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Addition of hydrogen peroxide to methylene blue conjugated to β-cyclodextrin in photodynamic antimicrobial chemotherapy in *S. mutans* biofilm

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ABSTRACT

Objective: This study evaluated the effect of hydrogen peroxide addition on β-cyclodextrin-conjugated methylene blue in antimicrobial photodynamic therapy(a-PDT) in *S. mutans* biofilm model using laser or light emitting diode (LED) (λ = 660 nm).

Methods: A preliminary assay was performed to evaluate the cytotoxicity of hydrogen peroxide in oral fibroblasts by the colorimetric method (MTT). Afterwards, groups were divided into (n = 3, in triplicate): C (negative control), CX – chlorhexidine 0.2% (positive control), P (methylene blue/β-cyclodextrin), H (Hydrogen Peroxide at 40 μM), PH, L (Laser), LP, LH (Laser + Hydrogen Peroxide), LPH, LED, LEDP, LEDH, and LEDPH. The biofilm was formed in 24 h with BHI + 1% sucrose (w/v). Light irradiations were conducted with laser, 9 J, 323 J/cm^2, 113 s or with LED, 8.1 J, 8.1 J/cm^2 for 90 s. Microbial reduction was evaluated by counting the viable microorganisms of the biofilm after the respective treatments, in a selective culture medium, and laser confocal microscopy evaluation.

Results: LP, LH, LPH, LEDP, LEDH, and LEDPH groups statistically reduced the counts of *S.mutans* compared with the C group and the log reductions were of 1.87, 1.94, 2.19, 0.91, 0.92, and 1.33, respectively; the addition of hydrogen peroxide did not potentiate the microbial reductions (LPH and LEDPH) compared with the LP and LEDP groups.

Conclusion: The association of hydrogen peroxide with the conjugated β-cyclodextrin nanoparticle as photosensitizer did not result in an enhanced effect of a-PDT; hydrogen peroxide behaved as a photosensitizer, since it reduced the number of *S. mutans* when associated with laser light.

1. Introduction

Dental caries, a chronic disease prevalent in adults and children worldwide can be prevented if the microbiota associated with caries, such as *Streptococcus mutans*, Actinomyces, *Lactobacillus*, and *Bifidobacterium* species, is controlled on the tooth surface and, consequently, inhibit biofilm formation [1,2]. Another way is controlling sugar intake, since excessive and frequent sugar intake associated with poor oral hygiene is responsible for the substitution of the initial, nonpathogenic biofilm, for a shifted acidogenic and aciduric colonized biofilm [3]. However, controlling sugar intake in the population is rather difficult. Therefore, innovative therapies should be studied to discover new alternatives to control biofilm [4], since the used conventional methods are not always effective. Dental brushing depends on

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the patients' collaboration [5], who often do not comply with a daily biofilm control consistent with good oral health.

In this perspective, photodynamic antimicrobial chemotherapy, which focus on the reduction of microorganisms, has been used as adjuvant therapy in the microbiological control of oral biofilms [6] and also as a complementary therapy after selective caries removal [7–9] with good perspectives in clinical use, including light sources such as lasers, LEDs and novel technologies in modern Dentistry [6].

A = PDT or PACT is based on the use of photosensitizers, molecules that absorb light and initiate a photochemical reaction when exposed to the light of specific wavelength. This process leads to the formation of reactive oxygen species, including singlet oxygen and free radicals that can cause irreversible damage to essential elements of bacterial cells, alter cell metabolism, and cause bacterial death [10,11] without affecting the host [12]. One advantage of PACT is that it can be an alternative to the use of chlorhexidine, since this antimicrobial agent shows limitations when in continued used [13] and also because it might develop drug resistance to the oral microorganisms [14].

Many studies have already shown the efficacy of PACT on oral bacteria, reducing bacterial load in cariogenic biofilms [15–19]. On the other hand, some researchers have shown that PACT has not been effective [20–23], especially in the presence of sucrose, due to the bacterial capacity to use this carbohydrate to produce polysaccharides, turning this matrix-rich biofilm, difficult to be diffused by the photosensitizer [24]. To overcome this limitation and to potentiate the PACT effect, nanoparticles of β-cyclodextrin have been associated with photosensitive agents [25–27]. Such biocompatible association has been shown to increase bioavailability of photosensitizers, thereby increasing its potency and biological effect, due to the ability of the nanoparticle to solubilize small organic molecules [28].

