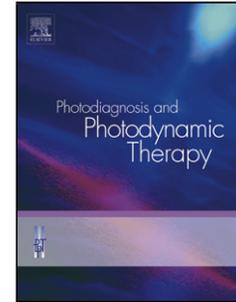


Accepted Manuscript

Title: Effectiveness of photodynamic therapy associated with irrigants over two biofilm models

Authors: Ricardo Abreu da Rosa, Manuela Favarin Santini, José Antônio Poli de Figueiredo, Fernanda Visioli, Jefferson Ricardo Pereira, Rodrigo Ricci Vivan, Francisco Montagner, Marcus Vinícius Reis Só



PII: S1572-1000(17)30431-3
DOI: <https://doi.org/10.1016/j.pdpdt.2017.10.003>
Reference: PDPDT 1031

To appear in: *Photodiagnosis and Photodynamic Therapy*

Received date: 30-8-2017
Revised date: 27-9-2017
Accepted date: 8-10-2017

Please cite this article as: Rosa Ricardo Abreu da, Santini Manuela Favarin, Figueiredo José Antônio Poli de, Visioli Fernanda, Pereira Jefferson Ricardo, Vivan Rodrigo Ricci, Montagner Francisco, Só Marcus Vinícius Reis. Effectiveness of photodynamic therapy associated with irrigants over two biofilm models. *Photodiagnosis and Photodynamic Therapy* <https://doi.org/10.1016/j.pdpdt.2017.10.003>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Effectiveness of photodynamic therapy associated with irrigants over two biofilm models

Ricardo Abreu da Rosa - DDS, MsC, PhD - Department of Conservative Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Manuela Favarin Santini - DDS, MsC, PhD - Department of Endodontics, Centro Universitario Franciscano, Santa Maria, RS, Brazil.

José Antônio Poli de Figueiredo - DDS, MsC, PhD - Department of Endodontics, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Fernanda Visioli - DDS, MsC, PhD - Department of Oral Pathology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Jefferson Ricardo Pereira - DDS, MsC, PhD - Department of Prosthodontics, University of Southern Santa Catarina, Tubarão, SC, Brazil.

Rodrigo Ricci Vivian - DDS, MsC, PhD - Department of Endodontics, São Paulo State University, Bauru, SP, Brazil.

Francisco Montagner - - DDS, MsC, PhD - Department of Conservative Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Marcus Vinícius Reis Só - DDS, MsC, PhD - Department of Conservative Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Highlights

- aPDT/saline reduced the bacterial load in canals infected with *E. faecalis*.
- aPDT reduced the biovolume of live cells but with no statistical differences
- aPDT did not reduce the total biovolume *in situ*
- The irrigant was decisive to dissolve multispecies biofilm.

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 chlorhexidine (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 hours 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.

Keywords: Photodynamic Therapy; *Enterococcus faecalis*; Root Canal Irrigants; Biofilm; Confocal Laser Scanning Microscopy.

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 properties 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]. Chlorexidine (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 monospecies 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 antibacterial 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 biofilm, and found cell disruption after aPDT using atomic force microscopy. Therefore, new information must be provided regarding bacterial killing and biofilm dissolution promoted by aPDT associated with irrigants both in monospecies 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 monospecies (*E. faecalis*) and multispecies (*in situ* induction) biofilms. The null hypotheses were: (1) there is no difference in CFU counts after the contact with irrigants and after aPDT over monospecies 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).

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 monospecies 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 assessed using colony-forming units (CFU) for monospecies 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).

Monospecies biofilm model (ATCC 29212)

Forty-six premolars were selected, cleaned, sterilized in autoclave (30 minutes, 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 1mm 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, 3M 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 1mL of *Brain Heart Infusion* (BHI) (BHI; Difco, Detroit, MI, EUA) and stored at 37°C for 7 days. Medium turbidity was assessed every 24 hours. 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 hours at 37°C. The cell suspension was adjusted through spectrophotometry to match the turbidity 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 minute and then transferred to tubes with 1mL 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 microaerophilic conditions at 37°C for 24 hours. 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: irrigation with 5 mL of the tested solution for 5 minutes 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 μ L of 0.01% methylene blue (Chimiolum 10; DMC, São Carlos, SP, Brazil). The photosensitizer was maintained within the canal and manually agitated using a K-file size 20 for 1 minute (pre-irradiation time). Next, Therapy XT diode laser (DMC, São Carlos, SP, Brazil), with $100 \pm 20\%$ mW and red continuous emission (650 ± 10 nm wavelength), with an intracanal fiber attached, was used. The root canals were irradiated for 60 seconds with an intracanal optical fiber (0.6mm 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 hours after each sample.

