IN VITRO LETHAL PHOTOSENSITIZATION OF *S. MUTANS* USING METHYLENE BLUE AND TOLUIDINE BLUE O AS PHOTOSENSITIZERS

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ABSTRACT

The purpose of this in vitro *study was to evaluate the antimicrobial effect of photodynamic therapy on Streptococcus mutans (ATCC 25175) suspensions, using a red laser for one minute in combination with toluidine blue O (TBO) or methylene blue (MB). Both photosensitizers were used in three concentrations (25, 10 and 5 mg/L). The activity of photosensitizers and laser irradiation were tested separately on the bacteria, as well as the irradiation of this light source in the presence of the TBO or MB. These groups were compared to a control group, in which the microorganism did not receive any treatment. The activity of both TBO and MB or laser irradiation, alone, were not able to reduce the number of S.mutans. In the groups of lethal photosensitization, a bacterial reduction of 70% for TBO and 73% for MB was observed when these photosensitizers were used at 25 mg/L and a reduction of 48% was observed for MB at 5mg/L. In other concentrations there were no significant differences in comparison to the control group. Both the TBO and the MB at 25 mg/L associated with a red laser had an excellent potential for use in PDT in lethal sensitization of S. mutans.*

Key words: photodynamic therapy, S. mutans, lasers, photosensitizer.

ESTUDO *IN VITRO* DO EFEITO DA TERAPIA FOTODINÂMICA SOBRE O *S. MUTANS* UTILIZANDO-SE AZUL DE TOLUIDINA OU AZUL DE METILENO COMO AGENTES FOTOSSENSIBILIZANTES

RESUMO

O objetivo deste estudo in vitro *foi avaliar o efeito antimicrobiano da terapia fotodinâmica em suspensões de Streptococcus mutans (ATCC 25175), utilizando um laser vermelho durante um minuto associado a dois agentes fotossensibilizantes: azul de toluidina (TBO) ou azul de metileno (MB). Os agentes fotossensibilizantes foram utilizados em três diferentes concentrações (25, 10 and 5 mg/L). A atividade destes agentes e da fonte de luz foi testada separadamente sobre a suspensão bacteriana, assim como a irradiação desta fonte de luz na presença de TBO ou MB (terapia fotodinâmica). Estes grupos foram comparados a um grupo controle, onde nenhum tratamento foi realizado. A aplicação dos dois fotossensibilizantes (TBO ou MB) e da fonte de luz, separadamente, não foi capaz de reduzir o numero de colônias viáveis do S. mutans. Nos grupos onde a terapia fotodinâmica foi aplicada, uma redução bacteriana de 70% foi observada para o TBO e de 73% para o MB, quando estes agentes foram utilizados na concentração de 25 mg/L. O uso do MB a 5mg/L causou uma redução de 48%. Para as outras concentrações testadas não se observou nenhuma redução em relação ao grupo controle. Pode-se concluir que tanto o TBO quanto o MB a 25 mg/L associados ao laser vermelho demonstraram um excelente potencial para promover a fotossensibilização letal do S. mutans.*

Palavras chave: terapia fotodinâmica, S. mutans, lasers, fotossensibilizantes.

INTRODUCTION

Dental caries is a disease which, after demineralization of the enamel has occurred, progresses slowly down into the dentine. The lesion consists of an advancing zone of demineralization behind which is a zone of partially demineralized dentine infected with bacteria[1]. The difficulties in determining the amount of tissue removal necessary clinically and the inadequacies of most restorative materials currently available in effectively achieving a long-term seal means that an effective means of disinfecting both the infected and affected tissue is highly desirable before completion of treatment. If bacteria in infected but only partly demineralized tissue could be killed, even more tissue could be retained[2].

It is well known that the accumulation of bacterial biofilms on tooth surfaces results in some of the most prevalent bacterial-induced human diseases, caries and inflammatory periodontal diseases[3]. The prevention of caries (primary prevention), and the control

of disease progression (secondary prevention), focus mainly on mechanical and/or chemical biofilm reduction[4] such as the use of antiseptics and antibiotics[3]. Antibacterial agents are widely used in the treatment of oral diseases, but problems of development of bacterial resistance mean alternative strategies are required to control bacterial plaque biofilm and treat caries, gingivitis and periodontal disease[5].

