

ORIGINAL RESEARCH

Comparative Effects of Photodynamic Therapy mediated by Curcumin on Standard and Clinical Isolate of *Streptococcus mutans*

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ABSTRACT

Aim: The aim of this study was investigate the effect of photodynamic therapy (PDT) using curcumin (C) as a photosensitizing agent irradiated with an LED (L) in the blue wavelength as a light source on a standard and clinical isolate of *Streptococcus mutans* (*S. mutans*) in a planktonic suspension model.

Materials and methods: Suspensions of both strains were divided into 4 groups as follows: absence of C and L (control group: C–L–), with C and without L (C group: C+L–), absence of C with L (L group: C–L+) and presence of C and L (PDT group: C+L+). Three different concentrations of curcumin (0.75 mg/ml, 1.5 mg/ml and 3 mg/ml) and three light fluences of studied light source (24, 48 and 72 J cm⁻²) were tested. Aliquots of each studied group was plated in BHI agar and submitted to colony forming units counting (CFU/ml) and the data transformed into logarithmical scale.

Results: A high photoinactivation rate of more than 70% was verified to standard *S. mutans* strain submitted to PDT whereas the clinical isolate showed a lower sensitivity to all the associations of curcumin and LED. A slight bacterial reduction was verified to C+L– and C–L+, demonstrating no toxic effects to the isolated application of light and photosensitizer to both *S. mutans* strains tested.

Conclusion: Photodynamic therapy using a combination of curcumin and blue LED presented a substantial antimicrobial effect on *S. mutans* standard strain in a planktonic suspension model with a less pronounced effect on its clinical isolate counterparts due to resistance to this alternative approach.

Clinical significance: Alternative antimicrobial approaches, as photodynamic therapy, should be encouraged due to optimal results against cariogenic bacteria aiming to prevent or treat dental caries.

Keywords: *Streptococcus mutans*, Photochemotherapy, Curcumin.

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INTRODUCTION

It is known that *Streptococcus mutans* (*S. mutans*) is a substantial part of the dental plaque microbiota and its importance in the dental caries etiology.¹

The mechanical removal of dental plaque is a fundamental aspect to decrease the levels of pathogenic microorganisms involved with the progression of this oral disease.² However, bacterial reservoirs can remain on tooth surface, since the dental plaque debridement is dependent of dexterity and compliance of the individuals.³ Thus, adjunctive methods are encouraged to control bacterial proliferation in the mouth environment, such as antimicrobial chemical substances.

Chlorhexidine has a greater *in vivo* immediate antibacterial effect and a greater substantivity than other antiseptics used in the oral cavity.⁴ However, probably due to difficulties in obtaining a long and significant decrease in the *S. mutans* resident cells after chlorhexidine regimen,⁵ the indiscriminate use of this substance can generates some side effects as alteration in taste, teeth and restorations staining, burning sensation and bacterial resistance.⁶

In the search for new strategies against microorganisms, photodynamic therapy (PDT) has been suggested as an alternative to chemical antimicrobial agents to avoid bacteria accumulation on dental substrata.⁷

Photodynamic therapy is a therapy modality that employs the combination of visible light, a drug (called

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photosensitizer – PS) and molecular oxygen present in the tissue. The used PS binds to the target cell and can be activated by light of a suitable wavelength.⁸ During this process, there is a production of free radicals and different reactive oxygen species (ROS), such as singlet oxygen that will lead a sequence of biological events resulting in microorganisms death.⁹ PDT represents an alternative antibacterial,¹⁰ antifungal,¹¹ and antiviral treatment for drug-resistant organisms.¹² It is not likely that bacteria would develop a resistance to the cytotoxic action of singlet oxygen or free radicals, due to different ROS action sites of damaged cells.⁹

Nowadays, there is a trend on the use of natural products in PDT field, since the PS used present high costs related to purification process (Photogem® and Photosan®)¹³ and due to possibility of tooth staining (toluidine blue O, methylene blue and malachite green).¹⁴

Curcumin, the major yellow pigment extracted from turmeric plant, is pointed out as a potent photoactivatable substance by recent investigations.¹⁵ This dye is commonly used as spice in traditional eastern cookery and exhibit a variety of pharmacological properties such as antitumor, anticancer, anti-inflammatory, antioxidants and antimicrobial activities, some of which could be enhanced by light application.¹⁵ In addition, curcumin presents a broad absorption peak in the 300 to 500 nm, which coincides with the blue light emission range.¹⁶

Several studies concerning PDT and curcumin present a high photoinactivation in cariogenic bacteria,¹⁵ candida species¹¹ in planktonic suspension models¹⁵ and on its biofilms counterparts.¹⁷ Although the literature have demonstrated positive outcomes related to effectiveness of PDT on laboratory strains, investigations regarding clinical isolates are able to demonstrate, with more reliability, the behavior of antimicrobial approaches close to clinical conditions.

