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MARY ANNE SAMPAIO DE MELO

**DETERMINAÇÃO DE PARÂMETROS EFETIVOS E SEGUROS PARA O USO DA
TERAPIA FOTODINÂMICA ANTIMICROBIANA EM DENTINA CARIADA:
ESTUDO *IN VITRO***

**FORTALEZA
2009**

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do Título de Mestre em Odontologia.

Área de Concentração Clínica Odontológica.

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RESUMO

A eliminação de bactérias presentes na camada de dentina desmineralizada poderia contribuir para uma abordagem mais conservadora no tratamento restaurador de lesões de cárie dentinária profundas. Desta forma, este estudo *in vitro* objetivou estabelecer parâmetros eficazes e seguros para a utilização de um LED ($\lambda = 620-660$ nm) associado ao fotossensibilizador azul de orto-toluidina (TBO) na desinfecção de lesões de cárie dentinária produzidas artificialmente. Para tal, blocos com 25 mm^2 de dentina oclusal planificada foram imersos por 5 dias em BHI caldo inoculado com *Streptococcus mutans* para indução de cárie. Depois da desmineralização, os blocos foram aleatoriamente distribuídos em 10 grupos experimentais (n=15), a saber: Controle5; Controle10; Controle15; TBO; LED5; LED10; LED15; TFD5; TFD10 e TFD15, que foram tratados ou não com TBO ($0,1 \text{ mg.ml}^{-1}$ por 5 minutos) ou solução de NaCl a 0,9% por 5, 10 ou 15 minutos, e submetidos ou não a irradiação com LED por 5, 10 ou 15 minutos (47, 94, e 187 J/cm^2). Amostras de dentina cariada foram coletadas antes e após os tratamentos e as bactérias foram semeadas para contagem de *Streptococcus mutans*. Adicionalmente, com um termômetro tipo K, a temperatura intra-pulpar e da área periodontal nos grupos TFD5; TFD10 e TFD15 foi monitorada em 10 dentes com cavidades oclusais confeccionadas em dentina profunda. Teste *t* pareado e ANOVA seguida pelo teste Tukey ($\alpha=5\%$) foram usados para verificação da redução microbiana promovida pelos tratamentos, bem como para verificação da alteração na temperatura durante a irradiação. Diferenças significativas estatisticamente na viabilidade do *Streptococcus mutans* entre contagens foram observadas nos grupos: Controle15; LED15; TFD5; TFD 10 e TFD15. A temperatura intra-pulpar e no periodonto foi inferior a 2°C , sendo a no interior da câmara pulpar maior para o grupo TFD15 quando comparado ao grupo TFD5. Dessa forma, nas condições experimentais atuais, a terapia fotodinâmica pode ser um tratamento eficaz e seguro a ser usado na desinfecção de dentina cariada, contudo a influência do tempo de irradiação ou exposição à solução salina na viabilidade do *Streptococcus mutans* deve ser melhor investigada.

Palavras-chave: Cárie dentária. *Streptococcus mutans*. Fotossensibilização. LED. Agentes antibacterianos

ABSTRACT

The elimination of bacteria inside the demineralized dentin layer might contribute for a more conservative approach in the restorative treatment of deep dentin caries lesions. This way, this *in vitro* study aimed to establish safe and effective parameters for using an LED ($\lambda = 620-660$ nm) associated to the photosensitizer toluidine blue O (TBO) in the disinfection of artificially produced dentin caries lesions. For this, slabs with 25 mm^2 of flatted occlusal human dentin were immersed for 5 days in BHI broth inoculated with *Streptococcus mutans* for caries induction. After demineralization, the slabs were randomly allocated to 10 experimental groups (n=15), as follows Control5; Control10; Control15; TBO; LED5; LED10; LED15; PDT5; PDT10 and PDT15, which were treated with TBO (0.1 mg.ml^{-1} for 5 min) or 0.9% NaCl solution for 5, 10 or 15 min, and submitted or not to LED irradiation for 5, 10 or 15 min (47, 94, and 187 J/cm^2). Dentin samples from caries lesions were collected before and after treatments and bacteria were then cultured for *Streptococcus mutans* counts. In addition, using a type K thermometer, the temperature inside the pulp and in periodontal area was monitored for the groups PDT5, PDT10 and PDT15 in 10 teeth with deep occlusal cavities. Paired *t* test/Wilcoxon matched pairs test ($\alpha=5\%$) were used to determine differences between microbial population before and after treatments, and ANOVA followed by Tukey test for comparing data of temperature and log reductions. Statistically significant differences in *Streptococcus mutans* viability were found for the groups: Control15; LED15; PDT5; PDT10 and PDT15. The temperature from intrapulpal and periodontal area were lower than 2°C , being higher inside the pulpal chamber for group PDT15 when compared to group PDT5. Thus, in the experimental conditions used in the study, photodynamic therapy may be a safe and effective treatment to be used for disinfecting carious dentin, however the influence of time of irradiation/exposition in the *Streptococcus mutans* viability should be better investigated.

Keywords: Dental caries. *Streptococcus mutans*. Photosensitization. LED. Anti-bacterial agents.

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1 INTRODUÇÃO GERAL

A terapia fotodinâmica baseia-se no uso de um corante (fotossensibilizador) associado a irradiação com luz de comprimento de onda compatível com a absorção do corante, onde o tratamento somente com a luz ou apenas o corante não produz efeito deletério (MITRA 2004). Quando a exposição ao corante vem seguida pela irradiação de luz, reações químicas são geradas conduzindo a uma cadeia de eventos citotóxicos que levam a alterações funcionais na célula-alvo e, conseqüente, perda de sua viabilidade (HABLIN; HASAN, 2004; KUBLER, 2005; JORI *et al.*, 2006; JORI; COPPELLOTTI, 2007).

O uso da terapia fotodinâmica na área da saúde aumentou consideravelmente no último século devido principalmente ao desenvolvimento científico e tecnológico (SIMPLÍCIO; MAIONCHI; HIOKA, 2002; ALLISON *et al.*, 2008). Assim a utilização desta modalidade terapêutica para o tratamento de injúrias pré-malignas, malignas e degenerativas é amplamente usada em diversas áreas médicas (LUKŠIENĖ, 2003; BAGNATO *et al.*, 2005; KUBLER, 2005; PERUSSI, 2007). Por também apresentar atividade antimicrobiana (ASHKENAZI; NITZAN; GÁL, 2003; WILSON, 2004; JORI; BROWN, 2004; LUKŠIENĖ, 2005; MAISCH, 2007), a possibilidade de sua utilização para destruição de diversos microorganismos, incluindo os relacionados a várias doenças orais tem sido tópico atual de pesquisa.

Além das reconhecidas medidas de prevenção da cárie dental, como os procedimentos mecânicos de remoção do biofilme cariogênico, a adequação da dieta e o uso do flúor (CHAVES; VIEIRA-DA-SILVA, 2002), tem-se investigado novas abordagens conservadoras, buscando-se evitar os prejuízos trazidos pela doença. Dentre as estratégias, tem-se destacado a redução de patógenos específicos relacionados à cárie por meio de agentes antimicrobianos capazes de inibir o crescimento de estreptococos do grupo mutans (MOUNT; NGO, 2000; MOUNT, 2007; FRANCO *et al.*, 2007).

Na Odontologia Restauradora, a terapia fotodinâmica antimicrobiana (TFA) é proposta como uma abordagem complementar a remoção do tecido cariado. Seu objetivo baseia-se na máxima redução de microorganismos na área de dentina remanescente que se encontra sobre a superfície pulpar em lesões de cárie profundas. Neste contexto, os estudos da TFA têm contribuído para a possibilidade de desenvolvimento de um tratamento capaz de eliminar as bactérias *in vivo* diminuindo a quantidade de tecido dentário a ser removido durante o preparo cavitário em dentina (BURNS; WILSON; PEARSON, 1995; KIDD, 2000; BONSOR; PEARSON, 2006).