A different way of potentiating PACT is the association of the photosensitizer with hydrogen peroxide (H_2O_2) to increase the death of microorganisms by two action mechanisms: 1) a chemical reaction between H_2O_2 and reactive oxygen species produced during the PACT that will improve the photosensitizer's photochemistry or 2. due to the presence of H_2O_2 which will facilitate the photosensitizer diffusion inside the microbial cells [29]. Another hypothesis is that during irradiation, the medium oxygen pressure decreases [30] leading to oxygen depletion and consequent degradation of the photosensitization reaction. Therefore, if we increase the oxygen dissolved in the medium [31], for instance, in H_2O_2 form, the oxygen depletion could be avoided, and the PACT reaction could be potentiated. Hence, associating hydrogen peroxide with a photosensitizer (methylene blue conjugated to β-cyclodextrin) in antimicrobial photodynamic therapy may be effective in reducing the number of microorganisms in a *S. mutans* biofilm model.

Therefore, we aimed to evaluate the effect of the addition of hydrogen peroxide associated with methylene blue conjugated to β-cyclodextrin on photodynamic antimicrobial chemotherapy in *S. mutans* oral biofilm using red laser or red LED light sources (λ = 660 nm).

2. Materials and methods

2.1. Experimental design

A preliminary assay was performed to evaluate the cytotoxicity of hydrogen peroxide in oral fibroblasts by the colorimetric method. *S. mutans* biofilms were produced in 96-well plates for 24 h. PACT was tested by the count of viable microorganisms in a selective medium and by laser confocal microscopy evaluation after the treatments, which were conducted in groups described in Table 1 (n = 3), in triplicate.

2.2. Cytotoxicity of hydrogen peroxide in human oral fibroblasts

The cytotoxicity of hydrogen peroxide (H_2O_2) was evaluated, in duplicate, in human oral fibroblast (ethical approval CAAE protocol # 65011017.9.0000.5418) by the enzymatic reduction of a yellowish and soluble tetrazolium salt, 3- (4,5-dimethyl thiazolyl-2) -2,5-diphenyltetrazolium bromide (MTT) (Thermo Fisher Scientific, USA), in purple and insoluble formazan crystal, performed by reductive enzymes of viable and metabolically active cells. Formazan is solubilized in ethanol and its concentration was determined by optical density (colorimetric reading), reflecting the numbers of viable metabolically active cells. To do so, human oral fibroblast cells at passage 8 (P8) were cultured in standard medium: DMEM medium (Gibco, USA) supplemented with 10% inactivated fetal bovine serum – FBS (Gibco, USA) and penicillin antibiotic solution (100 units/mL) / streptomycin (100 µg/mL) (Gibco, USA) in a 37 °C incubator at 5% CO_2. Cells were plated at an initial concentration of 7.5×10^4 cells/mL in a 96-well plate (200 µL volume/well) and cultured for 24 h for cell adhesion. After cell adhesion, cells were cultured for additional 24 h in DMEM medium in the absence of FBS for cell synchronization before H_2O_2 (Exodo cientifica, Brazil) exposure. Cells were then cultured in standard medium for 1 h in the incubator at several H_2O_2 concentrations (diluted in the medium), as follows (n = 8 wells/group): H5 (5 µM H_2O_2); H10 (10 µM H_2O_2); H25 (25 µM H_2O_2); H50 (50 µM H_2O_2); H100 (100 µM H_2O_2); H200 (200 µM H_2O_2); H300 (300 µM H_2O_2); H400 (400 µM H_2O_2); and H500 (500 µM H_2O_2). Cells cultured in the same period in standard medium without H_2O_2 served as Control (C), and cells cultured in standard medium with Triton 1% served as cytotoxicity positive control (C +) for the MTT test. After 1 h of H_2O_2 exposure, the medium was removed, cells were washed with phosphate buffer solution (PBS), and a solution of 0.3 mg/mL MTT (diluted in medium) was placed in each well, and the cells were incubated at 37 °C/5% CO_2 for 3 h for conversion of MTT to formazan crystals. Additional wells with medium + MTT, without cells, served as blank for reading (Blank); then, the MTT medium was removed from the wells and absolute ethanol was placed for formazan crystals solubilization. Finally, a colorimetric reading was performed (absorbance) with a 570 nm wavelength (VERSAMax Microplate Reader, Molecular Devices, USA).

2.3. Biofilm formation

Biofilms were formed following a single species biofilm protocol. Briefly, *S. mutans* (UA 159) was plated onto Mitis Salivarius agar and after incubation for 24 h at 37 °C and 10% CO_2, colonies were transferred to brain heart infusion broth (BHI - brain-heart infusion, Merck, Darmstadt, Germany) supplemented with 1% (w/v) sucrose for another 24 h at 37 °C and 10% CO_2. The suspension was adjusted to A550 nm = 0.5 (1.0×10^8 cells/mL) by spectrophotometry (Spectronic Unicam, Model Genesys 10 UV Rochester, NY USA) and this inoculum was further used in the biofilm formation process.