With ever-increasing levels of antibiotic resistance, light-activated antimicrobial agents (photosensitizers or PS) are becoming an attractive alternative to conventional antibiotics[6]. Photodynamic therapy (PDT) is an established treatment for localized tumors, involving the application and retention of an applied photosensitizing agent in malignant tissues and a substantial body of work has shown that this photodynamic approach can also be used to kill bacteria[7].

PDT is a therapy modality which employs the combination of visible light, a drug (called photosensitizer or dye) and molecular oxygen usually present in the tissue. This photosensitive agent can be a molecule normally present in cells and tissues, but in the specific case of PDT its administration is generally the first step in the treatment process. In the second step, the targeted tissue is exposed to visible light at a wavelength specific for each dye, which is absorbed by the photosensitive agent. The combination of the two agents in the presence of oxygen leads to the production of different reactive oxygen species such as singlet oxygen ($^{-1}O_2$), which will lead to a sequence of biological events resulting in the apoptosis of the cells or death of the microorganisms[8].

PDT has two main advantages over conventional antibiotic treatments. First, the bactericidal activity is confined to areas which have been treated using the photosensitizer and light—avoiding disruption of the indigenous microbiota at sites distant from the infected area. Second, the development of resistance to $^{-1}O_2$ by bacteria is unlikely due to its non-specific mode of action[6].

According to Wilson[9], several species of oral bacteria, in the presence of an appropriate photosensitizing agent, can be killed by light from a low-power laser. Susceptible species include the plaque-forming and cariogenic-species *S. sanguis, S. mutans, S sobrinus, L casei* and *A. viscosus*.

The aim of this *in vitro* study was to evaluate the antimicrobial effect of PDT on *S. mutans* suspensions, using a red laser in combination with TBO or MB as photosensitizers.

MATERIAL AND METHODS

Photosensitizers and light sources

TBO was dissolved in distillated water and stored in the dark and methylene blue was obtained from Quimiolux® (Brazil). Both photosensitizers were used at three concentrations: 25, 10 and 5 mg/L.

The light source used was a red laser (TwinFlex®, MM Optics, Brazil) in the wavelength of 660 nm, a power output of 40 mW and a light intensity of 1000 mW/cm^2. To reach a 60 J/cm^2 energy density, red laser was used for 1min.

Bacterial culture

The microorganism used in this study was *S. mutans* (ATCC 25195); it was maintained by subculture on *mitis salivarius* agar (Acumedia Manufacturers®, Inc. Lansing, Michigan) and 24 hours before the experiment it was placed to grow on brain heart infusion broth (BHI).

Standardized suspensions of *S. mutans* were prepared, adjusting the initial turbidity of the bacteria culture to A600 nm = 0.5 (~10^9 cells/ml), as described by Paulino et al.[8] using a spectrophotometer (SpectrumLab 22PC®). The BHI broth containing the overnight culture of *S. mutans* was added to a sterile peptone saline solution until it reached the desired concentration (~10^9 cells/ml), which was confirmed by the spectrophotometer measurement. The entire experiment was performed under aseptic conditions in laminar air flow chamber.

Photodynamic therapy

Ten groups with three samples each were tested (n=3). The control group (G1) contained 1 ml of the standardized suspension (inoculum), prepared as described above. To evaluate the light toxicity *per se* without any dye, 1ml of the standardized suspension was irradiated by red laser light for 1 min (G2). To evaluate the dye toxicity *per se*, the PS in their higher concentrations were left in contact with bacterial suspensions for 5 minutes in the dark, using MB on group G3 and TBO on group G4. Groups 5 to 10 were submitted to PDT, varying the PS concentration. In groups 5, 6 and 7, MB was used at concentrations of 25, 10 and 5 mg/L, respectively. For groups 8, 9 and 10, the PS was TBO, used at the same concentrations as mentioned above. For all PDT groups, exposure time was 1 min and before irradiation all these groups were maintained in contact with the PS for 5 min in the dark (pre-irradiation time).