Multispecies biofilm model

Sixty bovine dentin blocks (3×3 mm) were obtained from sterilized bovine roots. The samples were treated with 3 mL of 1% NaOCl for 15 minutes (the irrigant was renewed every 5 minutes) and further treated with 3 mL of 17% EDTA for 5 minutes to eliminate the smear layer produced during the sectioning process. Dentin blocks were stored in plastic tubes containing 5 mL of distilled water and sterilized by autoclaving for 30 minutes 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 hours, 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 minutes. Specimens which were photoactivated were immersed in 5 mL of 0.01% of blue methylene (Chimiolux 10; DMC) for 1 minute (pre-irradiation time). Next, photoactivation was performed using a diode laser unit (Therapy XT; DMC) as described in monospecies 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 microorganisms). 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 \times 512 pixels. Finally, thirty stacks were obtained in each experimental group (three images per specimen \times 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.

Statistical Analysis

The data found in monospecies 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 monospecies 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.

Results

Monospecies 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 (0.0041×10^4 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.

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 Figure 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 ($0.4 \times 10^6 \mu\text{m}^3$) ($P < 0.05$). On the other hand, Saline and Saline/aPDT presented the highest biovolume of live cells ($64.8 \times 10^6 \mu\text{m}^3$ and $40.5 \times 10^6 \mu\text{m}^3$, respectively) ($P < 0.05$).

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 monospecies 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 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 monospecies 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, 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 91–100% 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 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 ($33.9 \times 10^6 \mu\text{m}^3$ for CHX and $35.1 \times 10^6 \mu\text{m}^3$ 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 monospecies 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 the 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 multispecies 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 monospecies 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.

Acknowledgements

This study was supported by grants from the Brazilian funding agency CAPES and from Federal University of Rio Grande do Sul.

References

1. Ricucci D, Siqueira Jr. J. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *J Endod.* 2010; 36(8),1277–1288.
2. Costerton J. *The biofilm primer.* 1th ed. Berlin/New York; 2007.
3. Zand V, Milani AS, Amini M, Barhaghi M, Lotfi M, Rikhtegaran S, et al. Antimicrobial efficacy of photodynamic therapy and sodium hypochlorite on monoculture biofilms of *Enterococcus faecalis* at different stages of development. *Photomed Laser Surg.* 2014;32(5),245-251.
4. Del Carpio-Perochena AE, Bramante CM, Duarte MAH, Cavenago BC, Villas-Boas M, Graeff M, et al. Biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. *J Endod.* 2011 37(8);1134-1138.
5. Irala LE, Grazziotin-Soares R, Salles AA, Munari AZ, Pereira JS. Dissolution of bovine pulp tissue in solutions consisting of varying NaOCl concentrations and combined with EDTA. *Braz Oral Res.* 2010;24(3):271-276.
6. Só MVR, Vier-Pelisser F, Darcie M, Smaniotto D, Montagner F, Kuga MC. Pulp tissue dissolution when the use of sodium hypochlorite and EDTA alone or associated. *J Dent Sci.* 2011;26(2):156-160.
7. Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza-Filho FJ. Comparative study of the antimicrobial efficacy of chlorhexidine gel, chlorhexidine solution and sodium hypochlorite as endodontic irrigants. *Braz Dent J.* 2007;18(4):294-298.
8. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 2001;9(1):34–39.
9. Gursoy H, Ozcakir-Tomruk C, Tanalp J, Yilmaz S. Photodynamic therapy in dentistry: a literature review. *Clin Oral Investig.* 2013;17(4):1113-25.