Three independent 96-well plates (U-shaped) were used for biofilm formation to avoid light interference among the laser-irradiated groups when 2 columns were left empty. Therefore, the first plate represented the groups without light, and the second and third plates, the groups of the laser and LED light sources, respectively. Each plate well, in triplicate, received a 200 µL standardized suspension of *S. mutans*, containing BHI nutrient medium supplemented with 1% sucrose, which were incubated for 24 h at 37 °C in a 10% CO_2 partial atmosphere for the microorganism growth and multiplication [32].

2.4. Photodynamic antimicrobial chemotherapy (PACT)

The preparation of the encapsulated β-cyclodextrin methylene blue was performed in a spray-dryer equipment (Buchi, Switzerland, model B-290), where inlet and outlet air temperatures were kept at 130 °C and 81 °C, respectively, with a feed flow of 3.5 mL/min, aspirator volume of 35 m^3/h, and air volume flow of 84 L/h. In this encapsulation procedure, 10 g of β-cyclodextrin mass formulation (Sigma-Aldrich, Milwaukee, WI, USA) was dissolved in 1 L of distilled water and gently stirred for 4 h at room temperature. Afterward, 0.001 Kg of methylene

Table 1
Characteristics of the experimental groups (n = 3), in triplicate.

Group		LED (8.1 J/cm^2, 8.1 J, 90 s)	Laser (323 J/cm^2, 9 J, 113 s)	Photosensitizer (4.65 μM β-cyclodextrin + methylene blue)	Hydrogen Peroxide (40 μM of H_2O_2)
C	Negative control	–	–	–	–
CX	Chlorhexidine 0.2% - positive control	–	–	–	–
P	Photosensitizer / Nanoparticle	–	–	+	–
H	Hydrogen peroxide	–	–	–	+
PH	Photosensitizer / Nanoparticle + Hydrogen peroxide	–	–	+	+
L	Laser	–	+	–	–
LP	Laser + Photosensitizer / Nanoparticle	–	+	+	–
LH	Laser + Hydrogen peroxide	–	+	–	+
LPH	Laser + Photosensitizer / Nanoparticle + Hydrogen peroxide	–	+	+	+
LED	*light emitting diode*	+	–	–	–
LEDP	LED + Photosensitizer / Nanoparticle	+	–	+	–
LEDH	LED + Hydrogen peroxide	+	–	–	+
LEDPH	LED + Photosensitizer / Nanoparticle + Hydrogen peroxide	+	–	+	+

blue was added to the solution, which was stirred overnight and stored in the dark at 4 °C until use. The optical properties of the β-cyclodextrin encapsulated methylene blue were analyzed by ultraviolet visible (UV–vis) spectroscopy (Evolution 260 Bio system, Thermo Scientific Inc., USA), in order to characterize the absorption spectrum. The spectra were analyzed with INSIGHT 1.0.2006 software (Thermo Scientific Inc., USA) and the Lambert-Beer equation allowed us to obtain the actual concentration of 4.65 μM of methylene blue + β-cyclodextrin. The physical characterization of the β-cyclodextrin encapsulated methylene blue was performed by transmission electron microscopy (JEOL JEM 1400, Tokio, Japan) after preparation of a drop (25 μL) that was deposited on a 200-mesh formvar coated copper grids (EMS, USA). For TEM, the magnification ranged from 5000 to 25,000 fold, operating at an acceleration voltage of 120 kV. Digital images were captured using a digital camera system (Gatan, Pleasanton, CA, USA).

PACT was based on the minimum inhibitory concentration of the photosensitizer associated with the nanoparticle of 32 μM (pilot study). We considered the actual β-cyclodextrin encapsulated methylene blue concentration to be 4.65 μM, after the findings of the optical test UV–vis spectroscopy. A nontoxic hydrogen peroxide concentration to human cells was found to be below 50 μM, from a 30% solution [33]. As a safety measure, we chose to use a concentration of 40 μM.

After biofilm formation, the culture medium was removed from the wells, leaving only the biofilm, which was submitted to the treatments, according to Table 1. P, H_2O_2, both associated and chlorhexidine (0.2%) were added in an amount of 20 μL each to the biofilm for 5 min (pre-irradiation time), then removed to be irradiated or to continue the procedures (groups without light).

Groups that received irradiation with laser light (L, LP, and LPH) were exposed to the low-power laser light of GaAlAs (Laser duo – MM Optics, São Carlos, SP, Brazil), with a 660 nm wavelength. The beam area of the laser irradiation was 0.028 cm^2, with an average output power of 0.1 W, energy of 9 J for originally 90 s in continuous mode, determining an energy density of 320 J/cm^2 [8]. A power meter (Scientech 373 Model37-3002, Scientech Inc., Boulder, CO, USA) was used to measure the average power of the laser and since it presented 20% of power loss, the laser irradiation time was adjusted from 90 s to 113 s, in such a way that the delivered total energy would still be of 9 J and energy fluence of 323 J/cm^2.

