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Antimicrobial effect of the photodynamic therapy on caries lesion: an *in vitro* study

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ABSTRACT

The current evidence suggests that antimicrobial photodynamic therapy (aPDT) with the application of methylene blue as a photosensitizer and red laser activation is suitable for disinfecting carious tissue. However, since many dental practices lack access to lasers, a standard dental curing light could be an alternative light source. This study evaluated the use of fuchsin as a photosensitizer in aPDT and a blue light-emitting diode (LED) dental curing light for the disinfection of carious dentin. Sixty permanent third molars were selected, cut to obtain flat dentin surfaces, and subjected to a 15-day cariogenic challenge with *Streptococcus mutans*. The specimens were allocated into four groups (n=15): no disinfection; fuchsin; blue LED irradiation; and aPDT (fuchsin plus blue LED irradiation). *S. mutans* counts were obtained in the remaining carious dentin from all specimens across all groups. Results were analyzed by the Kruskal-Wallis test (Student-Newman-Keuls). The percentage reduction in microbial load was 96.07% for aPDT, 88.85% for LED alone, and 85.46% for fuchsin alone. SEM demonstrated a greater reduction in microbial burden after aPDT with fuchsin and blue LED light, with removal of the smear layer and exposure of dentin tubules. Fuchsin-mediated aPDT using blue LED irradiation significantly reduced microbial load and may represent a viable alternative for the disinfection of carious tissue, particularly in settings without access to laser devices.

Key words: blue methylene; dental caries; Streptococcus mutans; blue light.

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Introduction

Dental caries, a widespread chronic disease affecting humans, is understood to be a dysbiotic condition resulting from an imbalanced demineralization-remineralization process.[1] It is a chronic, multifactorial, highly prevalent disease associated with the presence of acidogenic microorganisms such as *Streptococcus mutans*, a sugar-rich diet, and host susceptibility.[2-4] Dentinal carious lesions can be divided into two distinct layers: infected dentin, characterized by disorganized collagen and a high bacterial load, and affected dentin, which maintains the potential for remineralization.[5]

Selective removal of carious dentin aims to preserve pulp vitality, avoiding unnecessary exposure of the pulp chamber and promoting biological repair of the dentin, as well as preventing wear that could further compromise and weaken the tooth structure.[6-9] Various methods for disinfecting carious dentin are being studied with the aim of avoiding the removal of carious tissue.

In this context, antimicrobial photodynamic therapy (aPDT) has emerged as an effective and minimally invasive disinfection method. This technique consists of the application of a photosensitizing chemical compound followed by irradiation with light of a specific wavelength, which generates reactive oxygen species (ROS) capable of destroying microorganisms by membrane lysis.[10]

Several effective aPDT protocols have been published in the literature, mostly using methylene blue (at concentrations between 0.005% and 0.01%) activated by low-level lasers in the red wavelength range; these have been shown to effect significant reductions in microorganisms such as *S. mutans* in dentinal caries.[11-14] This technique also has applications in other areas of dental practice, including endodontic and orthodontic procedures and biofilm control.[15-23]

In clinical practice, however, few dentists have laser units in their offices, and new aPDT protocols are needed to expand its application to a broader patient population. Therefore, the use of alternative photosensitizers and light sources, such as blue-light-emitting diode (LED) curing units, has been investigated.[24,25] Xanthene dyes, such as rose bengal, have demonstrated significant antimicrobial activity against *S. mutans* when activated by blue light, supporting their potential use in aPDT.[26-28]

Within this context, the present study sought to evaluate the utility of fuchsin as a photosensitizing agent. Although its use in aPDT has rarely been explored in the scientific literature, a recent study suggested it has photodynamic potential against *S. mutans* when irradiated with blue light.[29] Fuchsin is an inexpensive compound and is already widely used in dentistry as a plaque-disclosing agent,[30] which should facilitate its acceptability in clinical practice. Its absorption in the blue range of the spectrum means it can be activated with a standard blue LED curing light, a widely accessible and available device in dental practice, especially in resource-constrained settings.

Therefore, the objective of this study was to evaluate the effectiveness of aPDT, using fuchsin as the photosensitizer and blue LED light from a standard light-curing unit (LCU) as the activator, for disinfection of carious dentin. The null hypothesis was that fuchsin would not have photosensitizing potential.

