Effectiveness of photodynamic therapy associated with irrigants over two biofilm models

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ABSTRACT

Background: This study aimed to evaluate the antibacterial effect and the biofilm disruption promoted by antimicrobial photodynamic therapy (aPDT) associated with sodium hypochlorite (NaOCl) and chlorexidine (CHX) over monospecies and multispecies biofilms.

Methods: In monospecies model, forty-six premolars were inoculated with *Enterococcus faecalis* for 21 days and divided into three groups: saline, CHX and NaOCl. After irrigation, aPDT was performed. Samples were collected at baseline (S1) and after irrigation (S2) and aPDT (S3). Colony-forming unit (CFU) counts were performed. In multispecies model, sixty bovine dentin blocks were infected intraorally for 72 h and divided into six groups: saline, saline/aPDT, CHX, CHX/aPDT, NaOCl and NaOCl/aPDT. The percentage and the biovolume of live cells and the total biovolume were assessed using confocal laser scanning microscopy.

Results: CHX and NaOCl showed the lowest CFU counts (P < 0.05). aPDT reduced the bacterial counts in saline (S2–S3; P < 0.05). The lowest amount of live cells was observed in CHX, CHX/aPDT, NaOCl and NaOCl/aPDT. aPDT did not reduce the total biovolume (P > 0.05).

Conclusion: aPDT associated with saline reduced the bacterial load in root canals infected with E. faecalis. aPDT did not reduce the total biovolume in situ; however, the irrigant was decisive to disrupt multispecies biofilms.

1. Introduction

Microorganisms and their products are the main responsible agents for apical periodontitis [1]. A dynamical bacterial organization adhered to a surface and embedded in an extracellular polymeric matrix, which fills the space between microorganisms, is called biofilm [2]. Endodontic biofilms are capable of resisting alkaline stress and the action of antibacterial agents, especially in cases of mature biofilms [3].

Because the morphological features of the root canal systems, the use of irrigants with antibacterial proprieties is essential to dissolve the necrotic tissue and to reduce the number of viable microorganisms [4]. Although several irrigants have been proposed, sodium hypochlorite (NaOCl) have presented high effectiveness in eliminating endodontic pathogens, mainly due to its proteolytic action and its ability to dissolve organic tissue [5,6]. Chlorhexidine (CHX) consists of an alternative irrigant for NaOCl. Clinically, it presents some advantages over NaOCl such as: low toxicity, wide antimicrobial spectrum and substantivity [7]. The main drawback of CHX consists of its incapability to dissolve organic tissue, which impairs its clean ability [7].

Endodontic biofilms can resist the action of antibacterial irrigants using three mechanisms: failure of irrigant penetration into the biofilm, especially in deep portions; bacterial cells that present slow growing due to nutritional limitations; and adoption of phenotypes resistant to antimicrobial agents [8]. Thus, new methods have been proposed to enhance the disinfection process of the root canal system. Antibacterial photodynamic therapy (aPDT) is based on inactivation of cells, microorganisms and molecules induced by light [9]. aPDT acts through a non-toxic photoactivated dye (photosensitizer) which reacts with molecular oxygen [10]. Then, a cascade of oxidative events is produced that kill microorganisms by causing irreversible damage to essential

bacterial molecules such as proteins, lipid membranes and nucleic acids [11], thus leading to microorganism death [11]. A recent systematic review has stated that the available studies indicate the influence of aPDT in reducing the microbial load within the root canal system [12].

Previous *in vitro* studies have evaluated the effectiveness of aPDT in reducing the microbial load in canals previously infected with mono-specie biofilms, mainly with *Enterococcus faecalis* [13,14] and presented promising results. However, the majority of the investigations assessed the isolated use of aPDT without the influence of an irrigant with an-tibacterial properties. Additionally, the effect of aPDT over multispecies biofilm is not well established. Shrestha et al. [15] have evaluated the effect of antibacterial nanoparticles and aPDT over multispecies bio-film, and found cell disruption after aPDT using atomic force micro-scopy. Therefore, new information must be provided regarding bac-terial killing and biofilm dissolution promoted by aPDT associated with irrigants both in monospecie and in multispecies biofilms.

