Photodynamic Therapy Effect in Carious Bovine Dentin – An In Vitro Study

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Purpose: This randomized in vitro study determined parameters for using a light-emitting diode (LED) with toluidine blue O (TBO) for reduction of Streptococcus mutans counts inside carious dentin.

Materials and Methods: Seventy-two bovine coronary dentin slabs were immersed in Streptococcus mutans culture for demineralization. Dentin slabs were allocated to 6 groups (n = 12) as follows: control (treated with 0.9% NaCl solution for 5 min); TBO (treated with 0.1 mg/ml TBO for 5 min); LEDA (submitted to irradiation for 4.2 min); LEDB (submitted to irradiation for 6.5 min); photodynamic therapy A (PDTA; treated with TBO plus irradiation for 4.2 min) and photodynamic therapy B (PDTB; treated with TBO plus irradiation for 6.5 min). The energy densities used for 4.2 and 6.5 min correspond to 166 and 249 J/cm^2, respectively. Before and after treatments, dentin samples were analyzed with regard to S. mutans counts. The carious lesion depth produced by the microbiological model was analyzed by polarized light microscopy. ANOVA/Tukey tests were utilized to compare log reductions among groups (α = 5%).

Results: Bacterial reduction was observed when dentin was exposed to both TBO and LED at both irradiation times. However, no difference in S. mutans reduction was found between the two energy densities.

Conclusion: Although the use of LED combined with TBO was effective in reducing the Streptococcus mutans counts in carious dentin, this effect may not have clinical significance.

Keywords: LED, caries, bovine tooth, Streptococcus mutans, in vitro study, dentin.

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Photodynamic therapy is based on the use of photosensitizing agents, for instance, toluidine blue O (TBO), which are activated by irradiation with light at a specific wavelength to generate oxygen reactive species, including singlet oxygen and free radicals. These products are capable of damaging essential components of the cells or modifying metabolic activities in ways that may result in cell death.[1,2]

In this context, photodynamic therapy (PDT) may be a potential alternative approach for dentin disinfection of deep dentinal caries. The dentin carious lesion consists of two distinct areas:[3] an outer layer characterized by softened, wet, highly contaminated dentin and an inner layer that tends to be contaminated with a low number of microorganisms. This inner layer is thought to be susceptible to remineralization and may be preserved during cavity preparation,[4] which enables the

removal of less carious tissue and decreases the risk of pulp exposure.[5,6] However, distinguishing these layers in clinical settings is very difficult, and current methods of treating dentin lesions involve the removal of both dentin layers.[7] The amount of dentin tissue that must be removed or the number of microorganisms that can be left in the cavity without lesion progression has not been established.[8-12] Thus, the elimination of bacteria through application of photodynamic therapy inside the tubules and away from the remaining demineralized dentin[3] might contribute to the development of a more conservative approach to treat deep carious lesions.[13]

The antibacterial effect of PDT against oral microorganisms associated with dental caries has been previously shown.[7,14-17] This process achieved high bacterial kill of Streptococcus mutans in planktonic cells or biofilms, which demonstrated that PDT is a promising technique,[18-22] although the efficacy of PDT has not been shown when applied to multispecies biofilm.[17] However, studies of induced decontamination of different substrates are relevant because the penetration of a photosensitizer and light scattering are dependent on the illuminated medium. Consequently, the effect of PDT on oral microorganisms located in demineralized dentin may be reduced due to decreased penetration depth of the photosensitizer, diminished binding to bacterial cells, or attenuated light penetration for photoactivating the dye.[13,23]

In addition, a few studies have investigated the antimicrobial effects of photodynamic therapy on carious dentin tissue.[23-26] There are several limitations related to these studies, including the lack of control groups and innovative calculations to present the data,[24] as well as long irradiation times[25,26] that result in difficulties assessing the real effect of photodynamic therapy on dentin tissue and posterior application in clinical settings.

This study aimed to determine the necessary light-emitting diode (LED = 636 nm) parameters in combination with toluidine blue O (TBO) at two energy densities to reduce Streptococcus mutans counts in dentin caries.

MATERIALS AND METHODS

Experimental Design

For this study, 72 bovine incisors lacking previous lesions were used, and 10 bovine incisors were used for polarized light microscopy analysis.