A ação da TFA sobre bactérias orais crescidas em caldo de cultura já se encontra bem documentada na literatura (DOBSON; WILSON, 1992; BURNS; WILSON; PEARSON, 1994; WILSON; YIANNI, 1995; GRIFFITHS; WREN; WILSON, 1997; ZANIN *et al.*, 2002; WILLIAMS *et al.*, 2003) com resultados revelando altas taxas de redução microbiana.

Quanto ao seu comportamento frente a microorganismos crescidos em biofilme que normalmente apresentam uma redução significativa em suscetibilidade a agentes antimicrobianos comparados com culturas cultivadas em laboratórios (MAISCH, 2007); vários estudos (WILSON; BURNS; PRATTEN, 1996; O'NEILL; HOPE; WILSON, 2002; CHABRIER-ROSELLO *et al.*, 2005; ZANIN *et al.*, 2005, 2006; WOOD *et al.*, 2006) mostram resultados bastante promissores com consideráveis taxas de redução, apesar de serem em menor valor se comparados aos resultados obtidos sobre microorganismos em culturas planktônicas.

Porém, pouco tem sido cientificamente estudado para uma avaliação segura de sua eficácia antimicrobiana sobre lesões de cárie dentinária, necessitando de estudos mais detalhados sobre seu uso terapêutico (GIUSTI *et al.*, 2008). Para se instaurar esta terapia de forma eficiente, alguns parâmetros devem ser estabelecidos, uma vez que o conhecimento atual aponta para diversas variáveis que podem interferir no processo fotodinâmico quando realizados em um substrato diferenciado, a exemplo da dentina cariada. São variáveis que contribuem na redução do efeito sobre os microorganismos-alvo: menor penetração do fotossensibilizador nos túbulos dentinários, que influencia a capacidade de alcance das bactérias tanto por parte do corante como por parte da luz (BURNS; WILSON; PEARSON, 1995); o tempo de exposição e, conseqüentemente, densidade de energia (QUIN *et al.*, 2007); concentração de O₂ no meio (YAVARI, 2006) e a presença de uma diversidade microbiana (WILSON, 1993).

Apesar da influência de diversas variáveis na eficiência antimicrobiana da terapia, a densidade de energia se torna um dos parâmetros mais relevantes relacionando-se diretamente ao tempo de irradiação e se encontra aplicado com bastante variedade em estudos relacionados a TFA (HAMBLIN; HASAN, 2004; TEGOS; HAMBLIN, 2006; NUNEZ, 2007).

Especial atenção deve ser dada ao aumento da densidade de energia durante o tratamento. A possibilidade de elevação térmica associada a terapia em função da ativação do corante pode gerar danos térmicos ao órgão pulpar, pois uma maior energia irradiada será absorvida pelos tecidos dentais. Em acréscimo, a maioria das radiações de luz utilizada para ativação do corante se encontra em uma faixa próxima a região infravermelha do espectro de

luz, caracterizando-se em uma faixa capaz de produzir efeito fototérmico (calor) ao ser absorvida pelos tecidos dentais (ZACH; COHEN, 1965; LEE *et al.*, 2006). Tal fato é ainda mais importante em situações onde há mínima espessura de dentina como em cavidades profundas, uma das indicações proposta da terapia antimicrobiana.

Neste contexto, a investigação da eficácia antimicrobiana com mínimo efeito térmicos à polpa, deve ser ressaltada antes da realização de um estudo *in vivo*, onde outros fatores como microambiente, presença de biofilme organizado e distribuição da luz poderão afetar ainda mais os resultados obtidos.

2 PROPOSIÇÃO

Os objetivos do presente trabalho foram:

2.1 Objetivo Geral

Determinar por meio de um estudo *in vitro* os parâmetros efetivos e seguros para o uso da terapia fotodinâmica antimicrobiana para desinfecção de dentina cariada.

2.2 Objetivos Específicos

Avaliar o efeito antimicrobiano da terapia fotodinâmica, utilizando-se do fotossensibilizador azul de orto toluidina associado a um LED (λ predominante em 638,8 nm) com três densidades de energia diferentes, através de análise microbiológica e exploração de microscopia eletrônica de varredura.

Verificar *in vitro* se os parâmetros estabelecidos como eficazes são seguros para serem utilizados *in vivo* em cavidades profundas executadas em dentina no que diz respeito a alterações na temperatura pulpar e no periodonto.

3 CAPÍTULO

Esta dissertação está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria e co-autoria do candidato (Anexo A). Por se tratarem de pesquisas envolvendo dentes humanos, o projeto de pesquisa deste trabalho foi submetido à apreciação do Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade Federal do Ceará, tendo sido aprovado sob o Protocolo COMEPE n°143/06, conforme o Ofício n° 324/2006 de 31 de julho de 2006 (Anexo B). Assim sendo, esta dissertação é composta de um capítulo contendo um artigo com o idioma corrigido através dos serviços especializados do endereço eletrônico <http://www.journalexperts.com/> (Anexo C) e submetido para publicação no periódico “Laser in Surgery and Medicine”, (Anexo D) conforme descrito abaixo:

Safe and effective parameters for using photodynamic antimicrobial chemotherapy in carious dentin: an *in vitro* study.

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Safe and effective parameters for using photodynamic antimicrobial chemotherapy in carious dentin: an *in vitro* study

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ABSTRACT

Background and Objective: The development of a method to ensure bacterial-free surfaces without extensive cavity preparation would be highly useful to the field of Dentistry, since there is no currently available effective method for killing residual bacteria in dentinal tissue. Therefore, the purpose of this totally randomized *in vitro* study was to determine effective antimicrobial parameters for the use of toluidine blue O (TBO) in combination with a light-emitting diode (LED - $\lambda= 620-660$ nm) for the disinfection of lesions on dentin caries

Study Design/Materials and Methods: Slabs with 25 mm² of flatted occlusal human dentin were immersed for 5 days in brain heart infusion (BHI) broth inoculated with *Streptococcus mutans* for caries induction. After demineralization, the slabs were randomly allocated to 10 experimental groups (n=15), which were treated with TBO (0.1 mg.ml⁻¹ for 5 min) or 0.9% sodium chloride (NaCl) solution for 5, 10 or 15 min, and submitted or not to LED irradiation for 5, 10 or 15 min (47, 94, and 187 J/cm²). Dentin samples from caries lesions were collected before and after treatments and bacteria were then cultured for *Streptococcus mutans* counts. The temperature rise in the pulpal chamber and periodontal ligament during irradiation was also verified. Paired t test/Wilcoxon matched pairs test ($\alpha=5\%$) were used to determine differences between microbial population before and after treatments, and analysis of variance (ANOVA) followed by Tukey test was utilized to compare log reductions and temperature rises between the groups.

Results: Significant decreases in the viability of *S. mutans* in carious dentin were observed when slabs were exposed to both TBO and light at the three different doses. However, statistically significant microbial reduction also occurred when bacterial air-exposure time or irradiation time was 15 min, corresponding to 187 J/cm². The temperature increases were less than 2°C for either pulpal chamber or periodontal ligament.

Conclusions: Based on these results, LED combined with TBO is a safe procedure that was effective in reducing *Streptococcus mutans* population in artificially carious dentin. Nevertheless, additional studies should be conducted to determine the real influence of the exposition/irradiation time in *S. mutans* viability in dentinal surface/tubules.

INTRODUCTION

Dentin caries occur in different layers of the tooth. Can be divided in two different patterns: the outer layer is highly infected with bacteria that dissolve the mineralized tissue of dentin and damages the collagen matrix, which may result in an impossibility of remineralization, which should be completely removed during caries excavation and the inner layer where bacteria may dissolve the mineralized tissue but the cross-banded ultra structure of the collagen matrix will still remain.