Materials and Methods

All procedures were performed in compliance with relevant laws and institutional guidelines and with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. The study was approved by the Ethics Committee of *Pontifícia Universidade Católica de Campinas* (Certificate of submission for ethical appraisal: 5066322.1.0000.5481) on November 25, 2022. All volunteers received clarifications regarding the objectives, procedures, as well as possible risks and benefits of the study. The privacy rights of human subjects were observed, and each patient gave written informed consent to the study protocol prior to donating their teeth for research.

Specimens

The 60 permanent third molars were included in the current study. The teeth were extracted from patients aged between 18 and 25 years. Teeth with carious lesions and fractures were excluded. Impacted and unerupted teeth were also excluded.

Specimen preparation

The teeth were cut with a double-sided diamond disc at low speed (KG Sorensen, KG Sorensen Indústria e Comércio Ltda., São Paulo, Brazil) to expose the coronal dentin. The dentin surface was then sanded flat with a

silicon carbide disc (Norton, Norton Abrasivos Brasil Ltda., São Paulo, Brazil). The roots were sealed with epoxy resin (Araldite, Huntsman Advanced Materials LLC, TX, USA) and nail polish (Colorama, L'Oréal Brasil Ltda., Rio de Janeiro, Brazil), except for the coronal dentin, which was to be exposed to the cariogenic challenge. The specimens were then sterilized in an autoclave (Sercon, Sercon Indústria e Comércio de Equipamentos Ltda., São Paulo, Brazil).

Cariogenic challenge

Each specimen was attached to the inside of a jar lid with hot glue and orthodontic wire (Morelli Ortodontia, São Paulo, Brazil). The specimens were then submerged in brain heart infusion (BHI) medium supplemented with 0.5% yeast extract, 1% glucose, and 1% sucrose (Lab-Center) and *S. mutans* strain ATCC 25175 (Fundação André Tosello, São Paulo, Brazil). Incubation was carried out at 37°C for 15 days under anaerobic conditions. The culture medium was changed daily, and the pH was monitored. A pH level of £5 for 15 consecutive days was required for completion of the cariogenic challenge.[11,12,31-33]

Sample size calculation

For this study, the teeth were randomly divided into 4 groups using www.random.org. The sample size was calculated to be 15 specimens per group, using analysis of variance (ANOVA). Calculation was done in G*Power 3.1.9.4 (Franz Faul, University of Kiel, Germany), with =0.05, =0.80, and effect size (f)=0.45.

Study groups

The specimens were allocated into four groups (n=15 per group):

i) Control group: No tissue disinfection procedure was performed.
ii) Blue LED group: Irradiation with blue LED light using an LED LCU (DMC Equipamentos, São Paulo, Brazil).
iii) Fuchsin group: Active application of 0.005% fuchsin solution (Fórmula & Ação, São Paulo, Brazil) for 5 min using a microbrush (Microbrush International, Grafton, WI, USA).
iv) aPDT group: Active application of 0.005% fuchsin (Fórmula & Ação) for 5 min using a microbrush (Microbrush International), followed by irradiation with blue LED light delivered by an LCU (DMC Equipamentos).

The irradiation parameters for the blue-LED groups were as follows: D2000 dental curing light (DMC Equipamentos), power 1000 mW/cm^2, wavelength 460 nm, spot energy 9 J, duration 9 s. A flow diagram of the study methods is provided in Figure 1.