For microbiological analysis, teeth were randomly allocated using the lottery method to six test groups with 12 experimental units per group. This study involved six set conditions that were denoted as follows: control (carious dentin exposed to 0.9% NaCl solution for 5 min), TBO (carious dentin exposed to TBO for 5 min), LEDA (carious dentin exposed to LED for 4.2 min with an energy dose of 166 J/cm^2), LEDB (carious dentin exposed to LED for 6.5 min with an energy dose of 249 J/cm^2), PDTA (carious dentin exposed to TBO and LED for 4.2 min with an energy dose of 166 J/cm^2), and PDTB (carious dentin exposed to TBO and LED for 6.5 min with an energy dose of 249 J/cm^2).

UV-visible Spectroscopy Analysis

Ultraviolet visible (UV-vis) optical absorption spectrometry was performed in TBO solution before and after irradiation using an HP 8453 system spectrophotometer (Hewlett-Packard; Palo Alto, CA, USA) to characterize the TBO absorption spectrum and correlate this absorption with the LED emission spectrum and TBO photodegradation. The temperature was maintained at 25°C, and 0.01% TBO (w/v) was diluted with distilled, deionized water (pH 7.2) into a quartz cell with a 1-mm light path. Fractionated irradiation was performed for 5 min, and spectra were obtained at 0, 5, and 10 min. The spectra were analyzed with Origin Lab 8.0 software (Origin Lab Corporation, Northampton, MA, USA).

Specimen Preparation

Teeth were stored in 0.01% (v/v) thymol solution at 4°C prior to use. From each tooth, one slab of bovine coronal dentin (4 x 4 x 2 mm^3) was obtained using a water-cooled diamond saw and a cutting machine (IsoMet Low Speed Saw, Buehler; Lake Bluff, IL, USA). The buccal dentin face was used in this study, and the remaining slab surfaces were covered with an acid-resistant nail varnish, resulting in a dentin surface area of 16 mm^2 that served as a microbial surface on which carious lesions were produced. The dentin slabs were fixed in the piston of 3-ml syringes that were attached to the lids of glass containers, immersed in sterile distilled water, and then sterilized by gamma radiation (Gammacell 220 Excel, GC-220E, MDS Nordion; Ottawa, Canada) with 14.5 kGy for 30 h.[27]

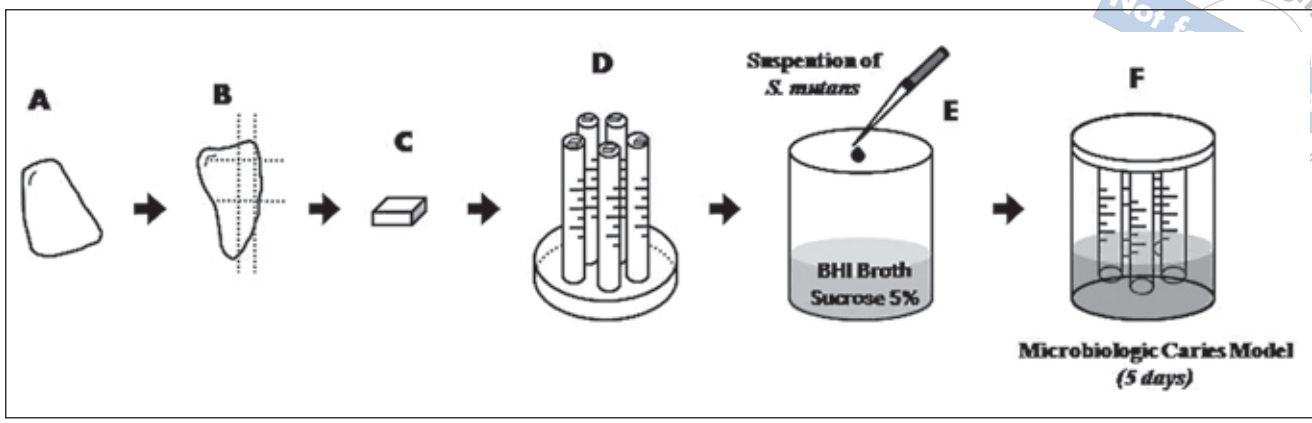

Fig 1 Schematic diagram illustrating preparation of dentin slabs, and representation of the devices used in the model to produce carious lesions in vitro. (A) sound bovine incisor; (B) cutting the slabs; (C) slab preparation; (D) fixation of the slabs in the device; (E) inoculation of the culture broth; (F) microbiological caries model (5 days).