Thus, the aim of this study was to evaluate the antibacterial effect and the biofilm dissolution promoted by aPDT associated with 2.5% NaOCl and 2% CHX over monospecie (*E. faecalis*) and multispecies (*in situ* induction) biofilms. The null hypotheses were: (1) there is no dif-ference in CFU counts after the contact with irrigants and after aPDT over monospecie biofilm; (2) aPDT does not alter neither the number of viable bacterial cells, nor the volume of multispecies biofilm assessed with confocal laser scanning microscopy (CLSM).

2. Materials and methods

In order to evaluate the antibacterial effects and biofilm dissolution promoted by aPDT associated with 2.5% NaOCl and 2% CHX, in this study, it was used two methods of biofilm induction and two methods of assessment. First, a monospecie biofilm model was induced, and root canals were infected with *E. faecalis* (ATCC 29212). The other method induced a multispecies biofilm intraorally. The obtained data were as-sessed using colony-forming units (CFU) for monospecie biofilms and using CLSM for multispecies biofilms. This study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul (09157313.4.0000.5347).

2.1. Monospecie biofilm model (ATCC 29212)

Forty-six premolars were selected, cleaned, sterilized in autoclave (30 min, 121 °C, and 1 atm), and stored in saline at 4 °C. Periapical radiographs were performed to confirm the presence of one root canal, absence of calcification, root resorption, complete root formation, and absence of endodontic treatment. All crowns were removed and the working length was established as being 1 mm lower than the apical foramen. To standardize the canal diameter, canals were prepared with an F2 instrument (ProTaper Universal®, Dentsply Maillefer, Ballaigues, Switzerland) under constant irrigation/aspiration with 2.5% NaOCl (Farmácia Marcela, Porto Alegre, RS, Brazil). Next, canals were rinsed with 17% ethylenediaminetetraacetic acid (EDTA) (Biodinâmica, Ibiporã, PR, Brazil) and manually agitated for three minutes to remove smear layer. After, they were rinsed with saline.

The apices were sealed with adhesive system (Single Bond, 3 M ESPE, USA) and composite resin (Magicfill Vigodent-Coltene, São Paulo, SP, Brazil), and the outer surface of the root was waterproofed with nail varnish. The next step was to fix the roots in 12-well cell culture plates and to perform the sterilization with ethylene oxide. The effectiveness of sterilization was confirmed by collecting one sample using three size 25 paper points (Tanariman, Manacapuru, AM, Brazil). Paper points were placed in polyethylene tubes (Eppendorf do Brasil Ltda, São Paulo, SP, Brazil) with 1 mL of *Brain Heart Infusion* (BHI) (BHI; Difco, Detroit, MI, EUA) and stored at 37 °C for 7 days. Medium turbidity was assessed every 24 h. After seven days, no turbidity was verified, which indicated the effectiveness of the sterilization process.

E. faecalis strains (ATCC 29212) were used to infect the specimens.

A suspension was prepared with 1 mL of pure culture of *E. faecalis* seeded in BHI agar plates and incubated for 24 h at 37 °C. The cell suspension was adjusted through spectrophotometry to match the tur-bidity of a McFarland 0.5 scale (1.5×10^8 CFU mL^{-1}). Fifty microliters of bacterial broth were inoculated into the root canals and all the roots were maintained for 21 days at 37 °C [16]. Every other day, 25 μL of this mixture was removed from each canal and 25 μL of pure BHI broth was inserted to guarantee cell nutrition and viability. A sterile cotton pellet was soaked BHI and placed at the root canal opening.

After incubation, the samples were randomly divided into three groups according to the following irrigants (N = 15): saline, 2.5% NaOCl, and 2% CHX (Farmácia Marcela, Porto Alegre, RS, Brazil). Sample collection and CFU counts were performed at three moments: before irrigation (S1), immediately after irrigation (S2), and after aPDT (S3). Each sample collection was obtained using three sterile paper points, which were inserted into the canals for 1 min and then trans-ferred to tubes with 1 mL of sterile saline. Decimal serial dilutions were made and aliquots were seeded in triplicate onto Petri dishes containing tryptic soy agar (TSA; Difco) and incubated in microaerophillic condi-tions at 37 °C for 24 h. Bacterial growth was measured by the CFU/mL counts of *E. faecalis*. Monoinfection with *E. faecalis* was confirmed by Gram staining and catalase testing.

