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Repeated exposures to blue light-activated eosin Y enhance inactivation of *E. faecalis* biofilms, *in vitro*

Karlo Marinic[a], Daniel Manoil[a], Anna Filieri[a], John C. Wataha[b], Jacques Schrenzel[c], Norbert Lange[d], Serge Bouillaguet[a],*

[a] *Endodontics Unit, Section of Dental Medicine, University of Geneva, Geneva, Switzerland*
[b] *Department of Restorative Dentistry, University of Washington, Seattle, WA, USA*
[c] *Service of Infectious Diseases, University Hospitals of Geneva, University of Geneva, Geneva, Switzerland*
[d] *Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, Switzerland*

Abstract

Background: In dentistry, antibacterial photodynamic therapy (a-PDT) has shown promising results for inactivating bacterial biofilms causing carious, endodontic and periodontal diseases. In the current study, we assessed the ability of eosin Y exposed to 3 irradiation protocols at inactivating *Enterococcus faecalis* biofilms, *in vitro*.

Methods: *E. faecalis* biofilms formed on hydroxyapatite disks were incubated with eosin Y ($10-80\,\mu$M), then activated with blue light using different irradiation protocols. Biofilms exposed to continuous exposure were incubated for 40 min before being light-activated for 960 s. For the intermittent exposure, biofilms were exposed 4 times to the light/photosensitizer combination (960 s total) without renewing the photosensitizer. For repeated a-PDT, the same light dose was delivered in a series of 4 irradiation periods separated by dark periods; fresh photosensitizer was added between each light irradiation. After treatment, bacteria were immediately labeled with LIVE/DEAD BacLight Bacterial Viability kit and viability was assessed by flow cytometry (FCM). Results were statistically analyzed using one-way ANOVA and Tukey multiple comparison intervals ($\alpha = 0.05$).

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* Corresponding author. Tel.: +41 22 379 41 07; fax: +41 22 379 41 01.
  E-mail addresses: Serge.Bouillaguet@unige.ch, cabinet.marinic@gmail.com (S. Bouillaguet).

Results: The viability of *E. faecalis* biofilms exposed to 10 μM eosin Y, was significantly reduced compared to controls (light only-eosin Y only). After a second exposure to blue light-activated eosin Y, viability significantly decreased from 58% to 12% whereas 6.5% of the bacterial biofilm remained live after a third exposure ($p < 0.05$). Only 3.5% of the bacterial population survived after the fourth exposure.

Conclusions: The results of this study indicate that blue light-activated eosin Y can photoinactivate *E. faecalis* biofilms grown on hydroxyapatite disks. Also, repeated exposures to blue light-activated eosin Y were shown to significantly improve efficacy. Further studies seem warranted to optimize the antibacterial activity of blue light-activated eosin Y on major oral pathogens.

Introduction

Endodontic infections are caused by the proliferation of bacteria inside the root canals of teeth that were exposed to the oral environment by carious or traumatic lesions [1]. The elimination of bacteria by means of mechanical instrumentation and endodontic disinfectants is therefore the primary objective of endodontic treatment. Although the use of sodium hypochlorite for disinfecting root canals remains the gold standard, antimicrobial photodynamic therapy (a-PDT) has been reported to be an effective adjunct to eliminate bacterial biofilms that survived after conventional irrigation with sodium hypochlorite [2,3].

Antibacterial Photodynamic therapy (a-PDT) uses a photo-absorbing molecule (called a photosensitizer) and a light source, both respectively absorbing and emitting within the same range of wavelengths. Absorption of the light triggers excitation of the photosensitizer that immediately produces highly reactive singlet oxygen or creates secondary reactive oxygen species (ROS) in presence of oxygen. There is evidence showing that ROS kill bacteria by damaging bacterial cell wall, membrane proteins and nucleic acids [4].