Serial dilutions of 10^4 UFC were obtained and aliquots of 100 µl were plated in triplicate on *mitis salivarius* agar. After incubation in a candle jar, in a microaerophilic atmosphere at 37°C for 36 hours, the number of viable colony forming units (CFU) was obtained by visual counting. The dependent variables were type and concentration of photosensitizer. To determine the significance of the irradiation alone, the presence of sensitizer alone and the combination of sensitizer and light, the data were analyzed by a variance analysis (ANOVA) model using the factorial (2x2) design. The Tukey test was chosen for evaluating the significance of all pairwise comparisons with a significance limit of 5%.

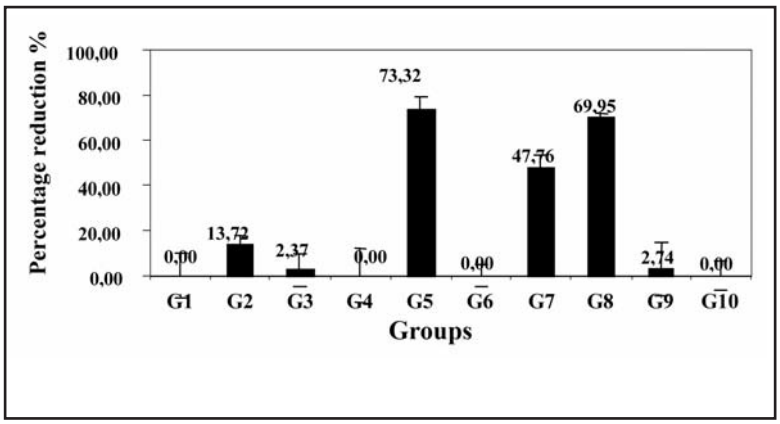

Fig. 1: Percentage reduction of the number of viable CFU in relation to control group (G1-inoculum). G2 (red laser alone); G3 (MB alone); G4 (TBO alone); G5, G6 and G7 (PDT: red laser + MB 25, 10 and 5 mg/L, respectively); G8, G9 and G10 (PDT: red laser + TBO 25, 10 and 5 mg/L, respectively).

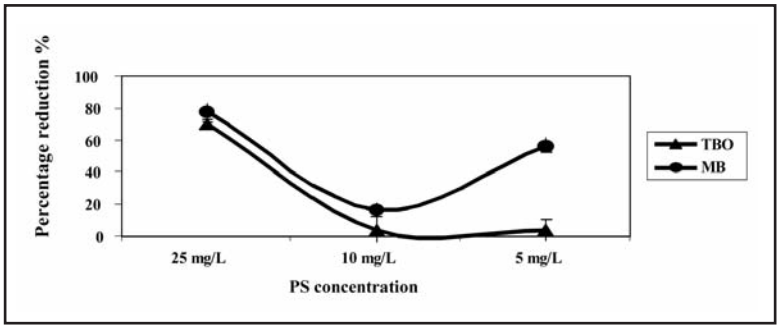

Fig. 2: Percentage reduction of the number of viable CFU with varying PS concentration. MB is represented by circles and TBO by triangles.

RESULTS

Neither irradiation of the suspensions in the absence of photosensitizer, nor incubation with dye alone had significant effects on the viabilities of the streptococcal suspensions ($p<0.05$). The control group (G1) and the groups without PDT (G 2, 3 and 4) showed no statistically significant difference, although G2 (red laser alone) showed a small decrease in the number of viable microorganisms (13%). The results are shown in Fig. 1.

Both photosensitizers caused a decrease in the number of colony forming units, when used at a concentration of 25 mg/L, demonstrating the bactericidal effect of PDT after one single application of red laser for 60 seconds. For that concentration, there was no significant difference between MB and TBO. The combination of MB at 25 mg/L and red laser (G5) produced a 73% reduction in the number of viable CFU. For TBO, in the same concentration and in the presence of light (G8), the reduction was 70% when the groups were compared to the control group.

When smaller concentrations were tested, the association of MB at 5 mg/L and red laser (G7) showed a percentage reduction of 48%, which was not as effective as the same photosensitizer used at 25 mg/L.

No significant reduction in the viability of *S. mutans* was presented either for MB at 10 mg/L nor for TBO (10 or 5mg/L), when these PS were used together with red light. Fig. 2 shows the percentage reduction in the number of viable CFU for varying PS concentration.