Nevertheless, clinically the differentiation between the zones of caries that are infected and affected by the carious process is extremely critical [3], since unnecessary cavity preparation may lead to the removal of dentin tissue and possible exposure of the pulp [4,5]. Accordingly, most current methods to treat a deep carious lesion involve the removal of all softened and infected dentin. The use of either hand instruments or rotary burs does not guarantee a cleaning of the infected dentin and residual bacteria, which are often present before the placement of restorations [6]. Therefore, the elimination of bacteria inside of the dentin tubules from the remaining demineralized dentin [4] might contribute to a more conservative approach for dealing with deep caries lesions [7].

In this context, photodynamic antimicrobial chemotherapy (PACT) may be a potential alternative treatment for dentin disinfection. This therapy involves photosensitizing agents, such as toluidine blue O (TBO), which are activated by irradiation with light of a specific wavelength to generate cytotoxic species, including singlet oxygen and free radicals. These products are capable of damaging essential components of the cells or modifying metabolic activities in an irreversible way, which may result in bacterial death [8–11]. Additionally, the use of approaches that are unlikely to result in the development of resistance by bacteria would be very valuable, in light of the emergent problem of bacterial resistance to conventional antimicrobials.

Photodynamic therapy is of great interest in the field of Dentistry, due to antibacterial effects [11–15], including the effectiveness against oral microorganisms related to dental caries [3,7,16–23]. This process has achieved high bacterial kill of *Streptococcus mutans* in planktonic cell or biofilms, demonstrating that PACT is a promising technique [21–26]. However, studies of decontamination induced in a particular tissue environment are relevant, since photosensitizer distribution and interaction with light is dependent on the medium. The

effect of PACT on oral microorganisms located in demineralized dentin can be reduced, due to lower penetration of photosensitizer or light that reaches the bacteria [7,27]. In addition, a few studies have investigated the antimicrobial effects of photodynamic therapy in dentin carious tissue [23], primarily in human dentin.

The aim of this investigated was to determine whether a range of bacteria (*Streptococcus mutans*) housed within lesions of *in vitro* caries could be killed by PACT.

The hypotheses under investigation were the following: 1) the null hypothesis that there is no difference in the antimicrobial effect of TBO, LED, or PACT with three different energy densities on artificial dentin caries disinfection, as assessed by bacterial counts and scanning electron microscopy, and 2) the null hypothesis that there is no difference in the increase of pulpal or periodontal temperature during irradiation for 5, 10, or 15 min.

MATERIALS AND METHODS

Experimental Design

An *in vitro* study was performed with one hundred and sixty five extracted human third molars that had more than two-thirds of the formed roots, and without any previous lesions were used. The teeth used in this investigation were collected from adults living in Fortaleza, Brazil, in conformity with the norms of the Research and Ethics Committee of Federal University of Ceara Medical School (Process #. 143/2006).

The teeth were randomly allocated to 11 test groups, with 15 experimental units per group, according to a computer generated randomization list [28]. The experimental design was performed in triplicate (n=5) at different time points to minimize the inherent bias related to microbiological procedures. In order to isolate the microorganism ability in dentin caries production, one group was only exposed to a non-inoculated BHI broth. This study involved 10 set conditions in which carious dentin was exposed to 0.9% NaCl solution for 5, 10, or 15 min; sensitized or not with TBO; and irradiated or not with a LED for 5, 10 or 15 min.: These conditions were denoted as follows:

Control5- Carious dentin exposed to 0.9% NaCl solution for 5 min.

Control10- Carious dentin exposed to 0.9% NaCl solution for 10 min

Control15- Carious dentin exposed to 0.9% NaCl solution for 15 min

TBO- Carious dentin exposed to TBO for 5 min

LED5- Carious dentin exposed to LED for 5 min

LED10- Carious dentin exposed to LED for 10 min

LED15- Carious dentin exposed to LED for 15 min

PACT5- Carious dentin exposed to TBO and LED for 5 min

PACT 10- Carious dentin exposed to TBO and LED for 10 min

PACT15- Carious dentin exposed to TBO and LED for 15 min

Specimen Preparation

The teeth were stored in 0.01% (v/v) thymol solution at 4°C prior to use in this *in vitro* study. From each tooth, one slab of coronal dentine (5 x 5 x 2 mm³) were obtained using a water-cooled diamond saw and a cutting machine (IsoMet™ Low Speed Saw, Buehler, Lake Bluff, IL, USA). Only the occlusal dentin face was used, and the remaining surfaces of the slabs were protected with resin epoxy adhesive Araldite Hobby for 10 min (Brascola, São Bernardo do Campo, SP, Brazil). This resulted in a 25-mm² dentin surface area that served as a microbial model to produce caries lesions, as assessed by visual examination. The dentin slabs were fixed in the lids of glass container vessels with plastic rods, immersed in sterile distilled water, and then sterilized by autoclaving to 121°C for 15 min [29].

For production of dentin carie-like lesion, a microbiological model were used [22] with the microorganism *S. mutans* CTT 3440. To prepare the inoculum, *S. mutans* was first grown in an overnight culture of brain heart infusion (BHI) (Difco Lab. Detroit, MI, USA) in a 10% CO₂ atmosphere (Thermo Fisher Scientific Inc, Waltham, MA, USA). After sterilization, the dental slabs were removed from distilled water and immersed in sterile BHI containing 5% sucrose (w/v). All BHI-containing recipients, except the control group (untreated), were inoculated with 80 µl of 1-2 x 10⁸ CFU.ml⁻¹ 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 only once on the first day, and the dentin specimens were transferred to a fresh medium every day for 5 days. A schematic representation of these stages is given in Figure 1. In addition, each BHI-containing recipient was streaked onto a new fresh BHI agar media

plated and incubated at 37°C in an atmosphere of 10% CO₂ for 24 h to check for purity. Catalase and morphologic Gram test were utilized for specie identification.

Photodynamic therapy

After the 5-day experimental period, the biofilm formed over the slabs was removed and the carious dentin tissue was exposed. Then, one sample of carious dentin was harvested to obtain a baseline value for tissue contamination and the treatment for each group was performed. The groups TBO; PACT5; PACT10; and PACT15 were incubated with 5 µl of TBO in the dark for 5 min (pre-irradiation time) without light exposure [21,30-32]. The groups TBO; PACT5; PACT10; and PACT15 were incubated with 5 µl of TBO in the dark for 5 min (pre-irradiation time) without light exposure [21,30-32]. Groups Control5, Control10, Control15, LED5, LED10, and LED15 were incubated with an equal volume of sterile 0.9 % NaCl solution instead of TBO

The photosensitizer TBO (Sigma-Aldrich Company Ltd. Poole, Dorset, United Kingdom) was dissolved in deionized water to obtain a final concentration of 0.1 mg.ml⁻¹ and was subsequently kept in the dark. Ultraviolet visible (UV-vis) optical absorption spectrometry was performed in TBO solution before and after the irradiation using a HP 8453 system spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA), in order to characterize TBO absorption spectrum between 550 and 680 nm and an absorption peak centered at 632±8 nm and correlation with LED emission spectrum. The light source used was a red light-emitting diode (LED; Laserbeam, Rio de Janeiro, RJ, Brazil), with a spectrum of emission ranging from 620 to 660 nm and a predominant wavelength of 638.8 nm. A fiber optic spot with a 9.5-mm cylindrical diffusing tip distributed the light. Irradiation was performed in a non-contact mode with a focused beam at 2.0 mm of working distance. A power meter Lasermate (Coherent Inc, Santa Clara, CA, USA) was used to measure the peak power, and a maximum output power of 40 mW was determined.