Most recent research indicates that dental curing units emitting blue light (400—500 nm) can successfully inactivate several oral pathogens when used in combination with appropriate photosensitizers. Paulino et al. used a dental blue light source to activate a xanthene derivate called rose bengal, and have reported a complete killing of *Streptococcus mutans* grown in planktonic suspensions [5]. The antibacterial properties of Photosan light-activated with a commercially available dental photopolymerizer emitting blue light were reported against the Gram-positive *S. mutans* and *Enterococcus faecalis* in planktonic cultures [6]. Other research indicates that *Aggregatibacter actinomycetemcomitans*, a Gram-negative pathogen causing periodontal disease, can be inactivated by rose bengal exposed to blue-light irradiation, both in planktonic and biofilm cultures [7]. Papastamou et al. assessed various formulations of ruthenium exposed to blue light for inactivating *Fusobacterium nucleatum* and *Porphyromonas gingivalis* and reported a significant reduction in viability of both pathogens after treatment [8]. More recently, Manoil et al. showed that blue light-activated curcumin can inactivate planktonic and biofilm cultures of *S. mutans* as evidenced by flow cytometry analysis of bacterial viability after treatment [9].

However, all above mentioned studies failed to demonstrate a complete killing of oral bacterial biofilms exposed to blue light mediated a-PDT. Thus, it is likely that several therapeutic parameters such as photosensitizer dose, photosensitizer-light interval and light conditions may require optimization to increase efficacy. A study by Street et al. has confirmed that the efficacy of a-PDT against three major periodontal pathogens was light dose dependent [10]. Also, there are reports showing that light dose fractionation may add some benefit to the treatment. Erythrosine-mediated PDT of *S. mutans* biofilms was previously shown to be enhanced by fractionation of light [11]. More recently, Sung et al. used intermittent and repeated irradiation to inactivate *Mycobacterium tuberculosis* by a-PDT. Their results indicate that intermittent and repeated a-PDT allowed improved inactivation of the bacilli compared to continuous light exposure [12]. In dermatology, light fractionation was also shown to lower the rate of photobleaching of the photosensitizer upon light irradiation and was therefore recommended to increase the efficacy of aminolevulinic acid (ALA) photodynamic therapy [13].

Therefore, in the current study, we assessed the ability of eosin Y exposed to three different irradiation protocols including continuous, repeated and intermittent light exposure to inactivate *E. faecalis* biofilms, *in vitro*. Eosin Y was selected as a photosensitizer because it absorbs blue light and was previously shown to inactivate Lactobacilli and *Candida albicans* upon light activation [14,15]. Intermittent irradiation consisted in exposing pathogens to light and photosensitizer first, then re-incubating pathogens for a certain period of time before repeating the irradiation. For repeated a-PDT, the same overall radiant energy was delivered in a series of shorter irradiation periods separated by dark periods; fresh photosensitizer was added between each light irradiation. Our hypothesis was that repeated or intermittent irradiations of eosin Y with blue-light would increase antibacterial activity against *E. faecalis* biofilms.

Materials and methods

A stock solution (1 mmol/L) of eosin Y (Sigma-Aldrich, Buchs, Switzerland) was prepared after dissolving the powdered chemical in 0.9% sodium chloride solution. A spectrophotometer (Cintra 40 UV/VIS, GBC, Dandenong, Australia) was used to confirm the absorbance of the solution in the blue range (380—500 nm). The stock solution was further diluted in 0.9% sodium chloride solution to evaluate the antibacterial activity of several concentrations of blue light-activated eosin Y (see below).

Enterococcus faecalis (*E. faecalis* 135737, culture collection of the University Hospitals of Geneva, CH) was selected

for the antibacterial assays because of its prominent role in endodontic infections [16]. Bacteria were cultured from frozen stocks onto Columbia agar plates (Oxoid, Pratteln, Switzerland), then transferred into 6 mL of brain heart infusion liquid medium (BHI) and incubated overnight aerobically at 37 °C. A spectrophotometer (Biochrom WPA Biowave II) was used to adjust the bacterial concentration in the culture medium at 600 nm (OD 1:5 × 10^9 bacteria).

Hydroxyapatite disks (9.5 mm diameter × 2 mm thickness), acquired from Clarkson Chromatography Products, (Williamsport, PA, USA) were autoclaved at 121 °C for 20 min before use. Hydroxyapatite (HA) disks were placed into 24 well culture dishes (TPP Techno Plastic Products AG, Switzerland) and inoculated with 200 μL of bacterial suspension. The bacterial suspension was placed on the top of each disk to allow cell attachment for 5 min before adding 1.8 mL of fresh BHI medium to the well. Specimens were incubated for 48 h at 37 °C in aerobic conditions without changing the culture medium. The culture medium was not renewed to obtain bacterial biofilms in the stationary phase of growth. Previous studies have shown that E. faecalis biofilms can develop within 48 h; the bacterial mode of growth was also shown to influence the response to antibacterial treatment [17,18].