Microbiological Caries Model

The microorganism S. mutans UA 159 was used as a model in these studies.[20] To prepare the inoculum, S. mutans was first grown in an overnight culture of BHI (Bacto Brain Heart Infusion, BD; Franklin Lakes, NJ, USA) using candle-extinguish jars with a 5 to 10% carbon dioxide atmosphere. After sterilization, the dental slabs were removed from distilled water and immersed in sterile BHI containing 5% sucrose (w/v). All BHI-containing recipients were inoculated with 80 µl of 1-2 x 10^8 CFU/ml overnight cultures of S. mutans. A specific optical density was determined using a spectrophotometer and used for all samples to adjust the inoculum to the same cell number. Inoculation of each BHI-containing recipient was performed on the first day, and the dentin specimens were transferred daily to fresh medium for 5 days. Figure 1 is a schematic representation of these stages. In addition, each BHI-containing recipient was streaked daily onto a fresh BHI agar plate that had been incubated at 37°C in an atmosphere of 10% CO_2 for 24 h to check for purity.

After lesion production, 10 dentin slabs were cut with a Series 1000 Deluxe Silverstone-Taylor hard tissue microtome (Sci Fab; Littleton, CO, USA) in the middle of the exposed dentin window to obtain 200 µm sections. These sections were then polished with 600- and 1200-grit abrasive paper to obtain sections of 100 ± 20 µm thickness. The sections were imbibed with water and observed with a Leica DMLP polarized-light microscope (Leica Microsystems; Wetzlar, Germany) coupled to a Leica FFC 280 digital system. Standard photomicrographs at 10X magnification were taken.

Treatments

After 5 days, the biofilm formed over the slabs was removed with a #15 scalpel blade, and the carious dentin was exposed. A sample of carious dentin was then collected to obtain a baseline value for tissue contamination, and the treatment for each group was performed. The TBO, PDTA, and PDTB groups were incubated with 5 µl of TBO in the dark for 5 min (pre-irradiation time) without light exposure.[28,29] The control, LEDA, and LEDB groups were incubated with an equal volume of sterile 0.9% NaCl solution instead of TBO during the same period of time.

The photosensitizer TBO (Sigma-Aldrich; St Louis, MO, USA) was dissolved in deionized water to obtain a final concentration of 0.1 mg/ml and subsequently kept in the dark. Toluidine blue O (TBO) is a well-known blue dye. The light source used was a red-light emitting diode (MMO; São Carlos, SP, Brazil), with a predominant wavelength of 636 nm in the spectrum of emission. A spot with a 3.5 mm cylindrical tip distributed the light. Irradiation was performed in non-contact mode with a diffused beam at a working distance of 3.0 mm. A power meter (Lasermate, Coherent; Santa Clara, CA, USA) was used to measure the peak power, and a maximum output power of 80 mW was determined.

For the LEDA and PDTA groups, slabs were irradiated under stable irradiation power (80 mW) for 4.2 min. Irradiation time was 6.5 min for the LEDB and PDTB groups. Incident energy doses of 166 and 249 J/cm^2 were obtained for groups A and B, respectively. The LED equipment presented no possibility of vary-