Groups irradiated with LED light (LED, LEDP, and LEDPH) were exposed to a device with a wavelength of 660 nm (Bridgelux, São Paulo, SP, Brazil) comprised by 12 LEDs. The illuminated area was 12.78 x 8.55 cm (~109 cm^2) (standard size of microplates) and the irradiation parameters of were 0.09 W/cm^2 of power density, 8.1 J of energy for 90 s, delivering an estimated average energy density of 8.1 J/cm^2. Distance between the LED and the wells allowed a uniform distribution of light. The power of the LED device was measured by an Ophir 10A-V2-SH power meter (Ophir Optronics, Har-Hotzvim, POB 45021, Jerusalem 91450, Israel) coupled to a NOVA microprocessor (Ophir Optronics, Har – Hotzvim, POB 45021, Jerusalem 91450, Israel), and no power loss was detected.

After the respective treatments, 50 μL of 0.9% saline solution were added to the wells containing the biofilms and the plates were sonicated in ice-cold deionized water for 90 s, then rested for 30 s with the appliance switched off and sonicated again for another 90 s to disperse the biofilms with a 11 W power (Fisher Scientific, Sonic Dismembrator, model 100; USA).

2.5. Viability analysis

After the plates were sonicated, 50 μL of biofilm plus saline solution was collected from each well and placed in the respective microtube containing 450 μL of 0.9% saline solution. The obtained suspension was diluted in 10^{-1} to 10^{-8} decimal series in saline solution, inoculated, in triplicate, onto Mitis Salivarius Agar (MSA), and incubated for 48 h at 37 °C in a 10% CO_2 partial atmosphere. *S. mutans* representative colonies were counted using a colony counter, and results were expressed as colony forming units (CFU/mL).

2.6. Confocal laser scanning microscopy (CLSM)

The organization of live and dead bacteria in biofilm surface from all groups was examined by laser scanning confocal microscopy. For this purpose, the Leica TCS SP5 microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) was used with an HCX APOL IUV 40X/0.8 numerical aperture water immersion objective. Biofilms were stained with the BacLight live/dead bacterial viability kit (Molecular Probes, Invitrogen, Eugene, Oregon, USA), prepared according to the manufacturer's instructions. Microplates containing the biofilm and the dye were incubated at room temperature in the dark for 15 min and examined under a laser confocal microscope, in which the green fluorescence indicated the viable cells and the red fluorescence, the dead/damaged ones. The ratio of the green sign over the sum of red and green signals was calculated and expressed as the percentage of vital cells [34,35].

2.7. Statistical analysis

For the assessment of treatment effects, the variables hydrogen peroxide cytotoxicity in human oral fibroblasts and microbial viability were analyzed and could not satisfy the assumptions of equality of variances (Levene's Test) and normal distribution of errors (Shapiro-Wilk test). Therefore, data were evaluated by the Kruskal-Wallis

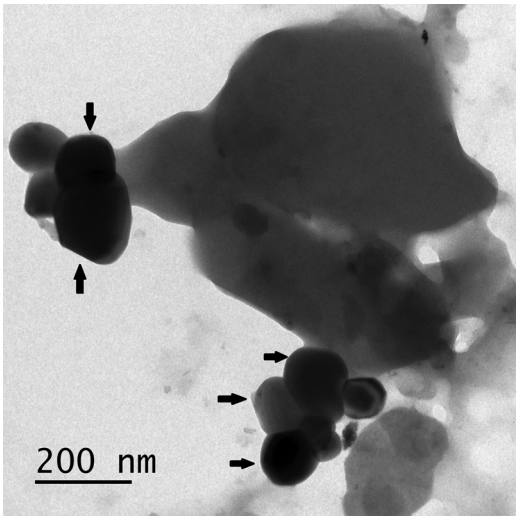

Fig. 1. Transmission electron microscopy of β-cyclodextrin encapsulated methylene blue in the concentration of 4.65 μM.

nonparametric test, followed by Dunn's *post hoc* test to evidence differences. The significance level was 5%.

3. Results

The nanoparticle characterization in Fig. 1 shows the β-cyclodextrin encapsulated methylene blue in the concentration of 4.65 μM with defined particles and size around 230 nm.

3.1. Cytotoxicity of hydrogen peroxide in human oral fibroblasts

The hydrogen peroxide cytotoxicity results in human oral fibroblasts are described in Fig. 2. The test was successfully performed, since in the positive control no cells survived and in the negative control group, all cells survived. In Fig. 2 we showed a significant statistical difference in the groups with cells cultured in 100, 200, 300, 400, and 500 μM when the hydrogen peroxide significantly reduced the number of metabolically active cells when compared with the negative control group.

