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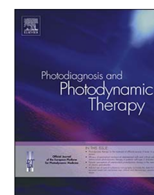

Evaluation of photosensitizer penetration into sound and decayed dentin: A photoacoustic spectroscopy study

Ingrid Gomes Perez Occhi-Alexandre[a], Mauro Luciano Baesso[b], Francielle Sato[b], Lidiane Vizioli de Castro-Hoshino[b], Pedro Luiz Rosalen[c], Raquel Sano Suga Terada[a], Antonio Medina Neto[b], Mitsue Fujimaki[a,*]

[a] Dentistry Department, State University of Maringá, Avenida Colombo, 5790 – Jardim Universitário, Maringá, PR, CEP 87020-900, Brazil
[b] Physics Department, State University of Maringá, Avenida Colombo, 5790 – Jardim Universitário, Maringá, PR, CEP 87020-900, Brazil
[c] Physiological Sciences Department, School of Dentistry of Piracicaba, University of Campinas, Avenida Limeira, 901 – Bairro Areião, Piracicaba, SP, CEP 13414-903, Brazil

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ABSTRACT

Background: Photodynamic therapy (PDT) may have topical indications. In those cases it is important for a topical photosensitizer to penetrate into the tissue to which it has been applied. This study aimed to compare the penetration of two different concentrations of erythrosine into intact and *in vitro* decayed dentin samples.
Methods: This *in vitro* study evaluated erythrosine (0.3 and 5%) penetration into sound (intact) and decayed dentin. A total of 11 dentin discs were prepared and divided into two equal halves, in order to keep one half sound while the other half was submitted to sterilization and an *in vitro* demineralization model for 5 days. Before erythrosine application, the organic and inorganic composition of all samples was evaluated by Fourier Transform Raman spectroscopy, and after erythrosine application for 30 min, the penetration depth was determined by Photoacoustic spectroscopy technique.
Results: The results indicated that 0.3% erythrosine showed a higher penetration depth into sound dentin ($p = 0.002$); and 5% erythrosine higher penetration into decayed dentin ($p < 0.001$). However considering clinical parameters, no statistically significant difference was found between any of the conditions tested.
Conclusions: Erythrosine demonstrated ability to penetrate into dentin, irrespective of sound or decayed condition. Photoacoustic spectroscopy can be considered a method for estimating the penetration into hard tissues, and in conjunction with Raman spectroscopy, these are effective methods for evaluating the spectral response of dentin. Considering that erythrosine is capable of penetrating into decayed dentin, clinical trials are needed to test the effectiveness of this photosensitizer in Photodynamic therapy and Antimicrobial Photodynamic therapy.

1. Introduction

Photodynamic Therapy has been tested as an adjuvant in carious lesion treatment with the aim of reducing bacterial load [1–4]. Dental caries is one of the most prevalent chronic diseases in the world [5], and when untreated, can result in loss of minerals from dental tissues [6]. PDT uses a combination of visible light at a suitable wavelength; a dye called a photosensitizer; and the molecular oxygen generally present in tissues [7]. Photodynamic Therapy has shown efficacy against microorganisms involved in the tooth decay process [1,2,8–10]. In Photodynamic Therapy, many dyes, such as methylene blue, toluidine blue orto, rose bengal, erythrosine, phthalocyanine and porphyrin have been used as photosensitizers against dental caries microorganisms [11]. Erythrosine, a dye already routinely used in dental practice for disclosing plaque [12], may act against Gram-positive bacteria; it is a hydrophilic dye and may have photodynamic effect even at low concentrations [11]. In spite of these chemical and biological advantages, it is necessary to know the penetration capacity of photosensitizing agents used in Photodynamic Therapy to ensure that they reach the full extent of carious tissue, thereby promoting the desired therapeutic effect [13].

Dentin permeability is partly determined by the nature of the dentin surface [14]. The resistance to fluid diffusion through dentin is dependent on the presence or absence of pulp tissue and the presence or absence of fragments on the surface [15]. Different methods have been used to measure the depth of penetration into dentin, and the spectroscopic techniques in both ultraviolet-visible (UV–vis) and mid-infrared (MIR) spectral regions have shown promising results in terms of dental structure and optical properties investigations [16–19].

* Corresponding author at: Department of Dentistry, State University of Maringá, Rua Luiz Gama, 144 apto 1601–Zona 1, CEP 87014-110, Maringá, PR, Brazil.
E-mail address: mfujimaki@uem.br (M. Fujimaki).

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Photothermal techniques have also been successfully applied in dental studies mainly justified by their characteristic of being highly sensitivity, to be minimally influenced by the scattered light and because they permit realization of depth profile analysis along the dental structures. Early caries diagnosis and the monitoring of erosion and demineralization of dental tissues have been successfully reported [20–22]. The conventional Photoacoustic Spectroscopy, one of the most traditional technique of the photothermal area, was adopted in the present work because of its ability to measure the penetration of formulations through biological tissues and specially due to the fact that its setup can be built using lamps instead of lasers. This is particularly important in terms of delivering lower intensity of excitation light in the sample, minimizing possible occurrence of photodegradation and/or evaporation of the dyes used in the measurements. In addition, the photoacoustic spectroscopy technique demands minimal sample manipulation and is one of the few methods that can be applied for *in vivo*, *ex vivo*, and *in vitro* studies [13]. Raman spectroscopy is a technique capable of providing information on the chemical structure of organic or inorganic compounds, thus allowing them to be characterized [18,19].

In the published literature, there are few studies about photosensitizer penetration into dentin [13,23], and only one of them simulated caries lesions to evaluate the penetration of photosensitizer toluidine blue-orto, by means of Confocal Raman Microscopy [23]. Therefore the present study aimed to quantify and compare the penetration of two different concentrations of erythrosine into sound and *in vitro* decayed dentin samples; and to assess the structural changes in dentin with 5-day experimental carious lesions.