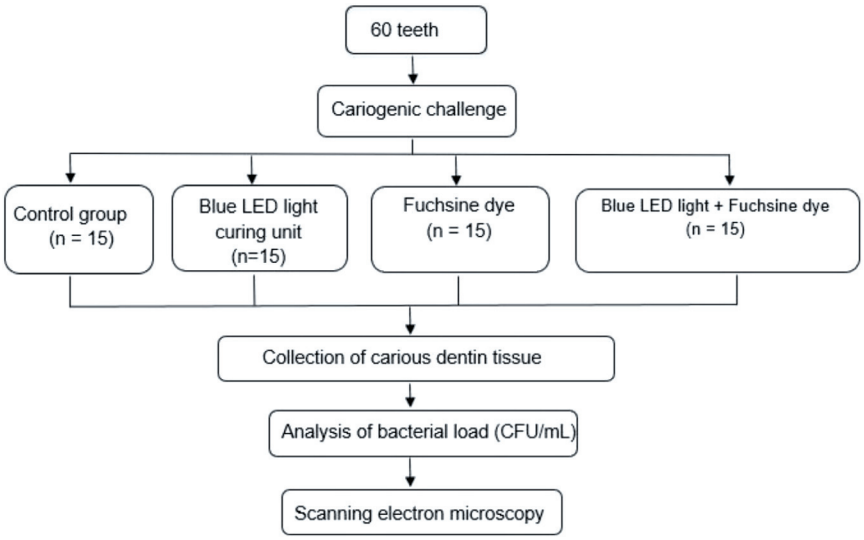


Figure 1. Flow diagram of the study methods.

Microbiological processing

After the interventions, all carious tissue was collected with a sterile dentin spoon (Golgran, São Paulo, Brazil), transferred to tubes containing peptone water (HiMedia Laboratories, Mumbai, India), and homogenized in a tube shaker (Phoenix, São Paulo, Brazil) for 30 s. Decimal dilutions up to 10^{-5} were prepared and 25-µL aliquots were seeded onto BHI agar plates (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with bacitracin (Merck KGaA, Darmstadt, Germany). The plates were incubated in an anaerobic jar (Oxoid) for 48 h at 37°C. Subsequently, colony-forming units (colony-forming units [CFU]/mL) were quantified as described elsewhere.[11,12,31]

Scanning electron microscopy analysis

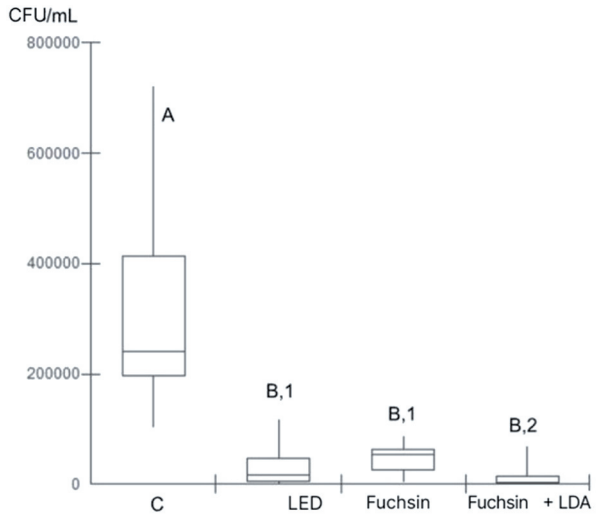
The scanning electron microscopy (SEM) analysis was performed for the qualitative assessment of carious tissue disinfection by each of the experimental protocols. The samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 6 h, followed by washing in ultrapure water and dehydration in a graded series of ethyl alcohol solutions (30, 50, 70, 80, 90, and 95%) for 5 min each, succeeded by 2 immersions in absolute ethyl alcohol, each lasting 10 min. The specimens were then treated with hexamethyldisilazane for 10 min under a fume hood and allowed to dry completely. Once dried, the specimens were mounted on aluminum stubs and coated with a ~25 nm-thick gold layer using an SCD 050 sputter coater. The analysis was performed using a LEO 430i SEM operated at 15 kV.

Statistical analysis

Results were analyzed in Biostat 5.3. The Shapiro-Wilk test was used to evaluate the normality of the distribution. As the sample was found to deviate from normality, the Kruskal-Wallis test followed by the Student-Newman-Keuls procedure was performed, with a significance level of 5%.

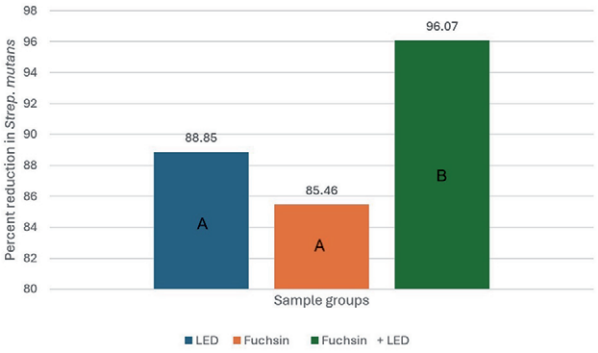
Results

The greatest reduction in *S. mutans* counts occurred in the aPDT group, with significant differences compared to the control group (p<0.0001), the fuchsin-only group (p=0.0452), and the LED-only group (p=0.0024). All experimental groups showed significant reductions in *S. mutans* counts in the carious lesions (p<0.01) (Figure 2). The percentage reduction in microbial load was 96.07% for aPDT, 88.85% for LED alone, and 85.46% for fuchsin alone (Figure 3).