Therefore, the aim of this present investigation is compare the effectiveness of PDT mediated by different curcumin concentrations irradiated with a blue LED at different dosimetries on *S. mutans* standard and a clinical isolate in a planktonic suspension culture. The null hypothesis formulated was that there are no significant differences between the tested groups in comparison with control groups.

MATERIALS AND METHODS

Light Source and Photosensitizer

A blue light-emitting diode (Edixeon, Edison Opto Corporation, New Taipei City, Taiwan) with a power intensity of 240.1 mWcm⁻² was used. This light provided

an absorption spectra with a central wavelength at 450 nm \pm 30 and the irradiances (energy fluency) tested were 24, 48 and 72 J cm⁻² following a formula used by a previous study.¹⁸ The work distance to achieve the desired power intensity was fixed at 5 mm (distance between the light source and bacterial samples).

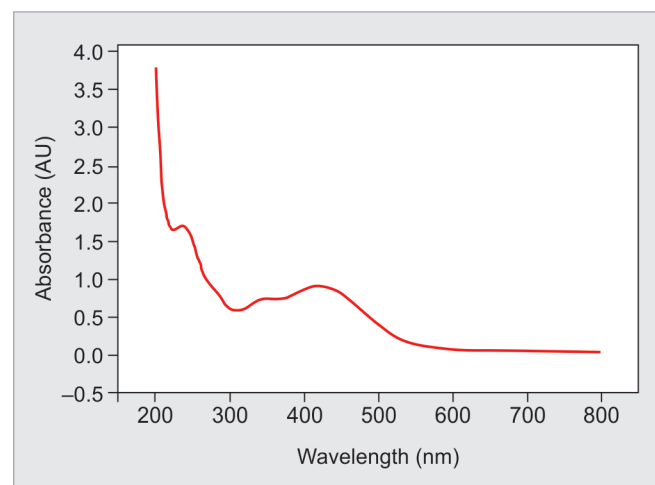
A solution of curcumin was prepared by PDT Pharma (Cravinhos – SP, Brazil) and dissolved in sterile distilled water to obtain the tested stock solutions at 0.75 mg/ml, 1.5 mg/ml and 3 mg/ml. The ultraviolet spectra of curcumin presented a maximum absorption at 430 nm, spectral region that absorbs efficiently the delivered light (Graph 1).

Preparation of the Microorganisms

A standard suspension of *S. mutans* (strain ATCC 25175) was inoculated in brain heart infusion broth (BHI, Becton, Dickinson Company, Sparks, MD USA) and incubated for 18 hours at 37°C under micro-aerophilic condition (10% of CO₂). This bacterial culture was then centrifuged at 3000 RPM for 5 min and the supernatant was discarded. Then, the cell pellet was re-suspended in 5 ml of sterile solution of 0.9% sodium chloride (NaCl).

The cell numbers were measured by means of a spectrophotometer (wavelength, at 600 nm using a 0.15-0.20 optical density unit equals $\sim 10^7$ cells ml⁻¹) in 1 ml cuvettes.

To clinical isolate sample, a patient presenting dental caries history attending at Pediatric Dentistry Clinic of Araraquara Dental School was elected. To participate in this present investigation both patient and respective guardians signed an informed consent (Proc. #54/10). A pooled plaque sample was collected with a sterile wood stick, transferred to a tube containing BHI and incubated by the same above conditions. A diluted sample was plated in agar plate and colonies similar to morphological



Graph 1: Absorption spectrum of curcumin dye

aspects of *S. mutans* were isolated, reincubated and re-plated to verify the growth of this specific bacterium. To confirm this finding, the colonies were analyzed by microscopic technique and Gram test. After this, *S. mutans* clinical isolate was submitted to same procedures to standardize the number of viable cells.