3.2. S. mutans reduction after PACT

Results of *S. mutans* viability (CFU/mL) are described in Fig. 3. *S. mutans* formed great amounts of biofilm in the negative control (C) and also in P, H, PH, L, and LED groups. On the other hand, the positive control group (treated with chlorhexidine 0.2%) demonstrated no bacterial growth, but with no statistical difference compared with the LP, LH, and LPH groups. The addition of hydrogen peroxide did not enhance the microbial reductions (LPH and LEDPH), compared with the irradiated groups in the presence of the photosensitizer + nanoparticle (LP and LEDP). Both light sources (laser and LED) combined with photosensitizer + nanoparticle (LP or LEDP) or with hydrogen peroxide (LH or LEDH) or with photosensitizer + nanoparticle + hydrogen peroxide (LPH or LEDPH) statistically reduced the *S. mutans* counts when compared with the negative control group (C). However, groups irradiated with LED light were not statistically different from L and P groups, except for LEDPH, which differed only from the P group. Groups irradiated with laser (LP, LH, and LPH) have demonstrated the greater bacterial reductions, differing from all nonirradiated groups.

3.3. Confocal laser scanning microscopy (CLSM)

The quali-quantitative images and the percentage of the ratio of live cells/live + dead cells for each group obtained from the CLSM are shown in Fig. 4, demonstrating the *S. mutans* cells viability in the biofilm after each treatment. Groups in the absence of light sources (C, P, H, and PH) showed no decrease in the viability of *S. mutans* cells present in the biofilm ($p > 0.05$, Dunns´s test). When the biofilms were irradiated with the light sources (L, LP, LH, LPH, LED, LEDP, and LEDPH), a numerical decrease in the *S. mutans* cells was observed, but only with significant difference for the LPH group. CX group (chlorhexidine 0.2% – positive control) substantially decreased the living cells present in *S. mutans* biofilm.

4. Discussion

The capability of PACT to inactivate oral microorganisms in biofilm has been widely researched and has shown to be an effective therapy [36,37]. Considering the importance of cariogenic biofilm control, this study was designed to potentiate the effect of photodynamic antimicrobial chemotherapy, by means of adding hydrogen peroxide, on the reduction of *S. mutans* in biofilms by using two different PACT protocols, associating the photosensitizer and the nanoparticle with either laser or LED as light sources. As a result, we showed that PACT treatment groups containing the hydrogen peroxide did not additionally reduce the numbers of *S. mutans* when compared with their irradiated groups without hydrogen peroxide. Although the addition of hydrogen peroxide to the photosensitizer irradiated with laser (LPH) decreased the amounts of *S. mutans* compared with the negative control groups, it was not statistically significant ($p < 0.05$) when compared with the group associated with the photosensitizer, but in the absence of the H_2O_2 (LP). These findings do not corroborate previous studies

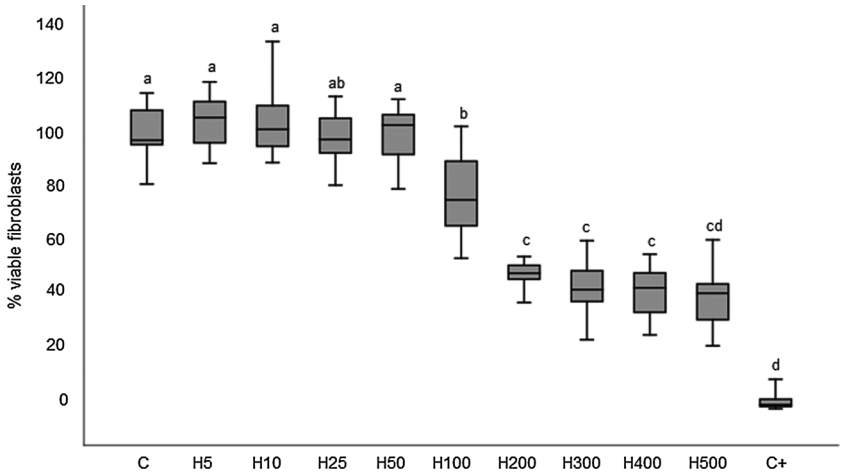

Fig. 2. Box plots represent the values of medians, 25th and 75th percentiles, and lower and upper bounds of 95% confidence interval of percentage of viable human oral fibroblasts with different concentrations of hydrogen peroxide: negative control (without H_2O_2 – C), 5 μM H_2O_2 (H5), 10 μM H_2O_2 (H10), 25 μM H_2O_2 (H25), 50 μM H_2O_2 (H50), 100 μM H_2O_2 (H100), 200 μM H_2O_2 (200), 300 μM H_2O_2 (H300), 400 μM H_2O_2 (H400), 500 μM H_2O_2 (H500), and positive control (Triton 1% – C+). Different letters (a, b, c, and d) indicate a statistically significant difference between the groups (Kruskal-Wallis followed by Dunn's *post hoc* test, $p < 0.05$).