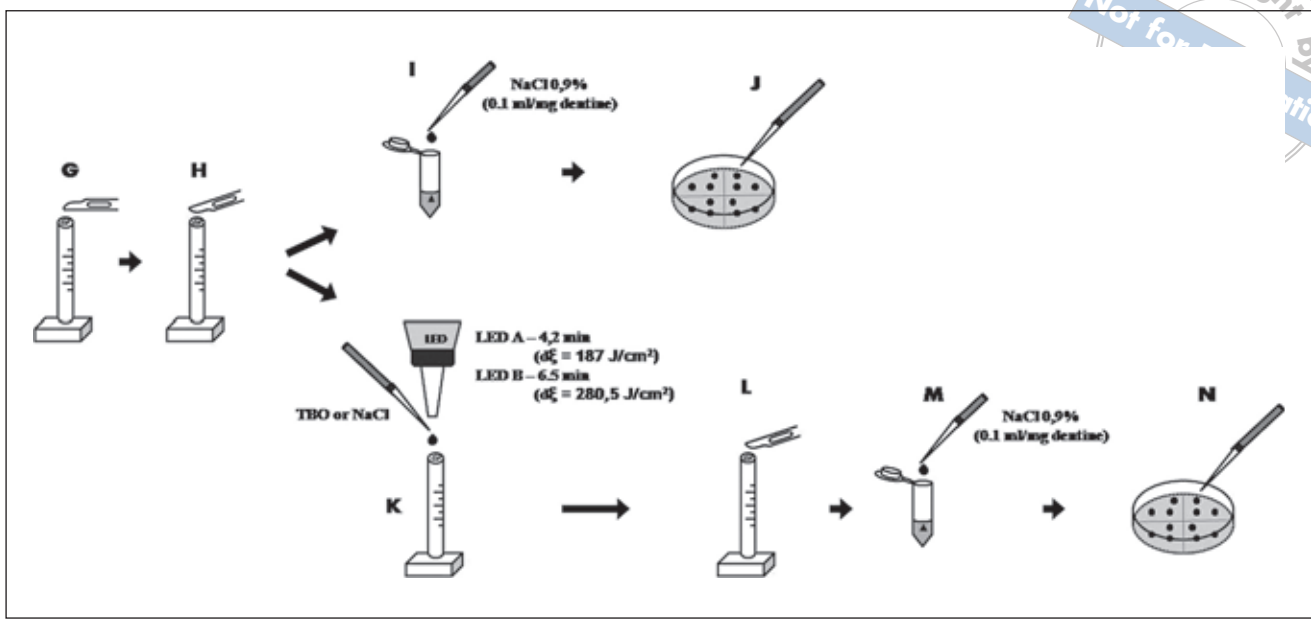

Fig 2 Schematic diagram illustrating collection of carious dentin, application of treatments, and microbiological analysis. (G) biofilm removal; (H) baseline sample collection; (I) adding NaCl solution to baseline dentin collected and weighed; (J) microbiological analysis; (K) treatments; (L) collection of post-treatment sample; (M) adding NaCl solution to post-treatment dentin collected and weighed; (N) post-treatment microbiological analysis.

ing the output power. Figure 2 is a schematic representation of the stages of treatment application and microbiological analysis.

Microbiological Analysis

For microbiological analysis before and after the treatments, carious dentin was collected from different portions of each slab using a #15 scalpel blade. The dentin samples were weighed in microcentrifuge tubes, and 0.9% NaCl solution was added to the sample (0.1 ml/mg dentin). The tubes were sonicated for 15 s in an ultrasonic processor (Hielscher, UP400S; Ringwood, NJ, USA) to detach the bacterial cells. Subsequently, the suspension was serially diluted (1:10 to 1:100,000) with 0.9% NaCl solution. Samples were plated in triplicate on Mitis Salivarius plus bacitracin (Difco, Mitis Salivarius Agar, BD) and incubated for 48 h at 37°C using candle-extinguishing jars under a 5 to 10% carbon dioxide atmosphere. Representative colonies with the typical morphology of S. mutans were counted using a colony counter (Phoenix, CP600 Plus; Araraquara, SP, Brazil). The log reduction results were calculated by subtraction of the initial from the final CFU/mg values after being $\log_{10}$ transformed.

Statistical Analysis

For assessment of treatment effects, the dependent variable log reduction was analyzed, and the equality of variance assumptions (Levene Test) and normal distribution of errors (Shapiro Wilks test) were verified. The data were analyzed with one-way ANOVA followed by the Tukey-Kramer test. The significance level was set at 5%. The software BioEstat 5.0 2007 (Instituto de Desenvolvimento Sustentável Mamirauá; Belém, PA, Brazil) was used.

RESULTS

UV-vis Spectroscopy

Figure 3 illustrates the emission spectrum of red LED, and Fig 4 illustrates the aqueous TBO absorption spectrum. A strong absorption band of TBO between 550 to 680 nm and an absorption peak at 632 ± 8 nm were observed. Additionally, Fig 5 shows a decrease in TBO absorption as a function of irradiation time.

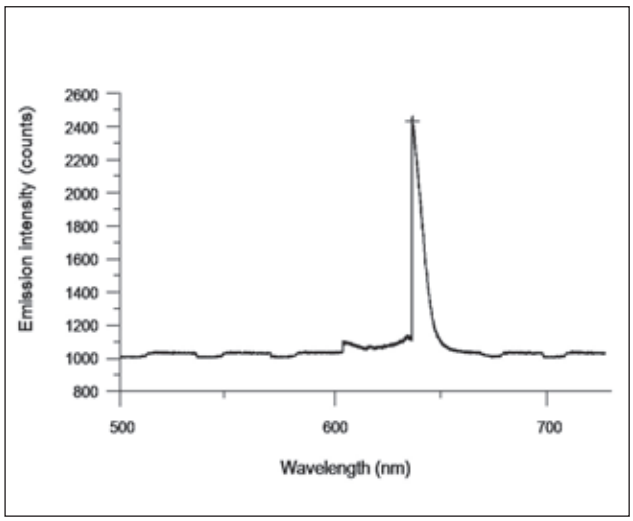

Fig 3 Emission spectrum of the red LED.