Afterward, irrigation protocols were performed as follows: irriga-tion with 5 mL of the tested solution for 5 min using a plastic syringe and a 30G NaviTip® needle (Ultradent Products, Inc., South Jordan, UT, USA). Once the irrigation process was accomplished, the canals were dried with aspiration tips (Endo Tips, Angelus, Londrina, PR, Brazil) and paper points. Then, a second sample (S2) was performed, and serial dilutions were performed as described before.

Immediately after S2, the canals were filled with 50 μL of 0.01% methylene blue (Chimiolux 10; DMC, São Carlos, SP, Brazil). The photosensitizer was maintained within the canal and manually agitated using a K-file size 20 for 1 min (pre-irradiation time). Next, Therapy XT diode laser (DMC, São Carlos, SP, Brazil), with 100 ± 20% mW and red continuous emission (650 ± 10 nm wavelength), with an intra-canal fiber attached, was used. The root canals were irradiated for 60 s with an intracanal optical fiber (0.6 mm diameter) placed 2 mm short of the working length as recommended by the manufacturer. After aPDT, canals were flushed with 1 mL of saline, and sample collection was performed (S3). One blinded evaluator performed the CFU counts 24 h after each sample.

2.2. Multispecies biofilm model

Sixty bovine dentin blocks (3 × 3 mm) were obtained from ster-ilized bovine roots. The samples were treated with 3 mL of 1% NaOCl for 15 min (the irrigant was renewed every 5 min) and further treated with 3 mL of 17% EDTA for 5 min to eliminate the smear layer pro-duced during the sectioning process. Dentin blocks were stored in plastic tubes containing 5 mL of distilled water and sterilized by auto-claving for 30 min at 121 °C, and 1 atm.

Prior to the *in situ* induction of oral biofilm, a Hawleys orthodontic device was performed on two volunteers (MFS and RAR). Then, dentin samples were fixed with sticky wax (DFL Indústria e Comércio, Jacarepaguá, RJ, Brazil) on the orthodontic device so that they were exposed to the oral environment 1 mm above the surface to allow the biofilm formation. The volunteers used the device for 72 h, except during regular hygiene procedures in order to induce a multispecies biofilm [4]. Regular daily food diet and hygiene procedures were maintained. In order to protect the samples, a grille was fixed on the device to prevent mechanical removal of the biofilm.

After intraoral biofilm formation, each sample was removed and stored in plastic tubes containing 1 mL of BHI broth at 37 °C for 24 h in aerobic conditions. This procedure was performed to promote the growing of the biofilm. Next, the dentin blocks were rinsed with 1 mL of distilled water to remove the culture medium and those cells that did

not adhere to the dentin surface.

Specimens were randomly divided into six groups (N = 10): Saline, Saline/aPDT, NaOCl, NaOCl/aPDT, CHX, and CHX/aPDT. Each sample was immersed individually in 5 mL of the irrigant in a 12-well culture plate for 5 min. Specimens which were photoactivated were immersed in 5 mL of 0.01% of blue methylene (Chimiolux 10; DMC) for 1 min (pre-irradiation time). Next, photoactivation was performed using a diode laser unit (Therapy XT; DMC) as described in monospecie biofilm. However, in multispecies biofilm, the optical fiber was not used. After immersion in methylene blue in groups without aPDT and after aPDT in other groups, dentin surfaces were rinsed with 1 mL of saline.

The analysis of biofilm viability was performed by using the SYTO 9 and propidium iodide technique (Live/Dead, BacLight Bacterial Viability, Invitrogen, Eugene, OR). SYTO 9 is a green-fluorescent stain that labels both live and dead microorganisms, whereas propidium iodide is a red-fluorescent nucleic acid stain that just labels cells with damaged membranes (dead microrganisms). First of all, 10 μL of each stain were mixed in a sterilized plastic tube. Then, a 4 μL aliquot was placed on the dentin surface. A confocal laser scanning microscope was used to assess the images (Olympus Fluoview 1000, Olympus Corporation, Tokyo, Japan). The respective absorption and emission wavelengths were 494/518 nm for SYTO 9 and 536/617 nm for propidium iodide. Three confocal *'stacks'* from random areas were obtained from each sample using a $100 \times$ oil lens (1 μm step size) and a format of 512×512 pixels. Finally, thirty *stacks* were obtained in each experimental group (three images per specimen × ten samples per group). Biofilm quantitative analysis was assessed using bioImage_L software (www.bioImageL.com). The outcomes evaluated were total biovolume (μm^3), biovolume of live cells (μm^3), and the percentage of live cells.