For the light exposed groups (LED5, LED10, LED15, PACT5; PACT 10, and PACT15), the slabs were irradiated for 5, 10 or 15 min under stable irradiation power (40 mW), which allowed for determination of the incident energy doses of 47, 94, and 187 J/cm², respectively. The LED equipment used presented no possibilities of varying the output power,

and therefore, relatively long irradiation times were used and the groups Control5, Control10, Control15 were included to verify the influence of these time limits on bacterial viability.

For microbiological analysis, before and after treatments, a sample of carious dentin was collected in different portions of each slab using a #5 carbide bur in a low speed drill (Labor dental, São Paulo, SP, Brazil). The dentin samples were weighed (± 1 mg) in pre-weighed microcentrifuge tubes, and 0.9% NaCl solution was added (1 ml/mg dentin). The tubes were agitated during for 2 min in a Disrupter Genie Cell Disruptor (Precision Solutions, Rice Lake, Wisconsin, WI, USA) with three 0.1 mm diameter glass beads to detach the bacterial cells. Subsequently, the suspension was serially diluted (1:100, 1:1000, 1:10000, and 1:100000) with 0.9% NaCl solution. Samples were plated in triplicate on BHI agar, and incubated for 48 h at 37°C in a 10% CO₂ atmosphere. Representative colonies with typical morphology of *S. mutans* were counted using a colony counter. The results were expressed in CFU/mg dentin.

Temperature change during exposure to light

The purpose of this analysis was to observe potential temperature variations in the pulp chamber and periodontal ligament caused by the LED irradiation associated with TBO usage. For this purpose, 10 intact extracted human teeth were used and deep standardized cavities were prepared in the occlusal surface, using cylindrical diamond bur # 1098 (KG Sorensen, São Paulo, SP, Brazil) in a high-speed hand piece. The existence of a 1-mm dentin layer over the pulp chamber was assured using radiographies. Additionally, 5 μ l of TBO was placed inside each cavity for a pre-irradiation time of 5 min without light exposure [21,30-32]. The temperature range was measured in triplicate using two type K thermocouple electrodes connected to a thermometer (Minipa, São Paulo, SP, Brazil), and coordinated by the software PS250 (Minipa, São Paulo, SP, Brazil). One thermocouple was introduced into the pulp chamber in close contact with the pulp chamber floor. The second one was attached to the periodontal ligament area.

The root surfaces of the teeth were submerged into a water bath at 37°C, during the testing procedure. This method effectively stabilized the internal baseline temperature at 37°C, minimized the effects of ambient temperature changes, and provided a consistent initial temperature for each data set [33]. LED irradiation was initiated when a constant temperature

for the pulp chamber, which was approximately 37°C, was obtained. Temperatures were recorded every second during the irradiation times of 5, 10 and 15 min, relating time to each energy density used in this study. The temperatures rise considered (Δt) were calculated as the difference between initial and final recorded temperatures.

UV-vis spectroscopy analysis

Ultraviolet visible (UV-vis) optical absorption spectrometry was performed in TBO solution before and after the irradiation using a HP 8453 system spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA), in order to characterize TBO absorption spectrum, correlation with LED emission spectrum, and photodegradation. Room 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 1-mm light path. Fractionated irradiation was performed for 5 min and the spectra were obtained at 5 min intervals for 30 min. The spectra were analyzed with Origin Lab 8.0 software (Origin Lab Corporation, Northampton, MA, USA).

Scanning Electron Microscopy (SEM)

SEM was utilized to evaluate the morphology of treated and untreated bacteria in dentin from one sample of the PACT10, PACT5 and Control10 groups. All specimens were immersed in modified Karnovsky fixative solution containing 2.5% cold glutaraldehyde in 0.1 mol/L cacodylate buffer at a pH of 7.4 for 8 hours. Samples were then serially dehydrated in graded ethanol solutions (50, 60, 70, 80, 90, 95, and 100% ethanol) at 45 min intervals, critical point dried by CO₂, mounted on aluminum stubs, sputter-coated with gold/palladium, and examined by a TESCAN SEM (Model VEGA\XMU, Brno, Czech Republic) with different magnifications at an accelerating voltage of 20 kV.

Statistical analysis

For assessment of the effect of the treatments, the dependent variables CFU/mg of dentin before and after treatments, log reduction, and ΔT were analyzed; the assumptions of

equality of variances (Levene Test) and normal distribution of errors (Shapiro Wilks test) were verified; and data were transformed in the case of assumption violation. For variables CFU/mg of dentin before and after treatment for group PACT15 and log reduction, equality of variances, normal distribution of errors, and absence of outliers were not satisfied; such that these data could not be normalized. A non-parametric test to compare dependent samples (Wilcoxon matched pairs test) was applied for group PACT15 and Kruskal-Wallis and Student-Newman-Keuls were used for log reduction comparison means. For the other groups, a paired *t* test was used to verify differences between CFU/mg of dentin before and after each treatment and identify treatments that reduced the microbial community. The log reduction results were calculated by subtraction of initial from final values of the CFU/mg for dentin after being transformed by Log_{10} . Next, ΔT values were calculated by subtraction of the final from initial values of temperature in the pulp chamber and periodontal ligament. These data were analyzed by a one-way ANOVA followed by a Tukey-Kramer test. The significance level was set at 5%. The software BioStat 2007 Professional (AnalystSoft Robust business solutions company, Vancouver, British Columbia, Canada) was used.

RESULTS

UV-vis Spectroscopy

The emission spectrum of red LED and the aqueous TBO absorption spectrum are shown in Figure 2. A strong absorption band of TBO between 550 and 680 nm and an absorption peak centered at 632 ± 8 nm were observed. Additionally, a decrease in TBO absorption as a function of irradiation time is shown in Figure 3.

Scanning Electron Microscopy (SEM)

All dentin slabs appeared to be infected with an abundant amount of *S. mutans*, as shown in Figure 4a and 4b. No morphological alterations were noted in the sample of Control10 (control group) (Fig. 5a and Fig. 6a). After receiving TBO application and an incident energy dose of 47 J/cm^2 , some cells lost wall bands and became uncoupled, as shown in Figure 5b and 6b. When the energy was elevated to 94 J/cm^2 , some alterations, such as

disintegration or an unclear cell wall, were observed (Fig. 5c). In addition, the presence of amorphous masses was found involving individual bacterial cells that merged (Fig. 6c).

Microbiological analysis

Mean and standard deviation of CFU/mg for carious dentin before and after treatments, and log reduction achieved by each treatment are shown in Table 1. Based on paired tests, statistically significant bacterial reduction was found after treatment for groups Control15, LED15, PACT5, PACT10, and PACT15.

The log reduction values ranged from 0.39 to 5.80, with the lowest value for the Control15 group and the highest for the PACT15 group. Statistically significant differences between the groups are described in Table 1. The groups treated with PACT presented statistically higher log reduction values when compared to the respective control groups, except for those groups irradiated, but not sensitized by TBO, indicating that LED resulted in some cytotoxicity.

Temperature change

For ΔT variation, all groups treated with photodynamic processes presented temperature rises lower than 2°C degree (Table 1). For pulp temperature, the increase ranged from 0.9 to 1.7, and exhibited a statistically significant difference for only the PACT5 and PACT15 groups. With regard to periodontal data, the values ranged from 0.3 to 0.6 and no statistically significant differences were found, as summarized in Table 1.

DISCUSSION

The *in vivo* eradication of bacteria in microbiota within or over the dentinal tubules of carious lesions will be very beneficial for dental procedures, and will probably allow for

potential remineralizing processes to proceed more rapidly. The lack of excessive instrumentation, unwarranted dentin removal, and use of caustic chemicals or antibiotics on remaining dentin may improve the long-term prognosis for the repaired tooth. Thus, characterization of standardization of photodynamic processes is an important clinical issue.

