Antimicrobial Photodynamic Action on Dentin
Using a Light-Emitting Diode Light Source

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Abstract

Objective: The aim of this study was the evaluation of two different photosensitizers activated by red light emitted by light-emitting diodes (LEDs) in the decontamination of carious bovine dentin.

Materials and Methods: Fifteen bovine incisors were used to obtain dentin samples which were immersed in brain-heart infusion culture medium supplemented with 1% glucose, 2% sucrose, and 1% young primary culture of Lactobacillus acidophilus 10⁸ CFU/mL and Streptococcus mutans 10⁸ CFU/mL for caries induction. Three different concentrations of the Photogem solution, a hematoporphyrin derivative (1, 2, and 3 mg/mL) and two different concentrations of toluidine blue O (TBO), a basic dye (0.025 and 0.1 mg/mL) were used. To activate the photosensitizers two different light exposure times were used: 60 sec and 120 sec, corresponding respectively to the doses of 24 J/cm² and 48 J/cm².

Results: After counting the numbers of CFU per milligram of carious dentin, we observed that the use of LED energy in association with Photogem or TBO was effective for bacterial reduction in carious dentin, and that the greatest effect on S. mutans and L. acidophilus was obtained with TBO at 0.1 mg/mL and a dose of 48 J/cm².

It was also observed that the overall toxicity of TBO was higher than that of Photogem, and that the phototoxicity of TBO was higher than that of Photogem.

Conclusion: Based on our data we propose a mathematical model for the photodynamic effect when different photosensitizer concentrations and light doses are used.

Introduction

PHOTODYNAMIC THERAPY (PDT) is a technique that involves the activation of certain dyes (photosensitizers) by light in the presence of tissue oxygen, resulting in the production of reactive radicals capable of inducing cell death. Until recently, the main application of PDT was to treat malignant and some benign lesions;1,2 however, the efficacy of this procedure for microbial reduction has been demonstrated in several studies.3–6 This emerging class of applications, which we refer to as antimicrobial photodynamic action (APDA), shows great potential.7–10

Currently, lasers are the most common light sources used to activate the photosensitizers. However, the development of bright light-emitting diodes (LEDs) provides an alternative light source for PDT.11–13 The light produced by an LED has characteristics different from those of laser light: it is a narrow-band non-coherent type of energy that is not diffraction-limited. In this respect it is similar to the light of a suitably filtered mercury lamp. LED devices have, in general, a lower cost and simpler technology compared to other laser devices, and moreover, they can be readily assembled in several configurations that are suitable for different types of anatomical illumination.

Studies from the 1990s have shown that a large number of types of oral bacteria, including periodontal pathogenic and cariogenic bacteria, are susceptible to photodynamic therapy.14–18 However, the use of LED-based light sources to activate photosensitizers for use against oral microorganisms is a recent development. Study of their use is important to promote the clinical use of LEDs instead of lasers for therapeutic applications in dentistry.19

Our study evaluated the efficiency of APDA using two different photosensitizers activated by LED energy for de-
Materials and Methods

Fifteen bovine incisors without any visible structural anomalies were used in this study. The periodontal tissue was scaled and the buccal enamel was removed using a conical diamond bur in a high-speed handpiece. From each tooth, four dentin fragments were obtained using a titanium trephine drill. Each dentin fragment was air-dried for 60 sec and weighed; the average weight was 23 mg. The fragments were autoclaved for 20 min at 121°C and transferred to individual test tubes, each containing 2 mL of a brain-heart infusion (BHI) culture medium 1 g/100 mL supplemented with glucose and 2 g/100 mL sucrose PA (Pro-analysis). For each 50 mL of medium solution, 5 mL of 10⁸ CFU/mL Lactobacillus acidophilus (ATCC #11842), and 5 mL of 10⁸ CFU/mL Streptococcus mutans (ATCC #33465) were added to induce bacterial colonization. The test tubes were maintained in a micro-aerobic environment at 37°C for 14 d, with the solution changed every 48 h. Following the 14-day period, the specimens were maintained under refrigeration at 4°C until treatment.

The specimens of the experimental groups were washed in sterile saline solution and then immersed for 60 sec in a solution containing either Photogem (Photogem, Moscow, Russia), a hematoporphyrin derivative, or toluidine blue O (TBO) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a basic dye. Three different concentrations of Photogem solution, 1 mg/mL, 2 mg/mL, and 3 mg/mL, and two different concentrations of TBO, 0.025 mg/mL and 0.1 mg/mL, were used. These solutions were prepared with distilled water at pH values of 7.4 for Photogem and 5.1 for TBO, as determined in previous studies using these photosensitizers.

A homemade light source based on an LED emitter (LXHL-PD01; Luxeon, San Jose, CA, USA) for photosensitizer activation was developed. The generated light was expanded, resulting in SF (D/H₁₀₀⁵) complete elimination of the colonies (SF = 0). These two limits are incorporated into equation [1], for the dependence of SF upon the drug concentration when no light is present.

\[ SF(D = 0) = \frac{1}{1 + \frac{C}{C_0}} \]  

For very small C/C₀, this expression can be mathematically expanded, resulting in SF (D = 0) = 1 - C/C₀, which implies a linear toxicity effect proportional to the concentration used. Here D is the fluence (in this case, zero), C is the concentration expressed in milligrams per milliliter, and C₀ is a constant.
constant related to the dark toxicity of the photosensitizer. The value of $C_0$ can be determined through a mathematical adjustment of equation [1] using experimental data for each photosensitizer used. $C_0$ is expected to depend intrinsically upon the photosensitizer.