C, control; LED, blue LED only; fuchsin, fuchsin only; fuchsin + LED, fuchsin *plus* blue LED (aPDT).

Figure 2. Box plot: medians and quartiles of *S. mutans* counts (CFU/mL) in the study groups. Kruskal-Wallis test with Student-Newman-Keuls procedure. A¨B and 1¨2 denote statistically significant differences.



LED, blue LED only; fuchsin, fuchsin only; fuchsin + LED; fuchsin *plus* blue LED (aPDT).

Figure 3. Percent reduction in *S. mutans* in dentinal caries after disinfection with blue LED light only, fuchsin only, and fuchsin followed by blue LED light. A¨B denotes statistically significant differences.

The SEM images showed significant contamination of the carious dentin by *S. mutans*, as well as the presence of a smear layer (Figure 4A). After irradiation with blue LED from an LCU or application of fuchsin dye, reductions in both *S. mutans* counts and the smear layer were observed (Figure 4 B,C). After aPDT with fuchsin and blue LED light, a greater reduction in microbial burden was seen, with removal of the smear layer and exposure of dentin tubules (Figure 4D).

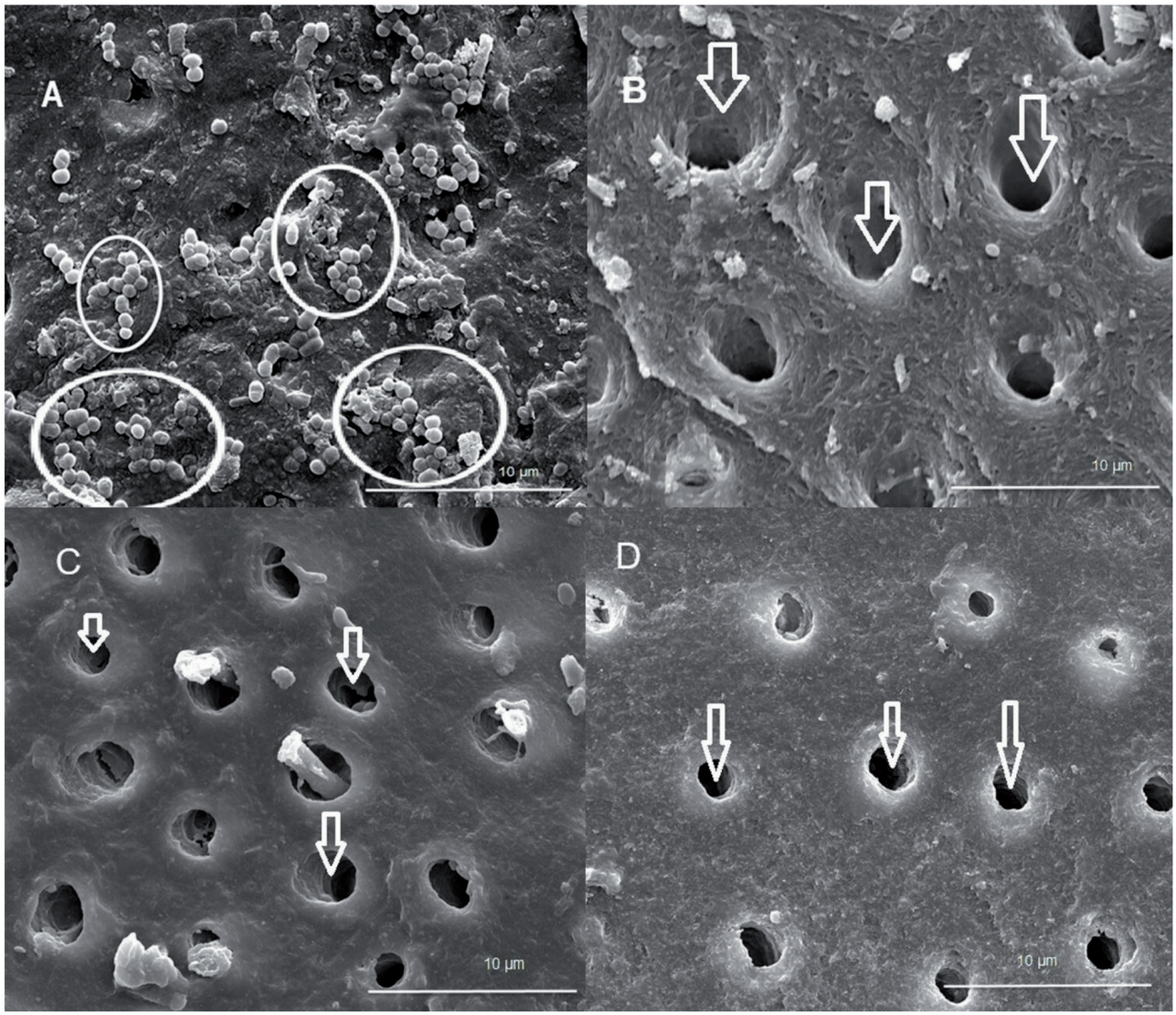


Figure 4. A) Carious dentin tissue. Note heavy microbial contamination and the presence of a smear layer. Circles: clusters of *S. mutans* in caries-infected dentin. **B**) Carious dentin tissue after LED irradiation. Note reduction of *S. mutans* contamination and removal of the smear layer, with exposure of dentin tubules (arrows). **C**) Carious dentin tissue after application of fuchsin. Note removal of the smear layer, exposure of dentin tubules (arrows) and reduced microbial burden. **D**) Carious dentin tissue after aPDT with fuchsin and blue LED light. Note clean dentin with near absence of contamination, removal of the smear layer, and exposure of dentin tubules (arrows).

Discussion