Photodynamic Therapy Application

Aliquots of 500 µl of both standard and clinical *S. mutans* suspensions were individually transferred to separate wells of a 24-well culture cell plate. On the day of the experiment, the curcumin solution was diluted in sterile saline concentrations and kept in the dark. An equal volume (500 µl) of each standardized micro-organisms were added to the wells to give final tested concentrations at 0.75 mg/ml, 1.5 mg/ml and 3 mg/ml. After dark incubation for 60 seconds, pre-irradiation time, the wells were illuminated by a blue LED device for 100, 200 and 300 seconds (corresponding to 24, 48 and 72 J cm⁻², respectively) (treated with curcumin and LED – PDT group: C+L+). To determine whether curcumin alone induced any toxic effects on bacterial viability, additional wells containing the bacteria suspension were exposed to curcumin under identical conditions to those described above, but not exposed to LED (treated only with curcumin: Group C+L-). Exposing cells to irradiation determined the isolated effect of LED with no previous exposure to curcumin (treated only with LED: Group C-L+). The control wells consisted of both *S. mutans* suspensions exposed to neither curcumin nor LED (no treatment: C-L-; control group). Tenfold serial dilutions of the contents from each well were obtained and aliquots were transferred in triplicate to Petri dishes containing BHI agar. After incubation (37°C for 48 hours),

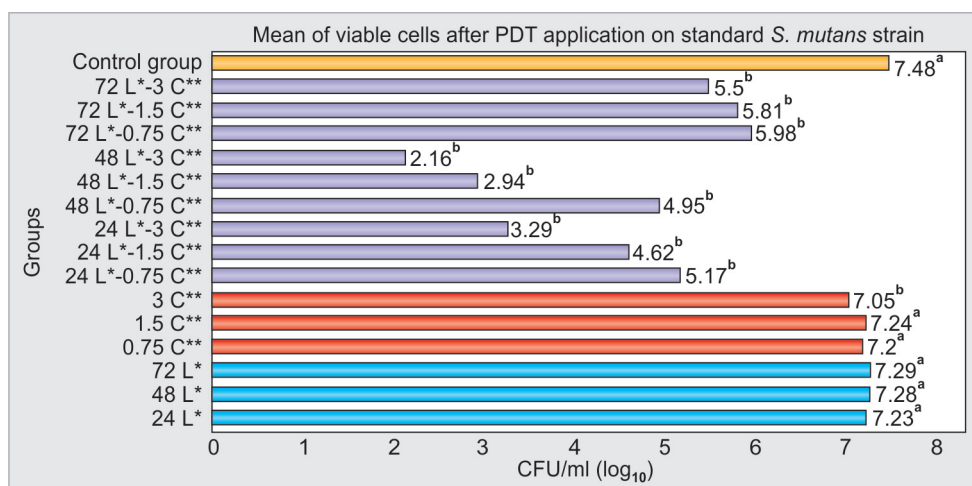
the total number of colony forming units (CFU) was determined by using a digital colony counter (CP 600 Plus, Phoenix Ind. Com. Equipamentos Científicos Ltda, Araraquara, SP, Brazil). The number of CFUs per millimeter (CFU/ml) was obtained and transformed into logarithm (log₁₀).

STATISTICAL ANALYSIS

In order to verify the differences between groups to both tested *S. mutans* strains, the variable reduction in viable bacterial colony counts promoted by each group was analyzed by two-way ANOVA and Tukey's test. The statistical significance cutoff level was set as $p < 0.05$. The BioEstat 5.0 software for Windows (Sociedade Civil Mamiara, Manaus, AM, Brazil) was used for data analysis.

RESULTS

Graph 2 shows the CFU values for each studied group to standard *S. mutans* strain. The group irradiated by blue LED in the presence of curcumin (PDT group: C+L+) presented a significantly lower number of viable bacteria ($p < 0.05$) compared to control group. Reductions of 60.66% and 71.07% were verified when curcumin used at 1.5 mg/ml was irradiated at dosimetries of 48 and 72 J cm⁻², respectively. On the other hand, *S. mutans* clinical isolate demonstrated a lower sensibility to PDT application. Graph 3 shows that to all the associations of curcumin and LED achieved the same CFU bacterial reduction (33.88%) ($p < 0.05$). The other groups, to both studied strains, revealed a slight reduction of viable bacteria without clinical significance in comparison to control group ($p > 0.05$). Thus, based on these findings, the null hypothesis was partially accepted.