Since the presence of light amplifies the toxic effect of the drug, we expect the toxicity to increase with increasing light dose. We added a term to equation [1] that represents the enhancing effect of the light, resulting in equation [2]:

$$SF(D, C) = \frac{1}{1 + \frac{C}{C_0} G \left( \frac{D}{D_0} \right)}$$

[2]

Here the function $G(D/D_0)$ must have the property of increasing value with $D$, and having the value of unity at $D = 0$. The simplest function that can be represented is $G(D/D_0) = 1 + D/D_0$, where $D_0$ is a constant related to the phototoxicity, and it was again determined by a linear regression fitting the experimental data. A higher value means that a higher light dose is necessary to kill the microorganisms. Alternatively, lower values of $D_0$ imply a stronger effect of light in the APDA application. So, finally another expression can be defined, equation [3]:

$$SF(D, C) = \frac{1}{1 + \frac{C}{C_0} \left( 1 + \frac{D}{D_0} \right)}$$

[3]

Results

The results obtained for the SF are shown in Fig. 2, where each data point is the average of three specimens. The error bars correspond to the standard deviation of the data from the average. The solid lines correspond to the best adjustment for the data using equation [2]. Fig. 2A and D (dark toxicity) show that the concentrations of photosensitizers used produced toxicity at different levels. Equation [2] can be adjusted to the experimental data resulting from $D = 0$, to $C_0 = 0.845 \text{ mg/ml}$ for Photogem and $C_0 = 0.127 \text{ mg/ml}$ for TBO. This indicates that TBO has a higher dark toxicity. Exposure to LED light at the dose of 24 J/cm² resulted in a smaller SF, as shown in Fig. 2B and E. Taking into consideration the value of $C_0$, equation [2] can be compared with the data, and the best agreement is obtained for $D_0 = 9.923 \text{ J/cm²}$ for Photogem and $D_0 = 2.081 \text{ J/cm²}$ for TBO. Since higher values of $D_0$ mean lower phototoxicity, TBO shows greater phototoxicity than Photogem.

It is possible to generate from equation [2] plots relating the concentration of Photogem or TBO to the light dose needed to achieve different levels of SF (Fig. 3A and B). The plots are divided into regions separated by various curves. Region A1 corresponds to the light dose and drug concentration required to produce a SF $< 0.01$; A2 yields $0.01 < SF < 0.05$; A3 yields $0.05 < SF < 0.1$, and A4 yields $0.1 < SF < 1$. Using these plots, one can determine the proper combination of photosensitizer concentration and light dose to kill $90\%$, $95\%$, or $99\%$ of the microorganisms, corresponding to SF values of $0.1, 0.05$, or $0.01$, respectively.

The solid lines in Fig. 2 correspond to the best mathematical adjustment using equation [3], to obtain optimal values of $C_0$ and $D_0$ for each photosensitizer tested.

Discussion

Photodynamic action has been used to kill oral microorganisms since the beginning of the 1990s, when studies demonstrated that some photosensitizers show an affinity for bacterial walls and can be photoactivated to cause the desired damage. Excited photosensitizer molecules can transfer energy to nearby molecules, resulting in the formation of reactive molecules as singlet oxygen, superoxide, and other free radicals, capable of causing damage and even death of cells and bacteria. Studies of biofilmshave shown the efficacy of TBO at concentrations of 0.01% and 0.1% when illuminated with HeNe laser energy, for reduction of the numbers of Streptococcus sanguis, Porphyromonas gingivalis, Actinobacillus actino- myctetemcomitans, and Fusobacterium nucleatum. An apparent bactericidal effect was observed after exposure to laser energy for 30 sec, with a light dose of 219 mJ and fluence of 16.5 J/cm². Burns et al., in 1993, observed that when a suspension of cariogenic bacteria (Streptococcus mutans, S. sobrinus, Lactobacillus casei and Actinomyces viscosus) was mixed with TBO at 50 µg/mL and exposed to 7.3 mW of HeNe laser energy, a considerable amount of cellular death was accomplished with a fluence of 33.6 J/cm². In the case of TBO, the results of our study are similar to those of Burns et al. If equation [2] is used with $C_0$ and $D_0$ values determined for TBO from our data (0.13 mg/mL and 9 J/cm², respectively), then $C = 0.05 \text{ mg/mL}$ and $D = 34 \text{ J/cm²}$, and an SF of $5.6 \times 10^{-2}$ is obtained for S. mutans. This is a much lower value than the reduction of viability observed by Burns et al. This difference arises mainly from intrinsic differences between the two light sources used. While Burns et al. used 500 mW/cm² of light centered at 632 nm, our experiment utilized approximately 400 mW/cm² distributed in a range from 615–638 nm, covering a much broader spectral band, and possibly influencing the resulting bactericidal effect.