The cariogenic challenge performed in this study used the microbiological method, as described by Le Goff *et al.*,[31] de Carvalho *et al.*,[33] Pinheiro *et al.*,[15] Santin *et al.*,[34] Moro *et al.*,[11] and Fernandes *et al.*[12] In comparison to the chemical model for creating carious lesions,[6,35,36] the microbiological method more faithfully simulates dental caries as it occurs in the oral cavity, since it combines bacterial metabolic activity, biofilm dynamics, and a cariogenic substrate.

A standard strain of *S. mutans* was selected for the cariogenic challenge in this study, as this species is considered the main cariogenic microorganism due to its high acidogenic and aciduric capacity, which facilitates demineralization and the progression of carious lesions.[3,4,37] Microbiological processing was carried out following the method described by Le Goff *et al.*,[31] consisting of dilution, homogenization, seeding, and counting of viable CFUs.[6,23,38]

The parameters used for the LED were adapted from the protocols using a low-power red laser for conventional aPDT with methylene blue as a photosensitizer.[11-16,21,39,40,41] An LED dental curing light was chosen as the light source due to its wide availability and low cost, enabling translation to clinical practice and increasing adherence to the disinfection protocol.[24,25,42]

Fuchsin has been investigated as a photosensitizer due to its ability to penetrate carious tissue and biofilm, a characteristic already explored in its clinical use as a plaque disclosing agent.[43,44] By highlighting the lesion, fuchsin helps visualize carious tissue. Therefore, as the fuchsin dye discloses where the carious lesion is, it can simultaneously be irradiated by the LED light source, increasing the precision of the photodynamic effect. A 0.005% concentration was used, in analogy to classic aPDT protocols with methylene blue as the photosensitizer.[15,16] Furthermore, fuchsin can be activated by the blue LED light of the LCU that dentists routinely use in their offices, allowing for greater use of aPDT, especially by practitioners who do not have access to a red laser unit.

