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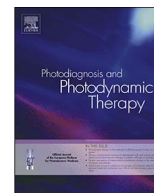

Antimicrobial photodynamic therapy on *Streptococcus mutans* is altered by glucose in the presence of methylene blue and red LED

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ABSTRACT

Background: Dental caries are a multifactorial disease that progressively produces tooth destruction as a result of bacterial colonization of enamel surface, especially *Streptococcus mutans*. The objective of this work was to investigate the role of glucose in antimicrobial photodynamic therapy (aPDT) on *S. mutans*.
Methods: *S. mutans* ATCC 25175 were cultured on microaerophilia at 37 °C for 48 h, and we tested aPDT in the presence of 50 mM glucose. Bacterial suspension was used to investigate aPDT with 100 μM methylene blue (MB) under LED emitting radiation at λ = 660 nm and parameters as following (P = 473 mW; I = 166.8 mW/cm^2, and doses of 5, 10 and 20 J/cm^2). A seventy-two hours biofilm was grown on 96 flat buttoned well-plate and irradiation was performed from 10 to 80 J/cm^2 at similar conditions.
Results: There was no dark toxicity nor bacterial death regarding LED irradiation on suspension and on biofilm. Nevertheless, aPDT presented expressive bacterial inactivation following 1 and 2 min of irradiation on cell suspension. On the other hand, there was no inactivation in the presence of glucose under the same conditions. Biofilm was completely inactivated by MB-mediated aPDT after 6 min of irradiation. However, the presence of glucose delayed the complete inactivation of the biofilm.
Conclusion: The presence of glucose in the suspension drastically delayed the effect of aPDT on *S. mutans* and this effect is more pronounced in bacterial suspension than on biofilm.

1. Introduction

Dental caries are considered a multifactorial disease characterized by localized and progressive destruction of the tooth, a process that results from colonization of the enamel surface by microorganisms, especially *Streptococcus mutans* [1,2].

The cariogenic properties of *S. mutans* are regulated by several genes involved in basic metabolic pathways: microbial adhesion, biofilm formation, extracellular polysaccharide synthesis, carbohydrate uptake and acid tolerance [3].

The bacterial biofilm is characterized as a community of bacteria from a single or several bacterial species, which adhere to a solid surface. For biofilm formation metabolic and physiological changes are necessary [2,4,5]. *Quorum sensing* mechanism is responsible for controlling various physiological processes. It is an intercellular communication mechanism that controls the expression of genes in response to population density. The ability of bacterial cells to communicate and behave collectively provides a number of benefits in terms of mass colonization, defense against other types of microorganisms, and cell differentiation [6,7].

S. mutans normally inhabits a complex biofilm. The bacterium produces a large amount of exopolysaccharides, especially in the presence of glucose facilitating the adhesion process [2,4,5]. Photodynamic therapy (PDT) emerges as an alternative treatment for diseases caused by biofilms, such as dental caries. This therapy combines a photosensitizer (PS), that will be absorbed by microbial cells followed by irradiation with resonant light, resulting in cell death [8–10]. The objective of this work was to investigate the role of glucose in antimicrobial PDT on *S. mutans*.

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2. Materials and methods

Experimental procedures were performed with *S. mutans* ATCC 25175 cultured under microaerophilia on Agar broth and heart (BHI) medium and incubated at 37 °C for a period of 48 h.

2.1. Photosensitizer

The methylene blue (MB) (Sigma-Aldrich, St Louis, USA) was used as photosensitizer. The powder was diluted in distilled water to a concentration of 10 mM and the mother solution was filtered through a sterile 0.22 μm membrane (Milipore, SP, Brazil) and stored at 5 °C protected from light. In the moment of the experiment, the mother solution was diluted 1/100 to generate final concentration of 100 μM MB.

2.2. Light source

The light source used was the LED (Light Emited Diode) developed by Prof. Dr. Alessandro Melo Deana from Nove de Julho University, emitting radiation at λ = 660 nm, with a power of 473 mW, an irradiance of 166.8 mW/cm^2 and radiant exposure of 5, 10 and 20 J/cm^2 (Table 1).

2.3. PDT on suspension

Bacterial cells were suspended in PBS pH. 7.2 by harvesting *S. mutans* cells from BHI agar. Bacteria were incubated with MB for 10 min in dark conditions and an aliquot was taken from each sample before irradiation to quantify the number of viable cells (on control and dark toxicity). All experiments were performed with and without 50 mM glucose and cells were incubated for 60 min. At 50 min of incubation with glucose, the PS was added (at a final concentration of 100 μM) performing total 10 min of contact with PS. Following incubation, the cell suspensions were placed into a flat-bottomed 24-well plate (Fisher Scientific) for irradiation. The experiments were divided into the following groups: Control (is the cells without any treatments), LED (is cells irradiated without any methylene blue), Dark toxicity (is time 0 with cells with MB and non-irradiated), and PDT (30, 60 and 120 s). After irradiation procedures, each group was serially diluted to give dilutions of 10^{-1}–10^{-7} times the original concentrations, and then 10 μL of each dilution were streaked over the surfaces of BHI plates in triplicate. The plates were incubated at 37 °C for 24–48 h under a 5–10% CO_2 atmosphere. The bacterial colonies were counted and converted into colony forming units (CFU) for analysis. The experiments were replicated in a minimum of 3 times in independent days.