Okamoto et al. also reported the death of various species of Streptococcus using a HeNe laser dose of 720 mJ and a fluence of 5.7 J/cm² with TBO at 7.5 µg/mL.

In 2005 Zanin et al. evaluated the antimicrobial effect of TBO in combination with either a HeNe laser or a LED, on the viability and architecture of Streptococcus mutans biofilms and observed that the bactericidal effect was light dose-dependent, an effect we also observed in this study. The reductions in viability up to 99.99% with both light sources were observed using energy densities between 49 and 294 J/cm², a pre-irradiation time of 5 min, and TBO concentration of 100 mg/L.

Other studies, including the one developed by Venezio et al. in 1985, demonstrated that hematoporphyrin
FIG. 2. (A and D) Dark toxicity of the photosensitizers. APDA results as evaluated by SF for 24 J/cm² (B and E) and 48 J/cm² (C and F) of laser irradiation.
derivatives show a bactericidal effect on *S. mutans* and other microorganisms.\(^{20,24,30}\) Additionally, hematoporphyrin derivatives were the first drugs authorized by the U.S. Food and Drug Administration for clinical use in PDT.\(^{7}\) Their cytotoxic effect is mainly carried out by the production of singlet oxygen. Gram-negative bacteria seem to be more resistant to this treatment, probably due to their more complex cell wall.\(^{26,31}\)

Figures 2A and D show the dark toxicity of Photogem and TBO. The toxicity of both photosensitizers in the absence of light increased with increasing concentration, but the effect is more pronounced for TBO. Figures 2B, E, C, and F show the phototoxicity of the photosensitizers activated with LED light with doses of 24 and 48 J/cm\(^2\). These graphs also indicate a higher phototoxicity of TBO. Malik et al.\(^{24}\) and Dobson and Wilson,\(^{15}\) using hematoporphyrin at 0.005% and a HeNe laser emitting 7.3 mW at 5.5 J/cm\(^2\), did not report any favorable results for gram-negative microorganisms, although Wilson et al.\(^{22}\) noticed lethal photosensitization of *P. gingivalis* with hematoporphyrin when these microorganisms were in suspension, but not in biofilms.

Many studies have shown TBO’s effectiveness as a photosensitizer, but were performed with bacteria in suspension.\(^{5,8,13,16,17,22}\) However, similar studies demonstrated that the reduction of *S. mutans* in carious dentin was less than that seen in suspension, and was also less than that attained in a collagen matrix. In that study, it was concluded that the time of contact between the bacteria and TBO was a critical factor.\(^{32}\)

In 2003, Williams et al.\(^{13}\) noted 100% death of *S. mutans* in a plankton suspension, using a diode laser emitting at 633 ± 2 nm with fluences ranging from 0.4–4.8 J with TBO as the photosensitizer. In 2004 the same authors\(^{32}\) using the same light source, with fluences varying from 1.8–14.4 J and TBO at 10 \(\mu g/mL\) obtained a reduction of *S. mutans* in a collagen matrix and carious human dentin, although the dentin results were more variable than those observed in the collagen matrix. Those results are similar to the results found in our study, although the presence of the biofilm on the dentin itself negatively affect the efficacy of the technique, because the success of the process depends on photosensitizer diffusion throughout the dentin, and on light penetration and scattering in the tissue.

We believe that an important contribution of our study is the use of the empirical mathematical equation [2] relating the light dose, photosensitizer concentration, and bactericidal effect. Our experimental results indicate that there was substantial reduction at all investigated parameters using the photosensitizers Photogem and TBO for treatment of *S. mutans* and *L. acidophilus*. Equation [2] allows the extrapolation of our results to parameters not yet investigated. In Fig. 3A and B we plot the SF determined by equation [2] as a function of light dose for different concentrations of Photogem and TBO. We note the general effect that toxicity, with and without exposure to light, increases with increasing concentration of photosensitizer. In addition, it is apparent that light alone, without any photosensitizer, has no bactericidal effect. The effect for a large range of parameters is more evident in Fig. 2A and B, where we can readily determine the combination of light dosage and photosensitizer concentration to achieve the desired level of bacterial reduction.

**Conclusion**

In conclusion, we have shown that the use of LED light in association with Photogem or TBO was effective for bacterial reduction of *S. mutans* and *L. acidophilus* in carious dentin. The greatest toxic photodynamic effect was obtained with TBO at 0.1 mg/mL and 48 J/cm\(^2\) of illumination. It was also observed that the dark toxicity of TBO is higher than that of Photogem. Similarly, the phototoxicity of TBO is higher than that of Photogem.
We used an empirical mathematical expression to correlate the measured bactericidal effect with drug concentration and fluence. The use of such a formula provides a great deal of data about the expected results for a large variety of parameters.

This expression may represent a simple tool to evaluate the efficacy of APDA for different microorganism species, and this concept may be used in the dosimetry planning of future antimicrobial clinical applications. The introduction of constants that quantify dark toxicity and phototoxicity is a good way to compare different photosensitizers operating under equivalent conditions.

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References