2. Materials and methods

2.1. Experimental design

This was an *in vitro* study and the factors under evaluation were the erythrosine penetration into sound and decayed dentin, with two different dye concentrations (0.3 and 5%), resulting in 4 experimental groups: G1–sound dentin and 0.3% erythrosine (n = 5); G2 – decayed dentin and 0.3% erythrosine (n = 5); G3 – sound dentin and 5% erythrosine (n = 6); and G4 – decayed dentin and 5% erythrosine (n = 6). From 11 teeth selected, 11 dentin discs were prepared and divided into two equal halves, properly identified, in order to keep one half sound while the other half of the sample was sterilized by gamma irradiation and then subjected to the *in vitro* demineralization model for 5 days. These two erythrosine concentrations were selected after a pilot test to obtain the minimal amount of this dye that could be detected by the photoacoustic spectroscopy, what was around 0.3%. The 5% group was for comparison purposes.

Before erythrosine application, samples of sound dentin and their respective halves were tested by Fourier Transform Raman spectroscopy to evaluate the organic and inorganic composition of each tooth, to enable exclusion of those with a composition differing from that of the others. After erythrosine application for 30 min in all groups, the penetration depths were determined by the Photoacoustic spectroscopy technique.

The dependent variable was *dye penetration* (μm) and the interdependent variables were: *dye concentration* (0.3 or 5%) and the *dentin condition* (sound or decayed). The null hypotheses tested were: there would be no differences in erythrosine penetration between the sound and decayed dentin, and between the 0.3% and 5% erythrosine concentrations.

2.2. Sample selection and preparation

Sound human third molar teeth with incomplete rhizogenesis were collected in conformity with an informed consent protocol reviewed and approved by the State University of Maringá Ethics Committee on Human Research (CAAE 14398113.2.0000.0104). All the teeth were cleaned with gauze and 0.9% physiological solution and then stored in distilled water to prevent dehydration. Dentin discs were produced by cutting the teeth into slices approximately 1 mm thick, using a diamond disc saw under cooling (Sout Bay Technology; Diamond Wheel, San Clement, California, USA) coupled to a switching device (IsoMet Low Speed Saw; Buehler, Lake Bluff, IL, USA). All discs were cut in the transverse direction to that of the dentinal tubules, in the region approximately 2 mm from the cement–enamel junction. From each tooth, a single slice of dentin was obtained and divided into two equal halves, after the enamel had been removed with abrasive discs (Sof-Lex Pop-On, 3 M ESPE®). The sample dimensions were adjusted to obtain blocks measuring 3 mm (long) × 2 mm (wide) × 1 mm (thick) and the occlusal dentin surface was identified in each sample. Dentin blocks were kept in saline solution and gently dried before starting the treatment.

Erythrosine powder, $C_{20}H_6I_4Na_2O_5$ (Vetec® Química Fina Ltda, Duque de Caxias, Rio de Janeiro, Brazil) was used and diluted in distilled water to concentrations of 3 or 50 mg/mL. An aliquot of 4 μL of the solution containing the photosensitizer was applied on the occlusal surface of all dentin samples, and was left to interact for a time interval of 30 min. After this period had elapsed, cotton buds were used to remove the remaining photosensitizer from the surface, then the samples were placed inside the photoacoustic cell to perform the measurements.

2.3. In vitro demineralization model

An adaptation of the model proposed by Cunha et al. [24] was used to induce artificial caries lesions in the dentin blocks [24]. At first, samples were subjected to sterilization by gamma irradiation (dose of 14.5 kGy) at the Center for Nuclear Energy in Agriculture (CENA-USP, Piracicaba, Brazil) in order to ensure the specificity of the inoculated microorganisms.

The biofilm of *Streptococcus mutans* UA159 (ATCC 700610, serotype c) was formed on saliva-coated sterile dentin blocks. Human whole saliva was collected from one donor, clarified by centrifugation (6350 rpm, 4 °C, 10 min), sterilized and diluted (1:1) in adsorption buffer (AB-50 mM KCl, 1 mM KPO4,1 mM CaCl2, 0.1 mM MgCl2, pH 6.5), and supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF) at a final concentration of 1 mmol/L. The dentin blocks were aseptically placed into 96-well plates with concave bottom and inoculated with approximately 2×10^6 CFU/mL in low molecular weight medium with addition of 1% (w/v) sucrose (200 μL/well) and incubated at 37 °C, 5% CO_2. The biofilms were initially grown undisturbed for 24 h, and then the culture medium was replaced daily for 5 days.

2.4. Evaluation of photosensitizer penetration (μm)

The Photoacoustic spectroscopy measurements were taken with a homemade setup. Experimental details of the procedure can be found elsewhere [25–27]. The spectra were taken between 300 and 750 nm, because in this spectral range erythrosine showed an absorption band. Photoacoustic spectra were taken from the pulp surface of each sample before erythrosine application, and from both dentin surfaces after erythrosine application. The modulated light on the sample surface might reach different depths into the sample depending on the frequency modulation – this is an advantage of Photoacoustic spectroscopy over others spectroscopies; therefore the penetration analysis was performed using this Photoacoustic spectroscopy feature. Treated dentin samples were placed inside the photoacoustic cell, with the occlusal surface, on which erythrosine was applied facing down, and the incident light reaching the sample on the pulp surface, with frequency modulation of 75 Hz (Fig. 1). If no erythrosine absorption band was detected by Photoacoustic spectroscopy, the pulp surface was manually sanded with gentle movements, (this procedure was chosen to avoid interference over the penetration of the erythrosine) until absorption band

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