DISCUSSION

This research found an effective reduction in the viable numbers of *S. mutans* after photosensitization, which is significant, as this species is amongst those reported to be most highly associated with caries in humans[10]. It was observed that both variables evaluated, type and concentration of the photosensitizer, could affect the results of PDT.

TBO is a cell membrane active photosensitizer[10], which can absorb red laser light and is bactericidal for multiple species, including organisms such as streptococci that are implicated in dental caries[1,11]. In other studies[3,12] this photosensitizer was found to be effective *in vitro*, even in the presence of dem-

ineralized dentine and collagen[13]. It has been shown that the light dose and the PS concentration required to kill bacteria treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts[3].

Methylene blue has been widely used by several European transfusion services in the photodecontamination of blood plasma and has been shown to be particularly effective in the inactivation of viruses[10]. According to Ivanov et al.[14] the concentration of photosensitizers as MB and TBO could not be higher than 0,1% (1000 mg/L) because above this concentration they demonstrate toxicity to cells and could stain the dentine, making the treatment esthetically unviable.

In others studies, it was demonstrated that significant killing of the cariogenic organism *S. mutans* by PDT was possible *in vitro*: Zanin et al.[3] demonstrated a 95% reduction in the viability of *S. mutans* biofilms after lethal photosensitization using a light-emission diode combined with TBO. In a study analyzing bacteria in supragingival plaque scrapings, Wilson et al.[15] found that substantial kills (97%) could be achieved by a helium/neon laser (HeNe) in the presence of TBO. It has also been shown previously that the viability of *S. mutans* biofilms can be reduced (99%) by TBO associated with a HeNe laser or a light emmiting-diode[12].

According to de Souza *et al* [16] a great number of variables may influence the number of microorganisms affected by photodynamic therapy, including the type and concentration of the PS, the microorganism's physiological stage, photosensitizer incubation period before the irradiation, pre irradiation time, light exposure period and density of laser energy. Compared to the data in literature, this study found a smaller percentage reduction of *S. mutans* for both photosensitizers tested. It could be explained by the fact that we used shorter pre-irradiation and exposure times than those used in other studies described in the literature, because we tried to test the efficacy of an antimicrobial therapy that could be reproduced *in vivo*. Moreover, we used a low-power red laser – a light source commonly used in dentistry – thus making PDT more easily accessible to dentists.

As reported by Zanin et al.[3], dental caries may be a disease well suited to photosensitization therapy. Caries is often a localized infection, and so the sensitizer could be applied to the lesion by means of a syringe and the light could then be delivered via an optical fiber. If bacteria within carious lesions could be eradicated by photosensitization *in vivo*, there would be beneficial consequences for dental health. Infected or damaged dentine could be better preserved, thereby making patient treatment easier (for both dentist and patient) by enabling lesions to be restored with minimal tissue removal, and improving the long-term prognosis for the repaired tooth. The potential advantage of this over conventional caries treatment would be the ability to kill the bacteria *in situ* and then restore the site without the removal of the softened and demineralized dentine. This is clinically attractive, as it would reduce the amount of tooth tissue required to be removed[13].

The facility for killing the bacteria within the lesion would allow more objective decisions as to the amount of dentine which should be removed. The practitioners could apply the dye to the carious lesion within the cavity and then irradiate the site, after which a protective bacteriostatic lining could be placed. This would reduce the risk of recurrent caries while providing the clinician with a more objective means for removing those bacteria which are known to contribute to spread of caries in dentine, at the same time maintaining the demineralized dentine structure. This in turn would reduce the amount of tissue to be removed during cavity preparation[1].

Further works are now being undertaken to determine the efficacy of PDT on carious dentin *in vivo*, because according to Ten Cate et al.[17], bacteria in a carious lesion have a physiology different from that of planktonic cells. Nevertheless, this *in vitro* study was useful to determine the best type and concentration of the PS, as well as the best pre-irradiation and exposure time to be used with a specific light source. The antimicrobial effect of PDT on *S. mutans* suspensions was demonstrated as the result of this study and it will be used to define a clinical protocol in order to apply the photodynamic therapy *in vivo*.

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