 LED.

2.7. Antimicrobial Photodynamic Therapy

Prior to the procedures of aPDT, unbound bacterial cells were removed by washing the biofilms in Cysteine Peptone Water medium [CPW, 5 g/L yeast extract, 1 g/L peptone, 8.5 g/L NaCl, 0.5 g/L L-cysteine HCl, and deionized water, pH 7.3]. This medium was applied to avoid destruction of viable bacteria, since many types of aerobic microorganisms die rapidly when suspended in saline solutions. Differently, peptone water or other organic nitrogenous substances afford adequate protection for bacteria during dilution in experimental essays [30].

According to their groups, the samples were rapidly transferred to 1.5 mL of TBO or sterile deionized water (TBO solvent), and incubated for 2 min in dark conditions. Then, the biofilms were once irradiated with a visible red light wavelength (625 ± 30 nm), and a power density of 40 mW/cm^2, using the fluences of 18.75 J/cm^2, 37.5 J/cm^2 or 75 J/cm^2, which correspond to 1.88 J, 3.75 J, and 7.50 J of total light energy. These parameters of irradiation were achieved by varying the