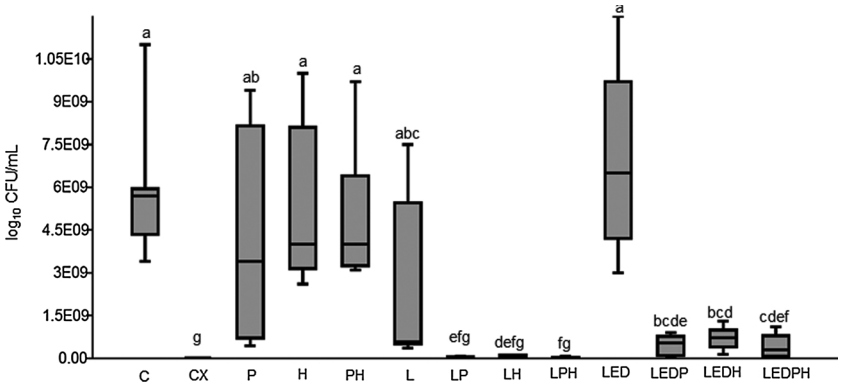

Fig. 3. Box plots represent the values of medians, 25th and 75th percentiles, and lower and upper bounds of 95% confidence interval of *S. mutans* viability (CFU/mL) obtained from the biofilm exposed to different experimental treatments: negative control (C), positive control (Chlorhexidine 0.2% – CX), photosensitizer + nanoparticle (P), hydrogen peroxide (H), photosensitizer + nanoparticle + hydrogen peroxide (PH), laser (L), laser + photosensitizer + nanoparticle (LP), laser + hydrogen peroxide (LH), laser + photosensitizer + nanoparticle + hydrogen peroxide (LPH), LED, LED + photosensitizer + nanoparticle (LEDP), LED + hydrogen peroxide (LEDH), and LED + photosensitizer + nanoparticle + hydrogen peroxide (LEDPH). Different letters indicate statistically significant differences among the groups (Kruskal-Wallis followed by Dunn's *post hoc* test, $p < 0.05$).

%	C-	C+	P	H	PH	L	LP	LH	LPH	LED	LEDP	LEDH	LEDPH
ratio live cells/live + dead cells	83.2	25.0	82.5	83.1	83.0	64.4	58.5	61.5	52.2	68.4	60.2	54.9	55.1