According to our results, the groups Control15, LED15, PACT5, PACT10, and PACT15 were capable of reducing *S. mutans* in carious dentin. These results confirm several studies, which have previously demonstrated that cariogenic bacteria in culture baths and biofilms are susceptible to PACT [3,7,13,15–20,30]. However, there are few studies that have investigated the use of this approach on different substrates. Recent investigations have evaluated the bacterial effectiveness of photodynamic therapy in an environment similar to carious tooth (collagen matrix, *ex vivo* carious dentin, and carious bovine dentin) [23,27]. Although these studies have demonstrated promising results for using photodynamic approaches in dentin, there have been limitations to these investigations, such as absence of required control groups, utilization of different calculations to verify efficacy, and use of bovine dentin [23] as well as bacterial irradiation through the dentin and not inside the tissue tubules [27]. In this context, our research demonstrated for the first time that *S. mutans* hosted in carious human dentin is susceptible to photodynamic therapy using TBO and a LED with energy densities ranging from 47 to 187 J/cm².

The results of this study also indicated that the red LED operating with 187 J/cm² may cause an inhibitory effect in bacterial cells of *S. mutans* present in carious dentin formed *in vitro*. This inhibition is thought to have occurred as a result of the length of the irradiation time (15 min), since treatment PACT15 also exhibited effectiveness in decreasing microbial population. However, this result does not agree with a previous study conducted with the same light source, which did not detect any influence on bacterial growth under irradiation times as long as 30 min [21]. These variable results are thought to be due to experimental differences in the studies, such as the use of biofilms instead of dentin for treatment groups. Thus, the complex and wet structure of biofilms might have provided an environment with more favorable conditions for bacterial surviving over longer time periods. Additionally, when the groups treated with PACT were compared to the respective control groups, the latter presented lower log reduction values, except for those groups irradiated, but not sensitized by TBO. This result indicates that LED treatment resulted in some cytotoxic effects. Log reduction analyses were performed to compare the efficacy among different treated groups and confirmed paired test results that 15 min irradiation can kill *S. mutans* in/on dentin tissue. The photocytotoxicity may be partially explained by the bacterial substrate, since bacteria

hosted in dentin were not protected from dryness by the presence of the polysaccharide matrix and higher amounts of water.

The use of high energy density (187 J/cm^2) to exhibit an antimicrobial effect in bacteria located inside dentin tubules has been suggested by Burns et al. [7]. Nevertheless, the significant reduction in viable counts after exposing bacteria to air for 15 min without light activation indicates that the time of treatment should not exceed 10 min for analysis of *S. mutans* viability in the current *in vitro* caries lesion model. The long irradiation time to achieve higher energy densities was one limitation of the chosen LED source used in this work. Additionally, group PACT15 demonstrated no contamination after treatment, which is not a realistic situation since the group Control15, which involved exposure of *S. mutans* the air for 15 min, was also able to disinfect dentin tissue without total eradication of the bacteria as PACT15. Consequently, the antimicrobial effect on the group PACT15 was improved by time of irradiation, resulting in a more effective therapy.

According to the UV-vis spectroscopy analyses, the photosensitizing mechanism of the LED on TBO 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 were obtained using conventional lasers to perform this therapy, although the power output can still be a limiting factor in their widespread application [34-36]. Use of this experimental methodology may result in technology simplification and the lower cost of treatment in comparison to complex laser systems. Moreover, LED sources provide more than a monochromatic form of irradiation, which is a special characteristic compared to lasers that increases the overlay of the spectrum of LED irradiation and the light absorption by TBO.

Conventional SEM observations revealed different forms of damage in irradiated groups as shown in Figures 5 and 6. The possible mechanisms underlying such cellular changes, such as loss of wall bands and the presence of amorphous masses, may be related to oxidation reactions of superoxide anions and hydrogen peroxide produced by the photodynamic reaction of LED irradiation on TBO over the bacterial cells. One of the mechanisms of PACT is based on the destruction of cell wall integrity [37]. In addition, Lee et al. [38] associated morphological changes of *S. mutans* to harmful external stimuli, such as laser irradiation. The extent of damage from PACT has been shown to be in line with the total density energy applied, as observed by Sharma et al. [39] in SEM images of the staphylococcal biofilms, which demonstrated that irradiation in the presence of $40 \mu\text{M}$ TBO led to a light dose-dependent disruption of the biofilms. In fact, the number of adherent

bacteria decreased and very few aggregated colonies were observed as the light dose increased.

Zach and Cohen in 1965 [40] discovered that an increase in temperature of 3.3°C resulted in reversible variations in pulpal histology, but that when the temperature increase exceeded 5.6°C, approximately 15% of the examined teeth had a loss of pulpal vitality. This possibility of temperature increase by diffusion of heat across the remaining dentin and the potential effect on pulpal tissue damage during the photodynamic process led to an additional investigation. The elevation of the temperature on the surface of the dentin depends on the thermal conductivity of this tissue, but also on the thickness and mass of these structures [38]. As the thickness of the dentin is the least in deeper cavity, with 1 mm or less of remaining dentin, the use of higher energy density should be careful to not exceed safe parameters.

Nevertheless, the results of this study demonstrated a low range of temperature variation. Difference related to the increase of pulpal or periodontal temperature during irradiation for the selected time periods was only accepted for the intrapulpal temperature, since the PACT15 group presented a more significant increase compared to the PACT5 group for the pulp temperature. However, all groups presented small temperature increases, not exceeding 2°C even for the highest LED density applied (187 J/cm²) and the increase was less than 1°C for the periodontal area, which is an even safer range. Thus, these findings indicate that PACT can be applied to carious dentin in deep cavity preparations without exposing pulp vitality to danger.

In conclusion, these results demonstrated that *S. mutans* housed within lesions of *in vitro* caries were susceptible to LED exposure in the presence of TBO, suggesting that this approach may be useful and safe in the treatment of dental plaque-related diseases. Therefore, PACT may be an adjunct tool to disinfect residual carious dentin, since decreasing the bacterial count under these circumstances could result in the difference between infection and contamination of dentin layers.

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Table 1: Mean and standard deviation of CFU per milligram of carious dentin before and after treatments, *p* value according paired tests, log reduction achieved by each treatment and temperature increase caused by photodynamic therapy treatments.

Groups	Pre-treatment (CFU/mg x 10 ⁶)	Post-treatment	<i>p</i> value (Paired test)	Log Reduction	Temperature rise	
					Pulp chamber	Periodontal area
Control5	4.58±4.72	3.18±3.36	0.311	0.39±0.45 ^f	-	-
Control10	2.61±2.72	10.7±34.5	0.370	0.58±0.59 ^f	-	-
Control15	55.4±73.7	1.67±2.88	0.006*	1.75±1.50 ^{cd}	-	-
TBO	29.1±5.08	9.32±23.5	0.137	0.77±0.78 ^{def}	-	-
LED5	9.11±9.98	9.54±16.8	0.915	0.68±0.89 ^{ef}	-	-
LED10	62.9±7.54	26.6±74.8	0.125	1.91±1.21 ^{bcd}	-	-
LED15	28.6±38.5	0.30±0.57	0.010*	3.47±2.02 ^{ab}	-	-
PACT5	53.2±76.2	9.25±26.2	0.036*	1.80±2.01 ^{cdc}	0.9±0.8 ^a	0.4±0.6 ^a
PACT10	50.7±78.1	5.30±13.5	0.023*	2.48±2.02 ^{bc}	1.3±0.9 ^{ab}	0.3±0.6 ^a
PACT15	3.76±8.89	0.00±0.00	0.000**	5.80±0.98 ^a	1.7±0.8 ^b	0.6±0.5 ^a

*Means statistically significant different by Paired t test, $\alpha = 5\%$.