The results of the current study demonstrate that the combination of fuchsin as a photosensitizer and blue LED as a light source is a feasible alternative to aPDT method, resulting in greater disinfection of dentinal caries than either method alone. This reduction in viable bacteria occurs due to the affinity of fuchsin for bacterial biofilm and its ability to generate ROS when activated by blue LED light, promoting significant bactericidal activity.[17,29,45] The SEM imaging of specimens treated with the fuchsin/blue LED aPDT method showed a reduction in *S. mutans* contamination of dentinal carious lesions, with removal of the smear layer and exposure of the dentinal tubules. However, the blue LED irradiation alone was also associated with significant reductions in *S. mutans vs.* the control group. This can be explained by the fact that LED irradiation activates endogenous photosensitizers present in the carious lesion, such as porphyrins. Additionally, the interaction between blue LED light and fuchsin promotes the generation of ROS, which contributes to cell death.[10,27,42] The significant reduction in *S. mutans* demonstrates that the presence of endogenous photosensitizers enhances the action of blue light on carious lesions.[24,25,42]

Therefore, fuchsin, a dye already used in dentistry as a plaque disclosing agent, can be a low-cost alternative photosensitizer; its wavelength of 545 nm, close to that of blue LED light (450 nm), makes it a favorable option for aPDT protocols. Also, rose bengal, another widely studied dye, has a similar wavelength (approximately 550 nm) and has already proven effective when activated with blue LED light.[26,45-48] Recent studies confirm the strong affinity of the xanthene dyes rose bengal and erythrosine for *S. mutans* biofilms. Upon irradiation with blue LEDs, these dyes generate ROS, leading to significant bactericidal activity.[45]

The results obtained in this *in vitro* study demonstrate that both fuchsin and blue LED light exhibit significant antimicrobial activity, but an aPDT protocol combining the two achieved the highest rates of bacterial reduction and the best dentin surface cleaning. Further studies, such as randomized clinical trials, should be conducted to generate robust clinical evidence on the use of a fuchsin/blue LED aPDT protocol for disinfecting carious lesions in dentin.

Despite the promising findings, some limitations of this study should be acknowledged. First, as an *in vitro* investigation, the experimental conditions do not fully replicate the complexity of the oral environment, including factors such as saliva, host immune response, and the presence of a multispecies biofilm. In addition, a single bacterial species (*S. mutans*) was used in the cariogenic challenge, which does not reflect the microbial diversity typically observed in clinical carious lesions. Another limitation is that the study evaluated short-

term antimicrobial effects, without assessing the long-term stability of the results or the potential for bacterial recolonization. Furthermore, variations in light penetration and photosensitizer distribution within deeper dentin layers were not investigated. Therefore, caution should be exercised when extrapolating these findings to clinical practice, and further studies, particularly *in situ* and randomized clinical trials, are needed to confirm the effectiveness and applicability of this protocol under clinical conditions.

Conclusions

The present study is particularly relevant, as it proposes a feasible, low-cost, and clinically accessible aPDT protocol based on materials and equipment already widely available in dental practice. By demonstrating that the combination of fuchsin dye and blue LED light enhances the reduction of *S. mutans* in carious dentin compared to isolated approaches, these findings contribute to the development of more effective strategies for caries disinfection. Moreover, the use of a microbiological model that closely simulates clinical conditions reinforces the translational potential of the results. This approach may facilitate the incorporation of aPDT into routine dental care, especially in settings with limited access to specialized laser devices, thereby expanding the applicability of minimally invasive strategies for caries management.
We conclude that application of fuchsin dye followed by irradiation with blue light from an LED curing unit is a feasible means of performing aPDT for disinfection of carious tissue.

Contributions: Ana Clara Miarelli Fortuna, José Roberto Rodini Caldari, Maria Fernanda Marchi, Maria Olivia Marcondes Pinto: data curation, investigation, methodology, project administration, resources, visualization, writing – original draft preparation, writing – review & editing; Carlos Eduardo Fontana: investigation, methodology, supervision, visualization, writing – original draft preparation, writing – review & editing; Victor Elias Arana-Chavez: investigation, methodology, visualization, writing – original draft preparation, writing – review & editing; Sérgio Luiz Pinheiro: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, supervision, validation, visualization, writing – original draft preparation, writing – review & editing. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors have no conflict of interest to disclose.

Ethics approval and consent to participate: all procedures were performed in compliance with relevant laws and institutional guidelines and with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. The study was approved by the Ethics Committee of Pontifícia Universidade Católica de Campinas (PUC-Campinas; Certificate of Submission for Ethical Appraisal: 5066322.1.0000.5481) on November 25, 2022. Each patient gave written informed consent to the study protocol prior to donating their teeth for research.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

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