2.3. Statistical analysis

The data found in monospecie and multispecies biofilm models were assessed using SPSS software version 16.0 for Windows (SPSS Co., Chicago, IL, USA). Normality assumptions could not be verified (Shapiro-Wilk test, p < 0.05), thus non-parametric tests were performed.

In monospecie biofilm model, inter-group analysis was performed using Kruskal-Wallis and Dunn post hoc tests in order to compare CFU counts in each experimental time. Intra-group analysis was conducted using Friedman's test in order to compare each group in different experimental times. For multispecies biofilm model, Kruskal-Wallis and Dunn post hoc tests were used to compare the total biovolume, biovolume of live cells (μm^3), and the percentage of live cells. The level of significance was set at 5% for all the statistical tests.

3. Results

3.1. Monospecie biofilm model

CFU counts were similar in S1 (P > 0.05). A reduction of CFU counts in all groups was observed, including Saline, after canal irrigation (S2) (P < 0.05). However, the main reduction of CFU counts occurred in CHX and NaOCl groups, with no differences between them (P < 0.05). aPDT presented a significant role in the reduction of CFU counts in Saline group (Saline S2–S3; P < 0.05). On the other hand, aPDT reduced the CFU counts in NaOCl and CHX groups (S2–S3); however, Kruskal-Wallis test did not point significant differences (S2–S3; P > 0.05). After aPDT (S3), the lowest CFU counts were observed in NaOCl (0) and CHX (0.0041 × 10⁴ CFU/mL) groups, with no differences between them (P > 0.05). Table 1 shows the median, 25th and 75th percentiles of CFU counts ($\times 10^4$ CFU/mL) in all experimental times and the reduction of bacterial load (%) in S1–S2 and in S2–S3.

Table 1

Median, 25th and 75th percentiles of CFU counts ($\times 10^4$ CFU/mL) at baseline (S1), after irrigation (S2) and after aPDT (S3), and the reduction of bacterial load (%) after each experimental time in monospecie biofilm model.

	S1	S2	S3	S1–S2 (%)	S2–S3 (%)
Saline	200 [Aa] (160–780)	11.6 [Ba] (6.6–21.5)	3.4 [Ca] (0.9–7)	94.1%	70.6%
NaOCl	100.7 [Aa] (37.7–200)	0.6 [Bb] (0–11.2)	0 [Bb] (0–7.1)	99.4%	100%
CHX	108.2 [Aa] (30.8–239.7)	1.6 [Bb] (0.4–3.6)	0.0041 [Bb] (0–1.5)	98.3%	99.3%

Footnotes: Different uppercase letters in the row denote significant differences after Friedman test (P < 0.05). Different lowercase letters in the column denote significant differences after Kruskal-Wallis and Dunn post hoc tests (P < 0.05).

3.2. Multispecies biofilm model

Median and 25th and 75th percentiles of total biovolume, biovolume of live cells, and the percentage of live cells after chemical action and aPDT procedures are presented in Table 2. Regardless the application of aPDT, NaOCl and CHX showed the lowest amount of live cells in comparison with Saline (P < 0.05), but with no differences between them (P > 0.05). Application of aPDT after saline (Saline/PDT) did not reduce the percentage of live cells (green) (P > 0.05). On the other hand, despite absence of significant differences, it can be noted that the percentage of live cells in NaOCl/aPDT (24.3%) decreased 62% in comparison with NaOCl (64.4%). Similar results were found in CHX/aPDT (29.9%). There was a reduction of 35% of live cells in comparison with CHX (46%).

aPDT was not able to reduce the total biovolume of biofilm (P > 0.05); however, the type of the irrigant was determinant to disrupt biofilm (P < 0.05). NaOCl and NaOCl/aPDT presented lower total biovolume values in comparison with Saline and Saline/aPDT (P < 0.05). CHX and CHX/aPDT showed intermediary biovolume of biofilm. Representative CLSM images are shown in Fig. 1.

aPDT reduced the biovolume of live cells; however, statistical differences were not observed (P > 0.05). The lowest biovolume of live cells was observed in NaOCl/aPDT ($0.4 \times 10^6 \mu m^3$) (P < 0.05). On the other hand, Saline and Saline/aPDT presented the highest biovolume of live cells ($64.8 \times 10^6 \mu m^3$ and $40.5 \times 10^6 \mu m^3$, respectively) (P < 0.05).