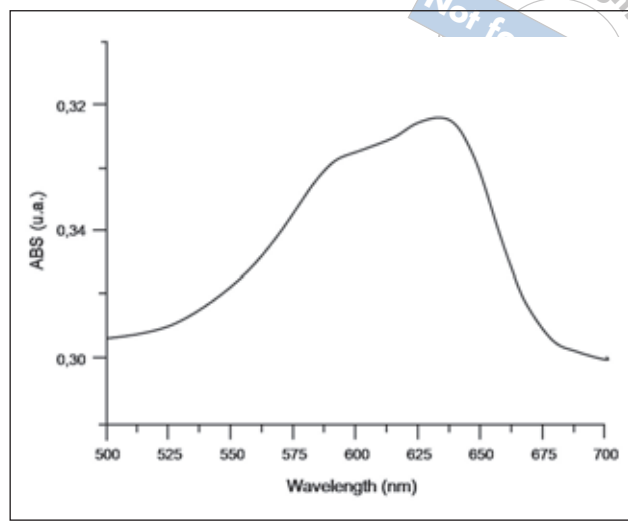

Fig 4 Absorbance spectrum of the TBO-water solution.

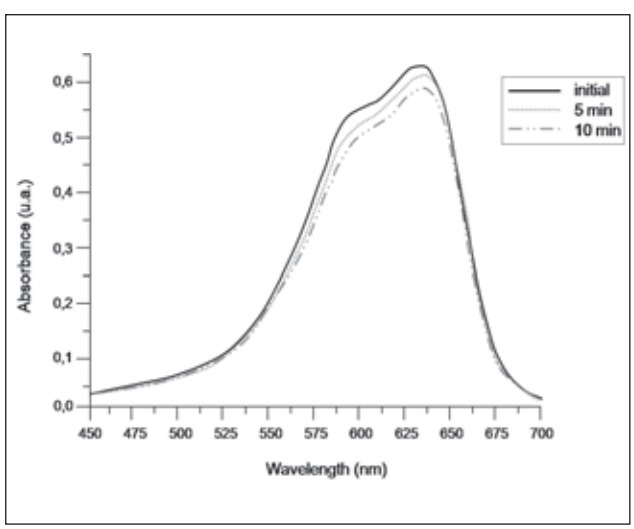

Fig 5 Absorption spectra after irradiation with the red LED (λ = 636 nm). Spectroscopic measurements of LED irradiation were collected every 5 minutes.

Fig 6 Cross section of representative artificial carious lesion in bovine dentin viewed under polarized light in water.

Microbiological Analysis

The weight of dentinal tissue collected from the slabs was 3.19 ± 0.97 mg (mean ± SD).

The mean lesion depth produced by the microbiological model used in this experiment was 253.7 ± 40.66 µm. Figure 6 shows the carious lesion image captured by polarized light microscope.

Table 1 shows the log reduction (mean ± standard deviation) achieved for each treatment and the statistical difference among groups. The log reduction values ranged from -1.63 to 2.43. The TBO group presented the lowest value, while the PDTB group exhibited the highest log reduction.

Table 1 Log reduction of S. mutans (mean ± standard deviation) achieved by each treatment

Group	Log reduction
Control	-0.04 ± 0.4[c]
TBO	-0.17 ± 0.76[c]
LEDA	0.07 ± 0.94[c]
LEDB	0.29 ± 0.92[bc]
PDTA	1.17 ± 0.64[ab]
PDTB	1.12 ± 0.97[ab]

Means followed by different superscript letters differ significantly (one-way ANOVA and Tukey-Kramer test, $\alpha = 0.05$).

PDTA and PDTB groups presented statistically significant log reductions compared to the control, TBO ($p < 0.01$), and LEDA ($p < 0.05$) groups but did not differ from each other. No difference was found among the control, TBO, and LEDA groups.