** Means statistically significant different by Wilcoxon matched pairs test, $\alpha = 5\%$.

Log Reduction means followed by distinct letters statistically differ by Tukey test, $\alpha = 5\%$.

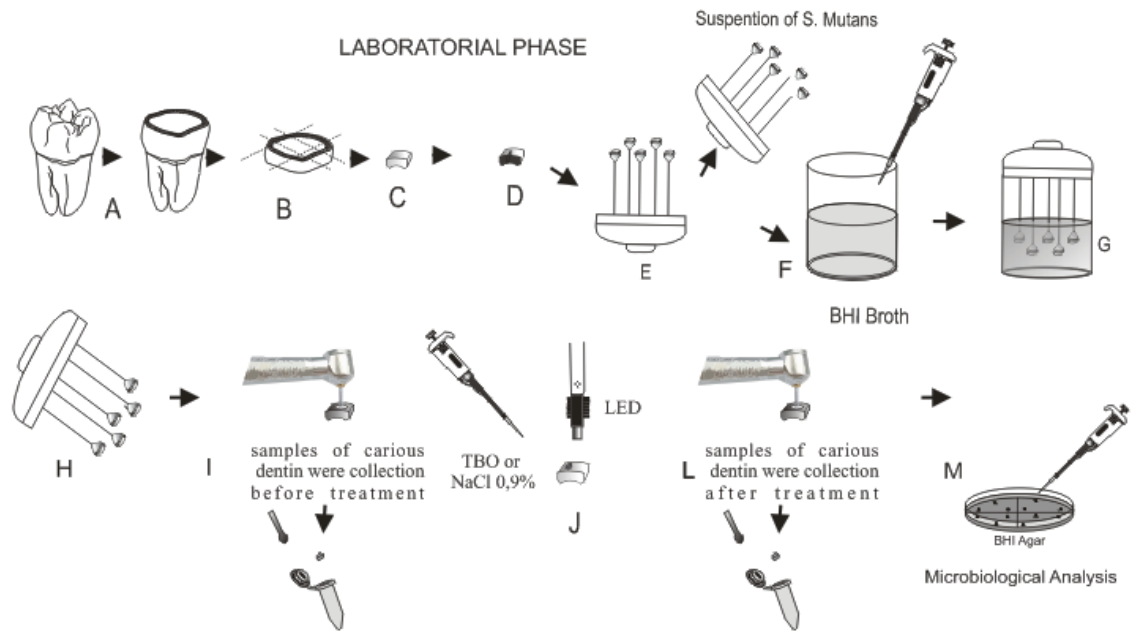


Figure 1- Schematic diagram illustrating preparation of dentin slabs and representation of the devices used in the model to produce caries lesions *in vitro*. A – Sound tooth and removal from the enamel of the occlusal face. B – Cut of the slabs. C - Slab prepared. D – Isolation of the occlusal area. E- Fixation of the slabs in the device. F - Inoculation of the culture broth. G-Device immersed in BHI broth. H-Carious slabs after 5 days. I- First collection of sample: before. J- Treatment according the group. L- Last collection of sample:after. M- Microbiological analysis

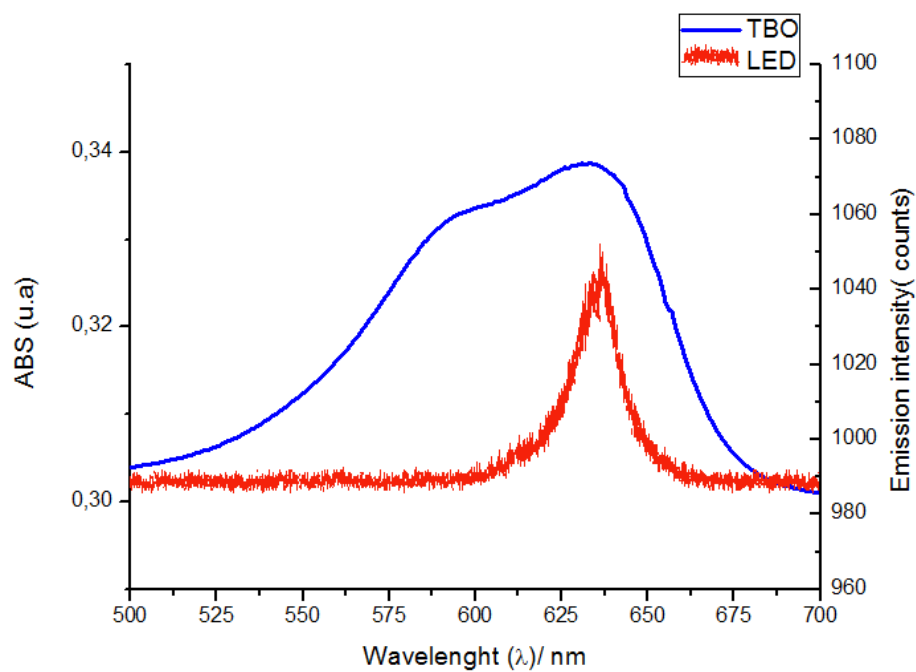


Figure 2: Spectrum of the red LED light source system and the absorbance spectrum of the TBO-water solution.

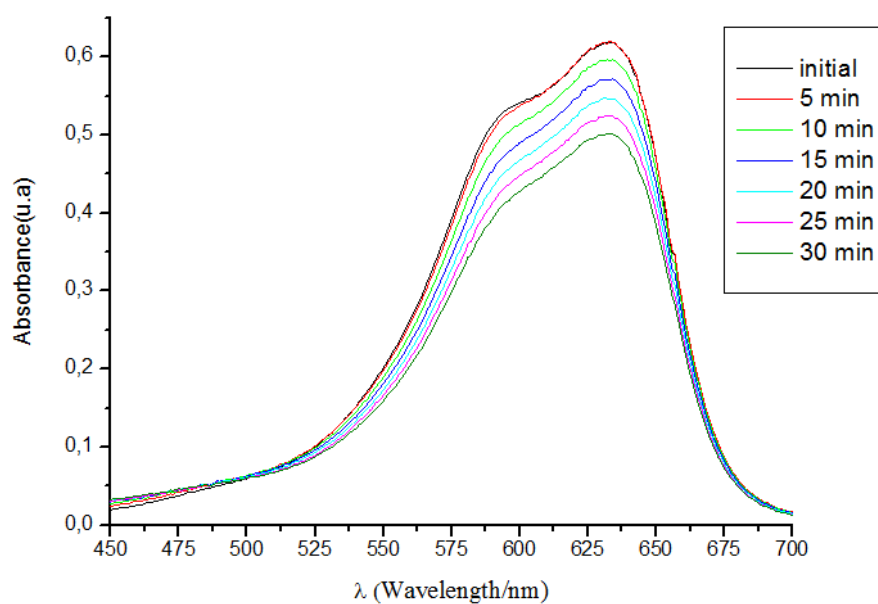
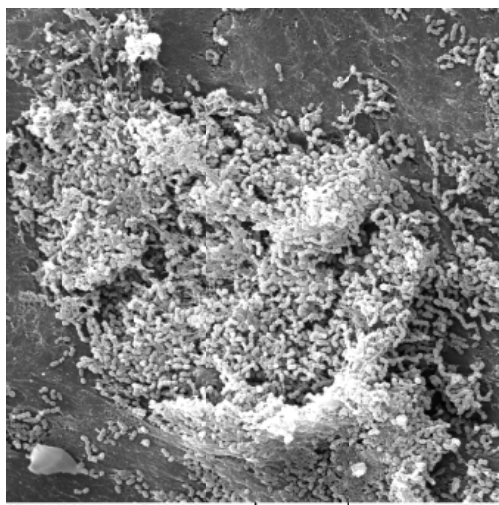
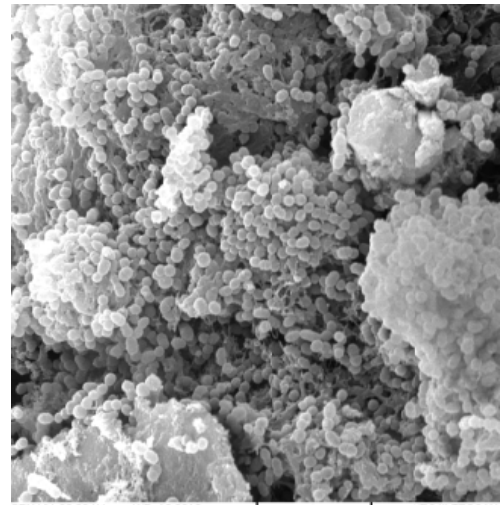