4. Discussion

Root canal disinfection is a challenge and sometimes other therapeutic modalities must be addressed to improve the reduction of bacterial loads. In this sense, aPDT has been proposed as an auxiliary method to treat the infection of the root canal system [10,12,17,18]. Because of methodological reasons, this study was designed to evaluate the antibacterial effect and the capability of biofilm dissolution of three irrigants and their association with aPDT over monospecie and multispecies biofilms. It must be highlighted that the irrigation is just one step of the endodontic treatment. Disinfection procedures are still composed of the mechanical action of the endodontic instruments and further placement of root canal dressings.

Previous studies have assessed the isolated effect of aPDT in root canal disinfection; however, they did not evaluate the effect of aPDT associated with endodontic irrigants with antibacterial properties such as NaOCl and CHX [13,19]. Recently, Ghinzelli et al. [13] compared the regular method of aPDT with ultrasonic activation of the sensitizer (*i.e.* methylene blue) previously to aPDT on the *E. faecalis* elimination from root canals infected *in vitro,* and found better results for the last method. aPDT must be performed after complete root canal preparation and not alone. Therefore, methodologies which investigate the combined effect of aPDT with antibacterial irrigants, endodontic instruments, and other

Table 2
Percentage, median, and 25th and 75th percentiles of live cells, total biovolume ($\times 10^6$ μm^3) and biovolume of live cells ($\times 10^6$ μm^3) for each experimental group in multispecies biofilm model.

	Saline	Saline/PDT	NaOCl	NaOCl/PDT	CHX	CHX/PDT
Percentage of live cells	93,1% [A] (91.6–94.7)	91.2% [A] (84.7–92.8)	64.4% [B] (40–72.7)	24.3% [B] (1.8–58.6)	46.0% [B] (41.2–60.2)	29.9% [B] (27.9–56.1)
Total biovolume	75.5 [A] (45.5–119.8)	44.4 [A] (36.4–94.0)	9.3 [B] (5.1–16.5)	10.3 [B] (0.6–12.5)	33.9 [AB] (32.4–47.1)	35.1 [AB] (27.3–66.7)
Biovolume of live cells	64.8 [A] (42.9–113.2)	40.5 [A] (33.2–80.9)	7.1 [B] (2.7–16.5)	0.4 [B] (0.07–7.4)	23.5 [AB] (14.2–27.8)	9.8 [B] (6.8–39.0)

Footnote: Different uppercase letters in the row denote significant differences after Kruskal-Wallis and post-hoc Dunn tests (P < 0.05).

disinfection techniques seem adequate. Furthermore, the reduction of bacterial load promoted by the irrigants or root canal preparation must be enough to achieve healing of the periapical lesion.

The first null hypothesis was rejected. There was significant difference in CFU counts after contact with the irrigants and after aPDT in monospecie biofilm model. At the baseline (S1), CFU counts were similar in all groups (P > 0.05). Such result plays an important role since it can identify homogeneous infection pattern using the applied methodology. This study showed the chemical action of the irrigant and the physical effects of irrigation/aspiration process and aPDT over *E. faecalis* biofilm. If the variable root canal preparation (*i.e.*, mechanical effect) was included in the study, probably the bacterial load observed at baseline would be too small and would minimize the chemical effect of the irrigants and aPDT, objects of this study [16]. Such aspect was observed by Dornelles-Morgental et al. [16]. These authors have performed sample collection after hand preparation up to size 50 K-files associated with 2.5% NaOCl or 2% CHX, among other irrigants and associations. Canals prepared and irrigated with 2.5% NaOCl and 2% CHX did not present CFU immediately after canal preparation, probably because of the chemical effect of the irrigants combined with the mechanical action of the instruments.