Graph 2: Mean of viable bacteria (colony forming units/ml) in log₁₀ to all the experimental conditions to standard *Streptococcus mutans* (ATCC 25175). Data represent mean values (n = 6). Different lowercase letters represent difference statistically significant ($p < 0.05$) (C*: Curcumin at mg/ml; L*: blue LED at J cm⁻²)

DISCUSSION

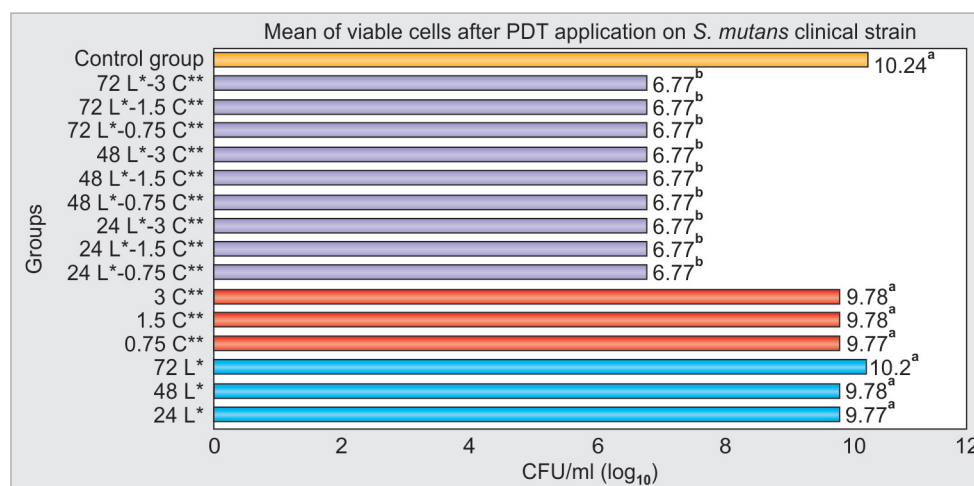
The outcomes of this present investigation demonstrated that the application of PDT mediated by curcumin activated with a blue LED was able to photoinactivate planktonic suspensions of *S. mutans* on both laboratorial standard and a clinical isolate strain. Although some studies have showed the success of this antimicrobial approach on cariogenic bacteria, none of these investigations has focused the behavior of PDT on clinical isolates of cariogenic bacteria. Recently, the success of curcumin-mediated PDT has been suggested by some authors. Araujo et al¹⁵ found a substantial bacterial reduction on planktonic suspensions of *S. mutans* and *Lactobacillus acidophilus* using 1.5 g/l at 5.7 J cm⁻² of a blue LED light source. In addition, Dovigo et al.¹¹ photoinactivated species of *Candida albicans* with 5.28 J cm⁻² in the presence of 20 µM of curcumin. Still, an *in vivo* approach was able to decrease the levels of *S. mutans* present in saliva after a curcumin mouthrinse and subsequently exposure to a light at 450 nm. Overall, due to simple manipulation, price and great effectiveness of curcumin associated with light source, PDT is a promising tool on dental practice.¹⁹

As a Gram-positive bacteria, *S. mutans* on both tested forms presented high sensitivity to PDT application. This behavior is explained by the structural differences of the outer bacterial cell wall between Gram-positive and Gram-negative bacteria, which decrease the efficacy of various photosensitizers. The 40 to 80 nm thick outer cell wall and up to 100 peptidoglycan layers of Gram-positive bacteria do not represent an effective permeability barrier. In contrast, the outer membrane of Gram-negative bacteria with a bilamellar membrane covering the only 3 nm thick peptidoglycan layer is able to prevent photosensitizer diffusion considerably, especially the negatively charged or neutral photosensitizers. Matiello et al²⁰ verified a

high resistance of *Actinobacillus actinomycetemcomitans* (A.a.) to photoinactivation in the presence of 0.01% of TBO irradiated with an AlGaInP diode laser light source in comparison to *Streptococcus sanguinis*, achieving a bacterial reduction of 61.53% and 84.32%, respectively. Furthermore, due to the possibility of interchange among microorganisms in oral cavity, clinical strains are normally more resistant to antimicrobials chemotherapeutic agents than laboratory strains. In accordance of this statement, it was achieved a photokilling rate more than 70% on laboratorial standard strain whereas a rate of less than 40% was observed on its clinical sample counterpart. To corroborate with our findings, a photoinactivation of laboratorial strain of *C. albicans* was achieved with the association of 20 µM of curcumin exposure to 5.28 J cm⁻² of a blue light source¹¹ whereas clinical isolates of its counterparts, *C. glabrata* and *C. tropicalis* to achieve a significant antifungal effect was necessary a exposure of more than three times of irradiance (18 J cm⁻²) in the presence of 40 µM of the same PS.²¹