2.4. PDT on bacterial biofilm

S. mutans was cultured into a 96 well-plate with BHI for 72 h period for biofilm formation. The medium was changed every day. Following the biofilm formation the BHI was removed from the wells. After that, the glucose groups were treated with 50 mM glucose for 60 min 100 μL of MB was added to the well and the dark toxicity time was set at 10 min. The experiment was divided into 5 groups without glucose and 5 groups with glucose as follow: control, PDT 0, 60, 240, 360, 390, 450, and 480 s. After irradiation procedures, the dilution and striation were performed on petri dishes as described before on *S. mutans* suspension. The plates were incubated in the same conditions and the CFU were obtained for analysis.

The data were presented as mean and standard deviation, and differences between groups were accessed using the One-Way variance test (ANOVA). The comparison of means was performed by the Tukey test and the significance was set at 5%.

Table 1
Irradiation parameters.

Wavelength (nm)	660 ± 15
Beam diameter (cm)	1.9
Beam area (cm^2)	2.8
Dark toxicity	10 min group without glucose 10 min group with glucose
Irradiation time (s)	0, 30, 60 120 s (suspension) 0, 60, 240, 360, 390, 450, 480 s (biofilm)
Radiant Output power (mW)	473
Irradiance (mW/cm^2)	166.8
Radiant exposure (J/cm^2)	5,10, 20 (*S. mutans* suspension) 10, 40, 60, 65, 75, 80 (on Biofilm)

3. Results

Bacteria suspension presented cell concentration of 1×10^8 CFU/mL in the control group. For all experiments, there were no dark toxicity nor antimicrobial effect of light (660 nm) without photosensitizer. In experiments without glucose, the PDT 30 s group showed a significant amount of cell death when compared to the PDT 0 s group (2 logs) (Fig. 1). Total bacterial killing of *S. mutans* was observed with the irradiation time of 2 min. On the other hand, experiments with irradiation at 660 nm for up to two minutes in the absence of photosensitizer had no effect on the number of colony forming cells (Fig. 1).

The results of the experiments performed on bacterial biofilms treated with PDT 0 min showed no significant difference when compared to the cell death presented by the control group of both experiments. In the experiments performed without glucose the group PDT 1 min presented cell death in a significant amount when compared to the group PDT 0 min (Fig. 2). Comparing the 4 min PDT group we also observed a significant amount of cell death compared to the 1 min PDT group. We observed a complete cell death at 6 min of irradiation in the experiments without glucose. However, complete cell death in experiments with glucose was observed only following 8 min of irradiation (Fig. 2).

4. Discussion

The relationship between sugars and oral health is dynamic. Bacteria are stimulated to produce acids and consequently to lower the pH. The ingestion of sugars is directly linked to the production of bacterial biofilms. Studies prove that oral bacteria are susceptible to PDT. Due to bacterial resistance the PDT has become feasible for treatments of diseases related to biofilms, such as dental caries [11,8].

The ability of *S. mutans* to rapidly transport and metabolize a wide range of sugars whenever they become available may be directly related to their survival in dental plaque and its cariogenic potential in humans [12]. Zanin et al. concluded that *S. mutans* biofilms are susceptible to LED light in the presence of toluidine blue O (TBO), suggesting that this approach may be useful in the treatment of dental plaque-related diseases [8].

Rolim et al., reports that MB-mediated antimicrobial PDT damages the outer cell membrane to a lesser extent than its ability to cause bacterial cell DNA damage. Furthermore, the lack of MB antimicrobial effect may be due to the photosensitizer pre-illumination time. The 5 min pre-irradiation time used by it may not have been long enough for the MB to reach its specific target. In our studies we used the 10 min pre-irradiation time, which resulted in no cell death of 99.9% at certain irradiation times, which is in line with what he stated in his studies [13].

As *S. mutans* is considered one of the main etiological agents of dental caries and is able to make the biofilm more cariogenic in the presence of glucose, we chose to use a medium that contained glucose. Glucose acts as a substrate for the synthesis of intracellular and