Light source

A quartz tungsten halogen dental light source (Optilux 501, KerrHawe SA, Bioggio, Switzerland) emitting blue light (380—500 nm) was used to activate the photosensitizer. The irradiance of the light source is 450 mW/cm^2. The light, which is delivered through a light guide of 10.4 mm in diameter, was placed 10 mm above bacterial biofilms to insure a complete irradiation of the culture dish. Each culture dish was irradiated separately.

Antibacterial assays

Different irradiation protocols were used to compare the effect of a single a-PDT exposure (continuous) versus repeated a-PDT or intermittent exposures on E. faecalis biofilm viability.

For the continuous exposure group, HA disks were rinsed twice with 0.9% NaCl to remove loosely attached bacteria and surrounding planktonic cells, then transferred into black polystyrene 24 well culture dishes (Greiner Bio-One, Milian, Switzerland) containing 1 mL of photosensitizer. Black opaque culture dishes were used to avoid light spreading to the next well during light irradiation. The concentrations of eosin Y tested in the continuous exposure group ranged between 10 and 80 μM. The incubation time was set to 40 min and each culture dish was irradiated separately for 960 s (Fig. 1). The dark toxicity of the photosensitizer was also measured on biofilms that were not exposed to blue light. Light and incubation conditions were selected to match those applied to the bacterial biofilms in the repeated and intermittent exposure groups. Control cultures received no photosensitizer and were either light irradiated for 960 s (light + conditions) or received no light (light-conditions).

For the repeated exposure group, biofilms on HA disks were transferred to black opaque culture dishes and incubated into 1 mL of photosensitizer (10 and 20 μM) for 10 min before light activation (Fig. 1). Each disk was separately light-activated for 240 s, except those used for dark toxicity evaluation. A set of 8 disks was sent to the FCM analysis to assess the effect of a first cycle of a-PDT whereas the remaining disks were transferred to new culture wells containing 1 mL of fresh eosin Y at the same concentrations (10—20 μM). Disks were again incubated for 10 min and irradiated for 240 s. After the second irradiation, 8 disks (including dark toxicity specimens) were collected for viability measurements of a two-cycle a-PDT. The remaining disks were placed into 1 mL of fresh photosensitizer, incubated 10 min and irradiated 240 s. After irradiation, 8 disks were used for assessing the effectiveness of a three-cycle a-PDT. A fourth cycle of a-PDT was performed on disks incubated for 10 min in fresh eosin Y solution and irradiated 240 s before FCM analysis. This experimental set-up allowed us to obtain 4 replicates per condition; the entire experiment was repeated three times.

For the intermittent exposure group, biofilms on HA disks were placed into black polystyrene 24 well culture dishes (Greiner Bio-One, Milian, Switzerland) containing 1 mL of photosensitizer at different concentrations (10—20—40—80 μM), and incubated 10 min before light activation for 240 s (Fig. 1). After irradiation, the disks remained in the same wells for 10 min and irradiated again for 240 s. After 10 min of rest in the dark, the disks were irradiated for 240 s. A fourth and last cycle of 10 min rest and 240 s irradiation was then performed. All specimens were collected for assessing bacterial viability. The entire experiment was repeated three times to assess the reproducibility of the assay as applied to the bacterial biofilms.