In contrast, LEDB treatment had an effect on bacterial reduction in dentin caries even in the absence of sensitizer, because log reduction data obtained with this group did not statistically differ from the PDTA and PDTB groups.

DISCUSSION

As shown in the UV-vis spectroscopy analyses, there is a match of emission and absorption spectra between LED and TBO that follows the same pattern as the majority of other light sources that generate singlet oxygen. Therefore, the use of an LED source is advantageous considering that the best previous results were obtained using conventional lasers to perform this therapy, although the power output can still be a limiting factor in their widespread application.[30,31] The use of this experimental methodology may result in technological simplification and lower treatment cost in comparison to complex laser systems. Moreover, LED sources provide more than a monochromatic form of irradiation, which is a special characteristic of these light sources compared to lasers that increase the overlay of the spectrum of LED irradiation and the light absorption by TBO. Parameters must still be standardized to perform dentin disinfection by photodynamic therapy using LED sources, which may contribute to a more conservative approach for dealing with deep caries lesions.

According to our results, the PDT at 166 and 249 J/cm^2 (PDTA and PDTB, respectively) was capable of reducing S. mutans in carious dentin. These results confirm several studies demonstrating that cariogenic bacteria in culture baths and biofilms are susceptible to PDT.[7,20,22,28,32] However, few studies have investigated the use of this approach on different substrates. Recent investigations have evaluated the anti-bacterial effectiveness of photodynamic therapy for dentin decay.[23-26] Although these studies have demonstrated promising results for using photodynamic therapy to treat dentin, there have been limitations to these investigations, such as the absence of required control groups, utilization of different calculations to verify efficacy,[24] and long irradiation times.[25,26]

Our research demonstrated that S. mutans hosted in carious bovine dentin is susceptible to photodynamic therapy using TBO and a LED source at energy densities of 166 and 249 J/cm^2. These energy densities were achieved using irradiation times of 4.2 and 6.5 min, respectively, while the previous studies used 10 min[25] to reach 94 J/cm^2 or 15 min[26] to reach 144 J/cm^2. Considering the penetration of the photosensitizer and light scattering into dentin tissue, parameters must be standardized to reduce the irradiation times and increase the energy densities emitted by the LED sources.

The results of the present study agree with those of Giusti et al,[24] Lima et al,[25] Melo et al,[26] and Williams et al,[23] which demonstrated an antibacterial effect of PDT against oral bacteria in dentin carious lesions. However, our results showed a lower log reduction of S. mutans when compared to recent in situ and in vitro studies performed with human dentin.[25,26] The decreased effectiveness of PDT in the present study might be due to the different methods used for dentin collection because a scalpel blade was used to collect dentinal tissue, while previous studies used carbide burs in a low-speed drill. Accordingly, a deeper dentin layer may have been removed in this study, which would be less contaminated with bacteria compared to the outer layer removed by Lima et al[25] or Melo et al.[26]

Log reduction analysis was performed to compare the efficacy of photodynamic therapy in reducing S. mutans counts among different treated groups and confirmed that a treatment of 6.5 min irradiation (LEDB group) was able to reduce S. mutans in carious dentin. This photocytotoxicity effect may be partially explained by the fact that bacteria hosted in dentin were not protected from dryness by the presence

of the polysaccharide matrix and higher amounts of water. These results agree with those found in previous studies,[25,26] because a cytotoxic light effect was also found when the LED was used without TBO at 94[25,26] or 144 J/cm^2,[26] which demonstrates that the isolated effect of red light on S. mutans should be further investigated.

A surprising result of the current study was that the increase in energy density did not exhibit any beneficial effect in reducing the S. mutans count after PDT; no numerical or statically significant difference was found between the PDTA and PDTB groups. These results are supported by a recent in situ study.[25] Giusti et al[24] found a higher level of S. mutans inhibition under the highest energy density. However, the latter study was performed using 48 J/cm^2. Therefore, PDT in the present study may have achieved a threshold antibacterial effect because very high energy densities were used. Further investigation must be performed considering that the light source and the photosensitizer were applied under the same characteristics for the two groups. Consequently, only the irradiation time changed the energy densities. The number of S. mutans CFU before irradiation was approximately 2.43 x 10^7. Although PDT reduced the bacterial number, the CFU still remained high, as the caries formation process could not be controlled.

CONCLUSION

The use of LED combined with TBO was effective in reducing the Streptococcus mutans counts in carious dentin, but this effect may not have clinical significance.

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