Significant reduction in CFU counts was observed in S2 in all groups, including Saline (P < 0.05). This finding occurred because of the physical action of the irrigation/aspiration process, which is responsible for the reduction of planktonic microorganisms (observed by collect methods and further culture). Physical action combined with the antimicrobial potential of 2.5% NaOCl and 2% CHX promoted the lowest CFU counts in S2 (P < 0.05) with no significant differences between them (P > 0.05). The antimicrobial potential of these irrigants is well established, especially when the evaluation method is based on sample collection using paper points [16,20]. Additionally, the

literature widely indicates 2.5% NaOCl and 2% CHX to disinfect necrotic canals [21]. Rôças and Siqueira [21], in a clinical study using forty-seven teeth with chronic apical periodontitis, found similar reduction of microorganism phenotypes and their levels when canals were treated with 0.12% CHX and 2.5% NaOCl.

Previous studies have confirmed the bactericidal effect of aPDT, mainly when associated with antibacterial irrigants [17,19]. A recent systematic review alerts to the shortage of clinical information regarding antimicrobial potential related to aPDT; however, it points to an effectiveness ranging from 91 to 100% in disinfecting the root canal system [12]. This investigation found a reduction of bacterial load of 70% in Saline after aPDT (S3) (P < 0.05). Similar values to those were obtained by Foschi et al. [19], who observed a 77% reduction of bacterial load after using diode laser with optical fiber and methylene blue as photosensitizer.

On the other hand, when aPDT was performed after irrigation with NaOCl and CHX (S3), the CFU counts were lower than those obtained after irrigation alone, but with no significant differences (P > 0.05). The use of antibacterial irrigants alone was enough to reduce CFU counts so the effect of aPDT could not be statistically detected. An important finding that must be pointed is that after aPDT (S3), 50% and 60% of the NaOCl and CHX samples, respectively, did not present CFU. This finding is accordance with the findings of Garcez et al. [17] who found a significant decrease in bacterial loads after endodontic treatment using 2.5% NaOCl as irrigant. Thirty-three per cent of the samples treated with conventional endodontic treatment presented themselves free of microorganisms, while those treated with aPDT presented absence of microorganisms under the conventional culture methods.

The second null hypothesis was confirmed. aPDT did not reduce neither the amount of live cells nor the biovolume of multispecies biofilms under CLSM. In this study, antibacterial effect of aPDT when
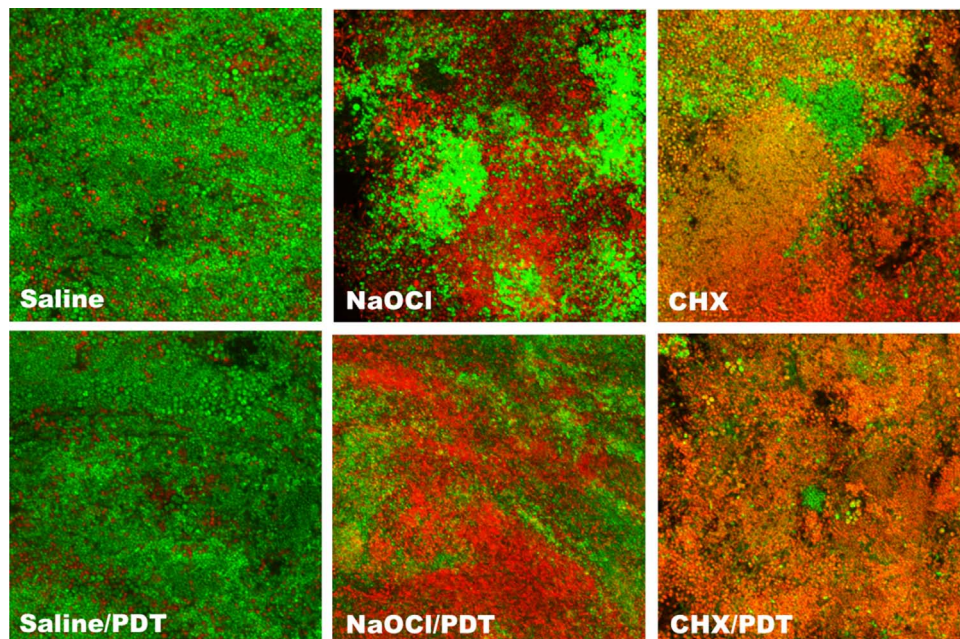

Fig. 1. CLSM images of live (green) and dead (red) cells in all experimental groups.

specimens were submerged in saline was not observed. The amount of live cells in Saline and Saline/aPDT was similar (93.1% and 91.2%, respectively). Saline is an inert solution, devoid of any antibacterial capacity. Thus, it was expected not to find antibacterial action promoted by saline, and for this reason, it was analyzed as a control group. In previous studies, saline was proposed as being a control group to compare its findings with those obtained with NaOCl, CHX and aPDT [3,18]. Methodology limitations can explain the 7% of dead cells (red) in Saline and 9% in Saline/aPDT. This occurred probably because of the saturation of the culture medium or during specimens processing for CLSM analysis.