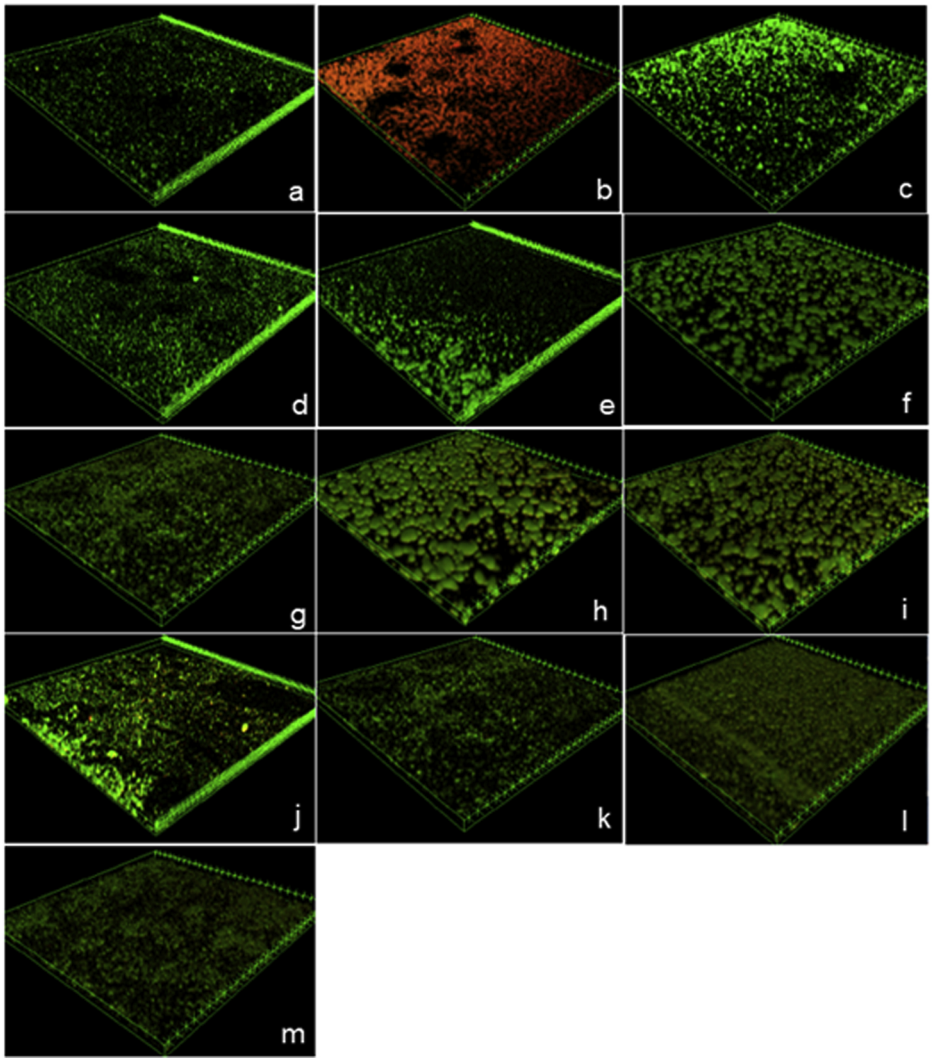

Fig. 4. CLSM quali-quantitative images and percentage of the ratio of live cells/live + dead cells for each group obtained from *S. mutans* biofilm exposed to different experimental treatments: (a) negative control (C), (b) positive control (Chlorhexidine 0.2% – CX), (c) photosensitizer + nanoparticle (P), (d) hydrogen peroxide (H), (e) photosensitizer + nanoparticle + hydrogen peroxide (PH), (f) laser (L), (g) laser + photosensitizer + nanoparticle (LP), (h) laser + hydrogen peroxide (LH), (i) laser + photosensitizer + nanoparticle + hydrogen peroxide (LPH), (j) LED, (k) LED + photosensitizer + nanoparticle (LEDP), (l) LED + hydrogen peroxide (LEDH), and (m) LED + \photosensitizer + nanoparticle + hydrogen peroxide (LEDPH). Viable cells emit green fluorescence, whereas dead cells emit red fluorescence.

whose authors studied the antimicrobial effect when using photodynamic therapy associated with methylene blue and hydrogen peroxide [29,38,39]. One interpretation could be the H_2O_2 concentration; although these researchers did not evaluate *S. mutans* reduction, they evaluated the decrease of *Pseudomonas aeruginosa* (Gram-negative) [38,39], *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) [29] with H_2O_2 concentration of 100 mM, much higher than the one we used (40 μM), in such a way they found a

significant decrease in the numbers of microorganisms when they associated H_2O_2 with methylene blue irradiated with laser light at 660 nm. Concentrations of hydrogen peroxide as high as 100 mM would be expected to induce the disruption of cellular or bacterial membranes and increase methylene blue accumulation in the cells [40], but we could speculate that not for H_2O_2 at 40 μM, the concentration we used. This could have contributed to the lack of PACT enhanced effect (photosensitizer + H_2O_2 + light), even though it decreased the viability of microbial cells.

A noteworthy finding of our study was that hydrogen peroxide associated with either laser or LED, without methylene blue conjugated to β-cyclodextrin, significantly reduced *S. mutans* numbers present in biofilm, whereas hydrogen peroxide alone, without light source activation, did not produce the same effect. Therefore, we could hypothesize that hydrogen peroxide have presented similar characteristics as a photosensitizer, since it reduced microorganisms only after interaction with a light source. Even though other studies [41–43] have also shown that photolysis of H_2O_2 produced a significant bactericidal effect against *S. mutans* biofilms (4–5 $\log_{10}$ reduction), they used higher H_2O_2 concentrations (1 M [41] and 0.88 M [42]) and lower light wavelengths (laser at 405 nm [41], or LED at 365 nm [42]), compared with our study. We chose laser and LED light sources with a wavelength of 660 nm due to the high absorption coefficient of the photosensitizer (methylene blue) at this wavelength. Of course that due to these differences, it is not possible to make comparisons on the antimicrobial properties of each protocol, endorsing that future studies need to be carried out to understand the bacterial reduction mechanism of action found in the present investigation. A possible explanation for the microbial reduction of groups irradiated in the presence of H_2O_2 could be the generation of hydroxyl radical that only takes place at the moment of laser application, immediately stopping at the end of light source irradiation [41]. Hydroxyl radicals react with bacteria and also with organic materials, such as extracellular matrix, killing the bacterial cells, even if they were present in the biofilm [41]. Therefore, the light source and hydrogen peroxide must be associated. In our study, we obtained a 1.94 $\log_{10}$ *S. mutans* reduction when we associated the laser with hydrogen peroxide (group LH). We might hypothesize that the bacterial killing mechanism of action can be attributed to the release of hydroxyl radicals after irradiating the biofilm in the presence of H_2O_2, since our study found significant microbial reduction even in the absence of the photosensitizer (groups LH and LPH).

We chose to use the concentration 40 μM of H_2O_2 because, according to our hydrogen peroxide cytotoxicity evaluation in oral fibroblast cells, concentrations higher than 50 μM were toxic to these cells. An attempt to kill more *S. mutans* present in the biofilm would be to increase the laser power, since increasing the H_2O_2 concentration is not feasible. Further studies need to be done in order to truly state that hydrogen peroxide can be used as an additional alternative instead of a photosensitizer in photodynamic therapy. If this is really proven, it would be a great alternative for microbial reductions, since it is colorless and methylene blue, in spite of having excellent results, can discolor the tooth due to its color [44].