In our study, neither curcumin nor light when used alone had a bactericidal effect. In fact, our results highlighted the need for dye-light conjugation to ensure the effectiveness of PDT. This outcome is in agreement with current photodynamic studies.^{11,15,20}

The increase of curcumin concentration and light dosages did not demonstrated a bactericidal cumulative effect to both studied strains, indicating a nondose response to variable increments. To laboratorial *S. mutans* strain, the highest log bacterial reduction was achieved when exposure to 48 J cm⁻² in the presence of 3 mg/ml (difference of 5.32 log₁₀ in comparison with control group). However, at same curcumin concentration but with increased light fluency resulted in a difference of 1.98 log₁₀. Additionally, however the clinical isolate have



Graph 3: Mean of viable bacteria (Colony-forming unit/ml) in log₁₀ to all the experimental conditions to *Streptococcus mutans* clinical isolate. Data represent mean values (n = 6). Different lowercase letters represent difference statistically significant (p < 0.05) (C*: Curcumin at mg/ml; L*: blue LED at J cm⁻²)

showed more resistance to PDT application, a difference of 3.47 log₁₀ in comparison with control group at all studied PDT situations was verified. A similar nondose dependent pattern was obtained by some authors^{11,20,21} using this same PS and light source. On the contrary, study of Chan and Lai,²² the exposure of periodontopathogenic bacteria to 665 nm diode laser with power output of 100 mW in the presence of 0.01% TBO resulted in a dose-dependent decrease of viable bacterial strains. Maintaining a constant power with an exposure time of 30 s and energy density of 10.6 J cm⁻² resulted in a bacterial decrease between 71 and 88%. Increasing the exposure to 60 s and energy density to 21.2 J cm⁻², there was a 99 to 100% reduction of *P. intermedia*, *P. gingivalis* and *S. sanguis*, while *A.a.* and *F. nucleatum* numbers decrease by 95 and 96%, respectively. On this same way, Paulino et al²³ treated *S. mutans* with different concentrations of Rose Bengal (RB) (0-50 µM) irradiated with a hand held photopolymerizer (400-500 nm) at different time periods showed that to concentrations from 0.1 µM of RB and a time illumination of 30 seconds achieved a dose dependent pattern, achieving 100% of cell death at 0.5 µM of RB. This difference among the mentioned studies can be explained by the different ways of action of used photosensitizers. It has been proved that curcumin, when submitted to longer irradiation times, produced lower quantities of free radicals or singlet oxygen due to high photobleaching rate.¹¹ There is a maximum of cell absorption related to PS or dyes utilized; an excess of dye in solution, corresponding to high concentrations, would blocks the light to reach bacteria, resulting in the optical quenching phenomenon.¹⁸

However, it is known that dental caries is resulted of biofilm accumulation on tooth surface²⁴ the present investigation focused the PDT application on planktonic phase, which resulted in reduction of bacterial counts of both standard and clinical isolate of *S. mutans* reinforcing curcumin as a potent photosensitizer and establishing PDT as one of the strategies for caries treatment and prevention.

CONCLUSION

In accordance of our findings, the present study demonstrated that both tested *S. mutans* strains presented sensitivity to this antimicrobial tool mediated by curcumin and a LED in the blue wavelength, although more resistance was found to clinical isolate of *S. mutans*. More extensive investigations to elucidate the effectiveness of curcumin-mediated PDT should be encouraged on *in situ* studies and over biofilms structures aiming to define more accurate parameters to future PDT clinical applications.

CLINICAL SIGNIFICANCE

Alternative antimicrobial approach, as photodynamic therapy, is demonstrating good results against cariogenic bacteria and could be used to decrease the number of microorganism in oral cavity aiming to prevent or treat dental caries.

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