Quantification of bacterial viability

Bacterial viability was measured immediately after a-PDT treatment, using flow cytometry (FCM). For FCM viability measurement, the HA disks were transferred into 15 mL tubes containing 2 mL of NaCl 0.9% solution and vortexed for 2 min to allow the detachment of the biofilms. Then, 200 μL of bacterial suspension was transferred into 1.5 mL tubes, sonicated for 20 s and mixed with 200 μL of LIVE/DEAD BacLight solution. Two nucleic acid stains (SYTO 9 and propidium iodide) compose the LIVE/DEAD BacLight solution (LIVE/DEAD BacLight viability kit, Life Technologies, Switzerland). SYTO 9 has excitation/emission wavelengths at 485/500 nm and can penetrate all cells. PI only enters membrane-damaged cells, and excitation/emission wavelengths are 535/617 nm. The labeled bacteria were incubated for 15 min in the dark before assessing cell viability by flow cytometry. The flow cytometry analysis was performed using a BD Accuri C6 flow cytometer (BD Accuri cytometers, Ann Arbor, Michigan, USA) with a 488 nm excitation laser. SYTO 9 and PI emissions were detected in the FL-1 channel (BP 530/30) and the FL-3 channel (LP ≥ 670) respectively. The emission spectral overlap has been compensated and the run limit determined at 20,000 events. A threshold set on the forward scatter (FSC-H) allowed the discrimination of bacteria from background. Doublets of bacteria have been excluded for analysis; singlets corresponded to 90% of the total population. The data
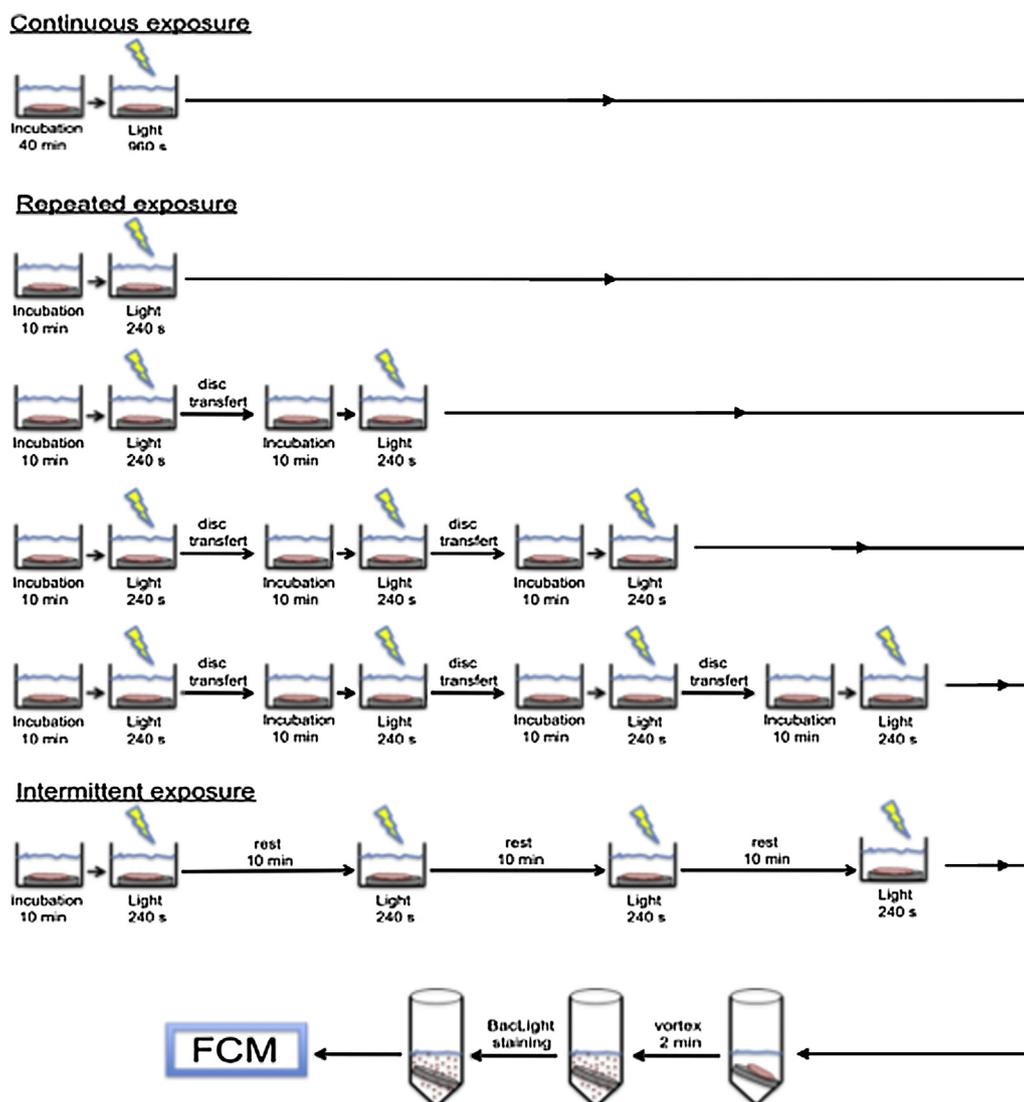
Fig. 1 Experimental set-up used in this study. For the continuous exposure group, biofilms were incubated for 40 min in presence of eosin Y (10—80 μM), irradiated with blue light for 960 s before assessing viability by flow cytometry. For the repeated exposure group, biofilms were incubated for 10 min with eosin Y (10—20 μM), then light-activated for 240 s. This procedure was repeated four times and antibacterial activity has been assessed after each incubation/irradiation cycle. For the intermittent exposure group, light activation for 240 s was repeated four times without renewing the photosensitizer (10—80 μM); bacterial viability was then assessed by flow cytometry.