10. Souza LC, Brito PR, Oliveira JC, Alves FR, Moreira EJ, Sampaio-Filho HR, et al. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of *Enterococcus faecalis*. J Endod. 2010;36(2):292-296.
11. Konopka K, Goslinski T. Photodynamic therapy in dentistry. J Den Res. 2007;86(8):694–707.
12. Chrepa V, Kotsakis GA, Pagonis TC, Hargreaves KM. The effect of photodynamic therapy in root canal disinfection: a systematic review. J Endod. 2014;40(7):891-898.
13. Ghinzelli GC, Souza MA, Cecchin D, Farina AP, Figueiredo JAP. Influence of ultrasonic activation on photodynamic therapy over root canal system infected with *Enterococcus faecalis* - an *in vitro* study. Photodiagnosis Photodyn Ther. 2014;11(4):472-8.
14. Jurič IB, Plečko V, Anić I, Pleško S, Jakovljević S, Rocca JP, Medioni E. Antimicrobial efficacy of photodynamic therapy, Nd:YAG laser and QMiX solution against *Enterococcus faecalis* biofilm. Photodiagnosis Photodyn Ther. 2016;13(3):238-43.
15. Shrestha A, Kishen A. Antibiofilm efficacy of photosensitizer-functionalized bioactive nanoparticles on multispecies biofilm. J Endod. 2014;40(10):1604-1610.
16. Dornelles-Morgental R, Guerreiro-Tanomaru JM, Faria-Júnior NB, Duarte MAH, Kuga MC, Tanomaru-Filho M. Antibacterial efficacy of endodontic irrigating solutions and their combinations in root canals contaminated with *Enterococcus faecalis*. Oral Surg Oral Med Oral Pathol. 2011;112(3):396-400.
17. Garcez AS, Nuñez SC, Hamblim MR, Suzuki H, Ribeiro MS. Photodynamic therapy associated with conventional endodontic treatment in patients with antibiotic-resistant microflora: a preliminary report. J Endod. 2010;36(9):1463-6.
18. Bago I, Plečko V, Gabrić Pandurić D, Schauperl Z, Baraba A, Anić I. Antimicrobial efficacy of a high-power diode laser, photo-activated disinfection, conventional and sonic activated irrigation during root canal treatment. Int Endod J. 2013;46(4):339-347.

19. Foschi F, Fontana CR, Ruggiero K, Riahi R, Vera A, Doukas AG, et al. Photodynamic inactivation of *Enterococcus faecalis* in dental root canals *in vitro*. *Lasers Surg Med*. 2007;39(10):782-787.
20. Gomes BP, Ferraz CC, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *Int Endod J*. 2001;34(6):424-428.
21. Rôças IN, Siqueira JF Jr. Comparison of the *in vivo* antimicrobial effectiveness of sodium hypochlorite and chlorhexidine used as root canal irrigants: a molecular microbiology study. *J Endod*. 2011;37(2):143-150.
22. Susila AV, Sugumar R, Chandana CS, Subbarao CV. Combined effects of photodynamic therapy and irrigants in disinfection of root canals. *J Biophotonics*. 2016;9(6):603-9.
23. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in endodontics. *Braz Dent J*. 2013;24(2):89-102.
24. Vianna ME, Horz HP, Gomes BP, Conrads G. In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. *Int Endod J*. 2006;39(6):484- 492.
25. Yamaguchi M, Noiri Y, Kuboniwa M, Yamamoto R, Asahi Y, Maezono H, et al. *Porphyromonas gingivalis* biofilms persist after chlorhexidine treatment. *Eur J Oral Sci*. 2013;121(3 Pt 1):162-182.
26. Ma J, Wang Z, Shen Y, Haapasalo M. New noninvasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy. *J Endod*. 2011;37(10):1380-1385.
27. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old *Enterococcus faecalis* biofilms in dentin canals. *J Endod*. 2014;40(4):509-514.
28. Siqueira JF Jr., Rôças IN. Diversity of endodontic microbiota revisited. *J Dent Res*.

2009;88(11):969–981.

29. Cheung GS, Ho MW. Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions. *Oral Microbiol Immunol.* 2001;16(6):332–337.

Tables

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 ($P < 0.05$).

Table 2 – Percentage, median, and 25th and 75th percentiles of live cells, total biovolume ($\times 10^6 \mu\text{m}^3$) and biovolume of live cells ($\times 10^6 \mu\text{m}^3$) 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 ($P < 0.05$).

Figure legends

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