Figure 3: Absorption spectra after irradiation with red LED ($\lambda=632$ nm). Spectroscopic measurements of LED irradiation were collected every 5 minutes

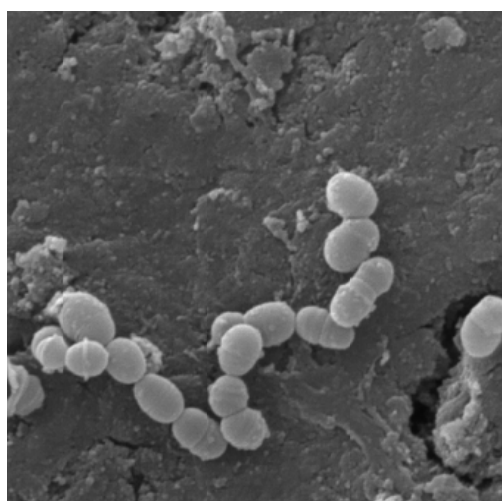


(4a)

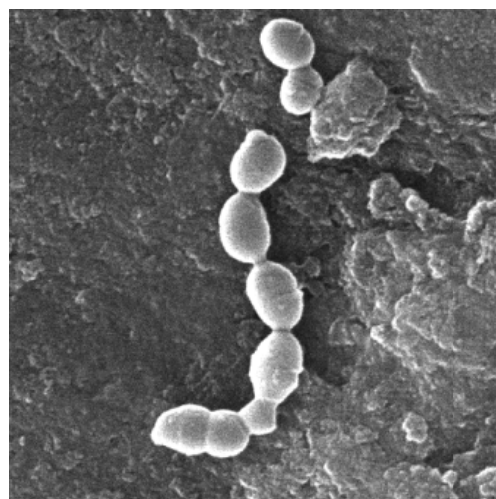


(4b)

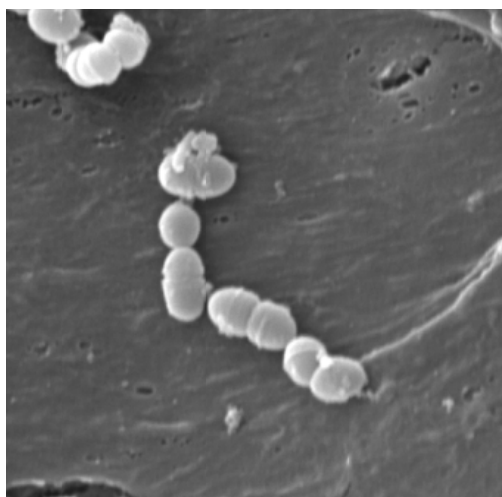
Figure. 4a. Sample of Control10 group **b:** Sample of PACT10 (Single flora of *S. mutans* is shown at lower magnification).



(5a)

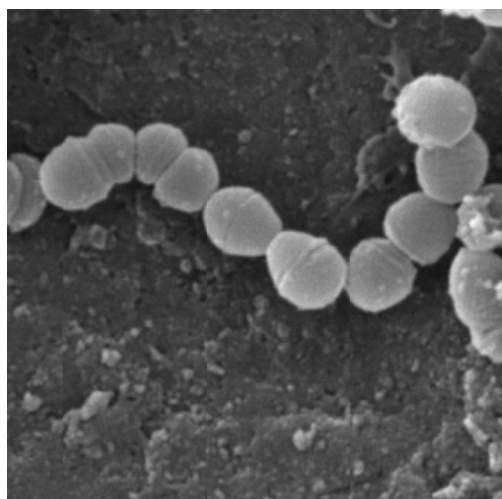


(5b)

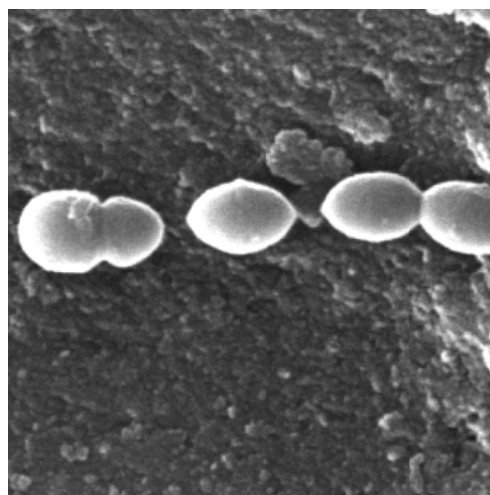


(5c)

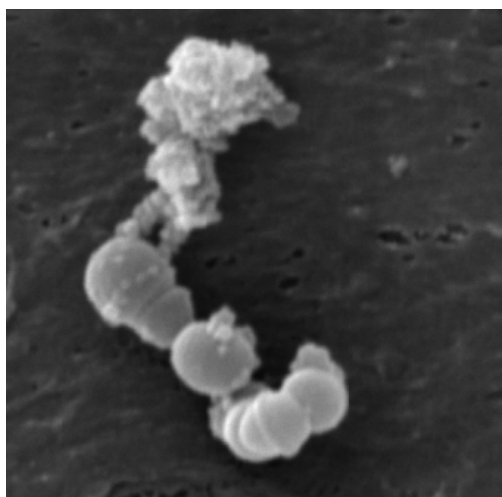
Figure. 5. a: Sample of Control10: normal morphology of *S. mutans* characterized by a short-rod shape. **b:** Sample of PACT5: small alterations in morphology, such same diplococcal desegregations. **c.** Sample of PACT10: some cells appear shriveled or fused.



SEM HV: 20.00 kV WD: 11.4970 mm VEGA\\ TESCAN
View field: 3.08 μ m Det: SE 500 nm
Date(m/d/y): 11/11/08 Bartolomeu Universidade Federal do Ceara (6a)



SEM HV: 20.00 kV WD: 11.2000 mm VEGA\\ TESCAN
View field: 3.05 μ m Det: SE 500 nm
Date(m/d/y): 11/11/08 Bartolomeu Universidade Federal do Ceara (6b)



SEM HV: 20.00 kV WD: 12.8350 mm VEGA\\ TESCAN
SEM MAG: 48.59 kx Det: SE 500 nm
Date(m/d/y): 10/29/08 guest Universidade Federal do Ceara (6c)

Figure. 6 a: Sample of Control10 at higher resolution. b: Sample PACT5: *S. mutans* displayed elongated forms with lost connections. c: PACT10: exhibited disintegration or fusion with amorphous masses.

4 CONCLUSÃO GERAL

Da avaliação dos resultados obtidos neste trabalho, pode-se concluir que:

- Nas condições desse estudo *in vitro*, o uso da terapia fotodinâmica foi capaz de atuar na viabilidade do *Streptococcus mutans* e de promover sua redução em cárie dental produzida artificialmente;

- A aplicação da terapia fotodinâmica apresentou parâmetros seguros em relação à temperatura não promovendo alterações que possam levar a danos pulparem com as densidades estudadas;

- A terapia fotodinâmica antimicrobiana pode ser um possível adjunto terapêutico no tratamento de lesões de cárie dentinária profunda.