recorded with the Accuri C6 were analyzed with FlowJo software (FlowJo for Windows, version 10.0.06, 2014, Tree Star Inc., Ashland, Oregon, U.S.A.) Results were statistically analyzed using one-way ANOVA and Tukey multiple comparison intervals ($\alpha = 0.05$).

Results

Results for the continuous exposure group are shown in Fig. 2. Light irradiation of control cultures without photosensitizer (0 μM) had no effect on biofilm viability. When *E. faecalis* biofilms were incubated for 40 min in presence of 10 μM of eosin Y, then light-activated for 960 s, bacterial viability significantly decreased ($p < 0.05$) compared to specimens irradiated without photosensitizer

(Fig. 2). Only 38.1% ($\pm7\%$) of bacteria remained live after light activation. When the concentration of blue light-activated eosin Y was increased to 20 μM, the antibacterial effect was not significantly different ($p > 0.05$); approximately 31% ($\pm7\%$) of cells remained live after treatment. At concentrations of 40 and 80 μM, bacterial viability remained similar with respectively 40% ($\pm12\%$) and 61% ($\pm15\%$) of bacteria live after treatment.

For the repeated exposure group, light irradiation of control cultures without photosensitizer (0 μM) had no effect on biofilm viability, independently of the number of exposures to light. As shown in Fig. 3, the viability of *E. faecalis* biofilms exposed to 10 μM of blue light-activated eosin Y was significantly reduced compared to controls (no photosensitizer- light irradiation only). Bacterial viability significantly decreased from 58% after the first irradiation
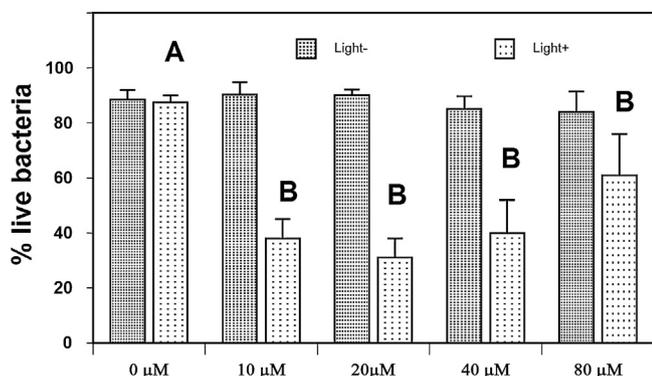
Fig. 2 Continuous exposure group. *E. faecalis* biofilms were either incubated for 40 min in presence of 10, 20, 40 or 80 μM of eosin Y that was light-activated for 960 s. Letters indicate statistical differences ($\alpha = 0.05$) between light + conditions (mean values ± standard deviation).
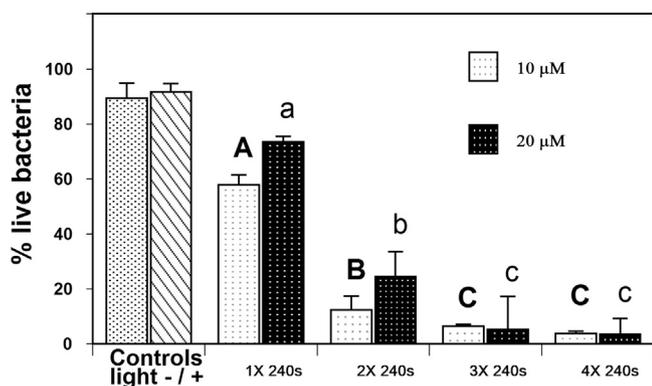