In those groups in which irrigants with antibacterial properties were used, aPDT seems to play a complementary antibacterial effect; however, statistical differences were not observed (P > 0.05). In NaOCl/ aPDT, there was a 62% reduction in the number of live cells when compared with NaOCl. On the other hand, in CHX/aPDT the reduction was 35%. The reason of why aPDT has a greater antibacterial effect after irrigation with antibacterial irrigants remains unclear.

Antibacterial effect of aPDT after using NaOCl and CHX can be explained because the irradiation over the photosensitizer may be more effective when only residual microorganisms remain in the root canal system after the first contact with the irrigant. Such aspect highlights the adjuvant role of aPDT on the disinfection process [17,22]. Furthermore, when the biofilm is well structured without being partially disrupted by the irrigant or the mechanical action of the canal preparation, aPDT seems not to present effectiveness (Saline and Saline/ aPDT, P > 0.05).

Several studies have evaluated the antibacterial effect but not the capability of biofilm dissolution promoted by photoactivated disinfection [3,10,12–15,17–19,22]. The ability to dissolve organic tissue of the irrigants is directly related to their chemical nature and, in the case of NaOCl, to its concentration [5,6]. This study is in agreement with previous reports in which the lowest volumes of biofilms were observed when specimens were irrigated with NaOCl [5,6]. In addition, authors have been unanimous in affirming that CHX is not capable of dissolve vital or necrotic pulp tissue or biofilms [23]. This investigation found intermediary values of total biovolume in groups treated with CHX (33.9×10^6 μm^3 for CHX and 35.1×10^6 μm^3 for CHX/aPDT); however, it must be considered that organic matter reported in this study consisted only of multispecies biofilm, and not of other organic contents such as pulp tissue and collagen fibers. The most reasonable explanation for this finding seems to be related to bactericide effect of 2% CHX. According to Gomes et al. [23], when 2% CHX is used, the precipitation/coagulation of bacterial cytoplasm occurs. This phenomenon generates cellular debris that may be easily removed after vigorous irrigation with saline [24]. In this study, the removal of this content probably occurred during the irrigation with 1 mL of saline after immersion in CHX. Moreover, the bactericide effect of 2% CHX on biofilms may be decreased due to the inactivation of the cationic bisbiguanides by organic content and their limited penetration through the extracellular polymeric matrix [25].

It must be pointed out that the methodology used in this study did not include the assessment of bacterial viability into the dentin tubules in monospecie biofilm model. This aspect can be evaluated in future studies, which aimed to assess the effect of the irrigants associated with aPDT within the dentin tubules and canal irregularities, especially in teeth with anatomical complexities. Moreover, multispecies biofilm model presents as a substrate a flat surface in which the biofilm grows. Because this study aimed to assess be viability and dissolution of multispecies biofilm formed over dentine and not within the dentin tubules bovine dentin blocks were used. Then, the access of the irrigants, the photosensitizer and the red light is easy. As a result, the antibacterial effect and biofilm dissolution promoted by the irrigants and by aPDT must be overestimated. In multispecies biofilm model, it is difficult to induce biofilm formation with consistent characteristics within dentin tubules because of variation in dentinal tubule size, nutrient supply,

and expression of key binding molecules [26] and for this reason the assessment of live cells was not performed. However, previous studies already reported the effect of irrigants over biofilms within dentin tubules after contamination by centrifugation [26,27]. Finally, the induction of mutlispecies biofilm model in aerobic conditions did not favor gram-negative anaerobes microorganisms which play important role in endodontic infections [28,29].

Based on the results of this study and considering its limitations, it can be concluded that aPDT, when used after previous irrigation with saline, reduced the CFU counts in canals infected with monospecie biofilm (*E. faecalis*). With regard to multispecies biofilm induced *in situ*, aPDT seems to reduce the amount and volume of live cells when associated with NaOCl and CHX. aPDT did not reduce the total volume of biofilm induced *in situ*, but the type of irrigant was determinant to dissolve it.

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