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APÊNDICE A

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

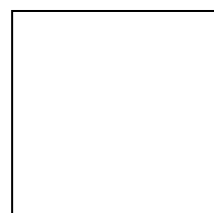
Título da pesquisa: **DETERMINAÇÃO DE PARÂMETROS EFETIVOS E SEGUROS PARA O USO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM DENTINA CARIADA: ESTUDO *IN VITRO***

Esta pesquisa tem o objetivo de avaliar a ação antimicrobiana da terapia fotodinâmica sobre dentes humanos. As amostras utilizadas nos experimentos serão de dentes que têm indicação para extração, conforme diagnóstico e planejamento do profissional responsável pelo atendimento clínico. Portanto, **não existe nenhum risco ao paciente que fará a doação destes dentes para a pesquisa**, já que os dentes serão utilizados apenas em testes laboratoriais. A identidade dos doadores dos dentes não será divulgada por qualquer meio, e o material recolhido será utilizado unicamente para a presente pesquisa. Qualquer esclarecimento adicional poderá ser obtido com os responsáveis pela pesquisa: Dra Mary Anne Sampaio de Melo (3254 38 41- 88641812); Dra Lidiany Karla Azevedo Rodrigues (3366 84 10-3366 84 26), ou com o comitê de Ética em pesquisa da UFC (4009 83 38)

Eu, _____, após ter sido devidamente esclarecido (a) dos objetivos da pesquisa acima mencionada, aceito voluntariamente participar, através da doação aos pesquisadores dos meus dentes que foram extraídos, e concordo que os mesmos sejam utilizados para os fins a que se propõe a pesquisa.

Fortaleza, ____ - ____ - ____

Testemunha



Assinatura paciente – doador

Mary Anne Sampaio de Melo
Pesquisadora responsável

Nº documento de identificação

APÊNDICE B

DECLARAÇÃO DE DOAÇÃO

Declaro ao Comitê de Ética em Pesquisa da Universidade Federal do Ceará (UFC), a concessão de _____ dentes humanos ao pesquisador(a) Mary Anne Sampaio de Melo, a fim de viabilizar a execução do Projeto de Pesquisa intitulado: “**DETERMINAÇÃO DE PARÂMETROS EFETIVOS E SEGUROS PARA O USO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM DENTINA CARIADA: ESTUDO *IN VITRO***”. Os dentes foram extraídos em _____, previamente à Resolução 196/96, do Conselho Nacional de Saúde e armazenados em solução fisiológica, sem identificação dos doadores.

Afirmo que a indicação da extração fundamentou-se em exames clínicos e/ou radiográficos, não tendo relação com o desenvolvimento da pesquisa científica.

As informações acima mencionadas são verdadeiras e de minha inteira responsabilidade, sendo que estou ciente das suas eventuais repercussões cíveis e penais.

Fortaleza, ____ de _____ de _____.

Nome: _____

Assinatura: _____

CRO: _____

ANEXO A – Seguimento do regimento interno

UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM

§2º - No caso de não cumprimento do prazo estipulado no §1º, o orientador deverá encaminhar, antes de seu vencimento e ouvido o aluno, solicitação de ampliação do prazo, mediante justificativa e descrição da etapa de desenvolvimento do projeto.

§3º - O aluno que não obtiver aprovação no Exame Geral de Conhecimentos, terá direito à nova oportunidade, desde que respeitados os artigos 4 e 5 das Normas para os Cursos de Pós-Graduação da UFC.

§4º - O aluno só poderá defender a Dissertação após aprovação no Exame Geral de Conhecimentos de que trata este artigo.

Artigo 46 – As dissertações apresentadas ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará poderão ser produzidas em formato alternativo ou tradicional. O formato alternativo estabelece: a critério do orientador e com a aprovação da Coordenação do Programa, que os capítulos e os apêndices poderão conter cópias de artigos de autoria ou co-autoria do candidato, publicados ou submetidos para publicação em revistas científicas, escritos no idioma exigido pelo veículo de divulgação.

§1º - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

§2º - A dissertação em formato tradicional ou as sessões gerais do formato alternativo deverão seguir as normas preconizadas pelo Guia para Normalização de Trabalhos Acadêmicos da Biblioteca Universitária disponível no site <http://www.biblioteca.ufc.br/servicos.html#apoio>. As partes específicas do formato alternativo deverão ser feitas em concordância com o *MANUAL DE NORMALIZAÇÃO PARA DEFESA DE DISSERTAÇÃO DE MESTRADO E TESE DE DOUTORADO NO FORMATO ALTERNATIVO do PPGO*.

Artigo 47 – Para cada aluno deverá ser constituída uma banca examinadora, que será formada por 03 (três) professores ou especialistas, com o título de Doutor, como membros efetivos e dois suplentes.

§1º - Os membros da banca examinadora de que trata o *caput* deste artigo constituirão a Comissão Julgadora, cuja presidência caberá ao orientador da Dissertação.

§2º - Dentre os membros efetivos da banca examinadora, 01 (um) deverá ser professor ou especialista de outra Instituição, com título de Doutor, sugerido pelo orientador e homologado pela Coordenação do Programa.

§3º - Dentre os membros suplentes da banca examinadora, 01 (um) deverá ser professor ou especialista de outra Instituição, com título de Doutor, sugerido pelo orientador e homologado pela Coordenação do Programa.

§4º - Quando na orientação da dissertação houver a participação de co-orientador, este não poderá participar da banca examinadora.

ANEXO B – Protocolo de aprovação COMEPE

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. Nº 324/2006

Fortaleza, 31 de julho de 2006

Protocolo nº 143/06

Pesquisador responsável: Lidiany Karla Azevêdo Rodrigues

Deptº./Serviço: Departamento de Odontologia/UFC

Título do Projeto: "Estudos *in vitro*, *in situ* e *in vivo* do efeito antimicrobiano da terapia fotodinâmica sobre lesões de cárie dentinária"

Levamos ao conhecimento de V.Sª, que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução nº196 de 10 de outubro de 1996 e Resolução nº 251 de 07 de agosto de 1997, publicadas no Diário Oficial, em 16 de outubro de 1996 e 23 de setembro de 1997, respectivamente, aprovou o projeto supracitado na reunião do dia 06 de julho de 2006.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório parcial e final do referido projeto.

Atenciosamente,

Dr. Fernando A. Frota Bezerra
Coordenador do Comitê
de Ética em Pesquisa
COMEPE/UFC

ANEXO C – Certificado de edição para língua inglesa do artigo



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American Journal Experts Editorial Certification

This document certifies that the manuscript titled "Safe and effective parameters for using photodynamic antimicrobial chemotherapy in carious dentine: an in vitro study" was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts. Neither the research content nor the authors' intentions were altered in any way during the editing process.

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Manuscript title: Safe and effective parameters for using photodynamic antimicrobial chemotherapy in carious dentine: an in vitro study

Authors: Mary A.S. de-Melo¹, DDS; Diego M. de-Paula¹, Dentistry undergraduate student; Juliana P. M. Lima¹, DDS; Fátima M.C. Borges¹, DDS; Carolina Steiner-Oliveira², MS; Eduardo B. Barros⁴, PhD; Marina Nobre-dos-Santos², PhD; Iriana C.J. Zanin³ PhD; Lidiany K.A. Rodrigues PhD.

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ANEXO D – Confirmação de submissão do artigo ao periódico



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Title: Safe and effective parameters for using photodynamic antimicrobial chemotherapy: an *in vitro* study

de-Melo, Mary
de-Paula, Diego
Lima, Juliana

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