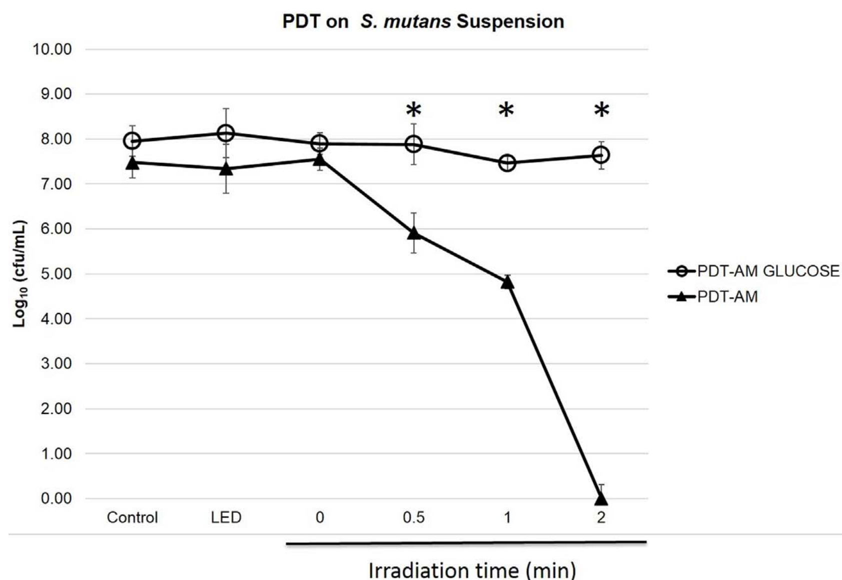

Fig. 1. Antimicrobial effect of aPDT on bacterial suspension. Open circles are *S. mutans* in the presence of 50 mM glucose; and black triangles are bacterial suspension without glucose. Control and LED groups did not presented statistical differences. Statistical significant differences ($p < 0.05$) between PDT vs PDT-GLUCOSE are presented as symbol (*). Each data are mean of at least 5 independent experiments in different days. Data are means and standard deviation.

extracellular polysaccharides, increasing acid production. The role of glucose in the dental demineralization process is well known, but there are few studies on the negative influence of different concentrations of this sugar on the processes of biofilm formation. Some authors state that the amount of glucose or sucrose supplied to *S. mutans* during biofilm formation should be limited, since in large quantities it may induce changes in biofilm metabolism, reducing its formation [14].

Fontana et al. stated that oral bacteria in biofilms are less affected by PDT than bacteria in the planktonic phase. The antibacterial effect of PDT is reduced in biofilm bacteria. In our studies we have also shown that in biofilms the PDT cell death is lower [15].

The metabolism of *S. mutans* involves several genes, membrane transporters of the ABC type and the Principal Facilitating Superfamily (MPS). These are capable of carrying a huge variety of natural or synthetic toxic products, being both endogenous and exogenous [16,17].

The ABC or MPS systems can cause MDR resistance, allowing it to function as an efflux pump, which will be able to eliminate large numbers and varieties of toxic compounds not chemically similar to the cell, characterizing a threat against the efficacy of Drugs [18,19]. Eflux is characterized as the process in which chemicals transporting bacteria, being they highly toxic compounds are thrown out of the cell. In photodynamic therapy the dyes and/or photosensitizers used are pumped out of the target cells through the efflux pumps [18]. The presence of glucose can increase ABC transport activity and increase MB eflux mechanism. It may explain the decrease of photodynamic effect in the presence of glucose. However, furthers studies are needed to explain the role of glucose on the photoinactivation of bacteria.

5. Conclusions

In conclusion, PDT is a viable solution for *S. mutans* inactivation and the presence of glucose delays the microbial inactivation. The effect of PDT is proportional to the radiant exposure of the light.

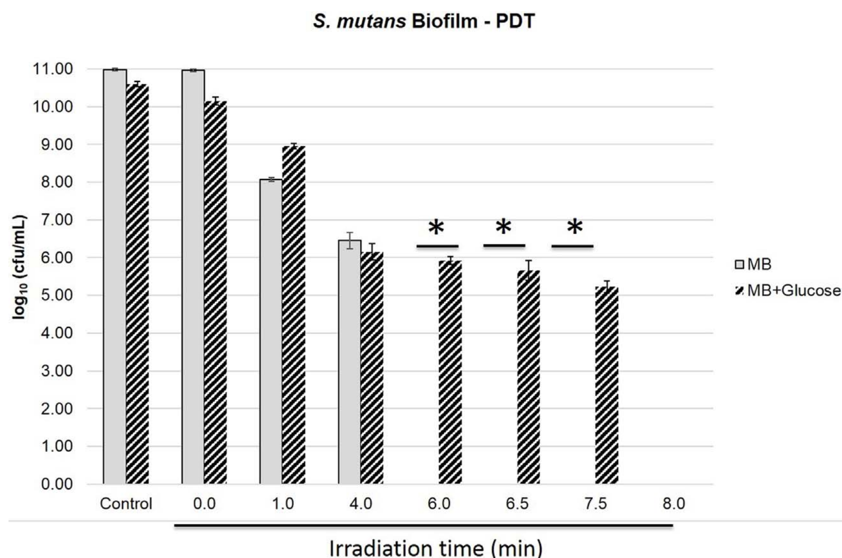

Fig. 2. Antimicrobial effect of aPDT on *S. mutans* biofilm. Dash barrs are *S. mutans* biofilm in the presence of 50 mM glucose; and gray barrs are the biofilm without glucose. Control and PS groups (0.0) did not presented statistical differences. Statistical significant differences ($p < 0.05$) between PDT vs PDT-GLUCOSE are presented as symbol (*). Each data are mean of at least 3 independent experiments in different days. Data are means and standard deviation.

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