According to the results of our study, LP, LH, LPH, LEDP, LEDH, and LEDPH groups showed a significant reduction in *S. mutans* number in relation to the negative control group. Although these reductions have been significant ($p < 0.05$), LPH group achieved the greatest log reduction of 2.19, but still not up to FDA- and ISO-established performance criteria, since we should have reached log reductions greater than 3 logs [36,45].

Chlorhexidine (0.2%) has well-known antimicrobial properties. It is able to reduce the bacterial viability and consequent polysaccharides production in the biofilm due to its ability to be attracted by the bacterial surface, promoting rupture and cell death, which is well defined in the literature [46]. Therefore, chlorhexidine is considered the “gold standard” against bacteria present in the biofilm, which explains its use as our positive control [46,47]. On the other hand, it may be cytotoxic to fibroblasts and macrophagic cells, besides staining restorations and changing the taste [48], thus the importance of studying innovative therapies, such as PACT, to control biofilm.

The photosensitizer used in our study was the methylene blue associated with the β-cyclodextrin nanoparticle. This was our choice because previous researchers have shown better results of cariogenic bacterial reduction present in biofilm when associating nanoparticles with a photosensitizer [25–27]. In addition, the methylene blue is absorbed in the red-light range of 600–660 nm [35], presents low toxicity to human cells [49], and is able to more-easily penetrate into the cell walls of microorganisms [18].

The laser or LED light sources associated with the conjugated photosensitizer separately used did not significantly reduce the amount of *S. mutans*, showing that for photodynamic therapy to be effective, both parts (light and photosensitizer) must be associated [50,51]. On the other hand, Saafan et al. (2018) [52] showed that laser alone can be effective to decrease the amount of *S. mutans*. However, they used a low power laser (power of 0.2 W), which could have produced some thermal effect, unlike our study, in which we used a laser power of 0.1 W. Méndez et al. (2018) [35], studying photodynamic therapy using the LED light source and the methylene blue photosensitizer, showed that the number of *S. mutans* was unaffected by the application of LED or methylene blue alone, which is in agreement with our findings. In addition, in our study, the use of laser associated with photosensitizer + nanoparticle, did not differ from the positive control group, demonstrating a trend of better results than the LED as a light source.

The viability of bacterial cells within biofilms can also be influenced by the bacteria disruption method chosen, because biofilm manipulation, such as in sonication, may impact the cell’s viability, especially for *S. mutans* that grow in dense microcolonies [53]. Robrish et al. (1976) [54] have shown that Gram-positive bacteria are more resistant to sonication disruption and that sonication energy as high as 28,400 W/s would kill 50% of a *S. mutans* population. Since we used around 0.12 W/s, in two sonication cycles, it is safe to think that the microorganisms from the biofilm were not affected by the sonication protocol and the viable count performed can be considered reliable.

The CLSM results confirmed the CFU/mL counts, showing that the ratio between red and green signals indicated that more bacterial death was achieved when the photosensitizer was irradiated by the light sources. In the images we could also observe that hydrogen peroxide behaved similarly as a photosensitizer, since when it was associated with laser and LED light sources, there was a reduction in cell viability (Figs. 3 and 4). By performing the quali-quantitative analysis, we also endorsed the findings that the greatest microbial reductions occurred in the LPH group ($p < 0.05$).

The combination of the photosensitizer with the light source stimulates events that result in the death of microorganisms. In our study, laser light source, when associated with methylene blue conjugated to β-cyclodextrin, significantly reduced the number of *S. mutans* when compared with the negative control group, corroborating Vasconcelos et al., 2019 [27]. Other authors have also observed reduction of *S. mutans* in the biofilm, using low power laser (energy density of 320 J/cm^2) combined with methylene blue [17].

This in vitro study cannot be extrapolated to the clinical practice yet, but has relevance for future studies using the PACT associated with hydrogen peroxide to control the *S. mutans* biofilm, consequently controlling the incidence of biofilm-dependent diseases such as caries. Different light sources parameters should be further studied as well as concentrations of photosensitizer associated with hydrogen peroxide. PACT is a promising alternative for biofilm control and an alternative to antimicrobials use to overcome disadvantages such as antimicrobial resistance and the action range of microorganisms.

Based on our results, the association of hydrogen peroxide with the conjugated β-cyclodextrin nanoparticle as photosensitizer did not result in an enhanced effect of PACT; hydrogen peroxide behaved similarly as

a photosensitizer, since it reduced the number of *S. mutans* when associated with laser light.

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Ethical approval

Informed consent form was obtained from participants included in the study under protocol # 65011017.9.0000.5418.

Declaration of Competing Interest

None.

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