Fig. 3 Repeated exposure group. *E. faecalis* biofilms were incubated for 10 min in presence of 10 or 20 μM of eosin Y that was light-activated for 240 s. The same light photosensitizer conditions were repeated 4 times and biofilm viability was measured after each successive a-PDT treatment. Letters (Superscript: 10 μM, Underscript: 20 μM) indicate statistical differences ($\alpha = 0.05$) between light + conditions (mean values ± standard deviation).

to 12% after a second exposure to light-activated eosin Y. Only 6.5% (±1.7%) of the bacterial biofilm remained live after a third exposure ($p < 0.05$) whereas 3.8% (±2.4%) of the bacterial population survived after the fourth exposure. For the 20 μM concentration, biofilm viability significantly decreased from 74% (±6.4%) after the first light activation to 24% (±11%) after the second activation. Biofilms exposed to a third light activation exhibited a viability of 5.3% (±2.4%) that further decreased to 4% (±4%) after the fourth and last light exposure (Fig. 3).

Results for the intermittent exposure group are presented in Fig. 4. As reported for the continuous and repeated exposure groups, light irradiation of control cultures without photosensitizer (0 μM) had no effect on bacterial viability. *E. faecalis* biofilms incubated with 10 μM of eosin Y that was light-activated 4 times for 240 s, exhibited a significant reduction in viability ($p < 0.05$) compared to controls (light irradiation without photosensitizer). Only 24% (± 4%) of bacteria remained live after the last light activation. When the concentration of blue light-activated eosin Y was
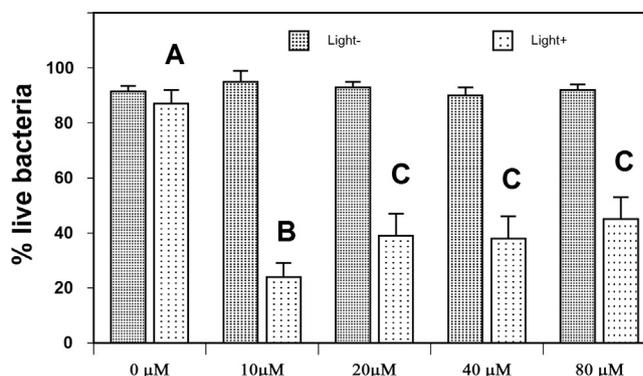

Fig. 4 Intermittent exposure group. *E. faecalis* biofilms were incubated in presence of 10, 20, 40 or 80 μM of eosin Y that was irradiated 4 times (4×240 s) with dark periods of 10 min between irradiations resulting in a total incubation duration of 40 min. Letters indicate statistical differences ($\alpha = 0.05$) between light + conditions (mean values ± standard deviation).

increased to 20 μM, the antibacterial effect was significantly less ($p < 0.05$) than observed for the 10 μM concentration; approximately 38% (±8%) of cells remained live after light-activation. At concentrations of 40 and 80 μM, bacterial viability remained similar with respectively 38% (±8%) and 45% (±8%) of bacteria live after treatment.

Discussion

In dentistry, a-PDT has recently gained interest for eliminating bacterial biofilms causing carious, endodontic and periodontal diseases [19]. Obviously, the use of blue light for a-PDT is an attractive concept because all dental offices possess high-intensity (500−1000 mW/cm^2) devices which can activate photosensitizers for disinfection of enamel surfaces, root surfaces or root canal dentin. Because all above mentioned substrates are only superficially infected by biofilms, light penetration through tissues is less of a problem compared with light penetration required for cancer therapy. There is evidence showing that blue light can penetrate across dentin despite thickness, diameter and density of dentinal tubules all affecting the light transmission properties of dentin [20]. Also, pilot experiments in our laboratory have shown that eosin Y can rapidly diffuse through dentin with penetration depths ranging between 100 and 600 μm (unpublished data). For comparison, the penetration of 1% sodium hypochlorite applied for 2 min on root dentin does not exceed 80 μm [21].

In this study, the antibacterial activity of blue light-activated eosin Y was assessed on bacterial biofilms grown on hydroxyapatite disks because biofilms are known to be more resistant to antimicrobial treatments than planktonic bacteria. *E. faecalis* was used as a target microorganism because this pathogen is frequently retrieved from root canals with persistent endodontic infection and resists elimination with conventional antimicrobial agents [22]. However, the relevance to clinical conditions would be further improved by using multispecies biofilms as usually encountered in oral infections [23].

In microbiological research, the CFU assay remains the gold standard method to measure the efficacy of new
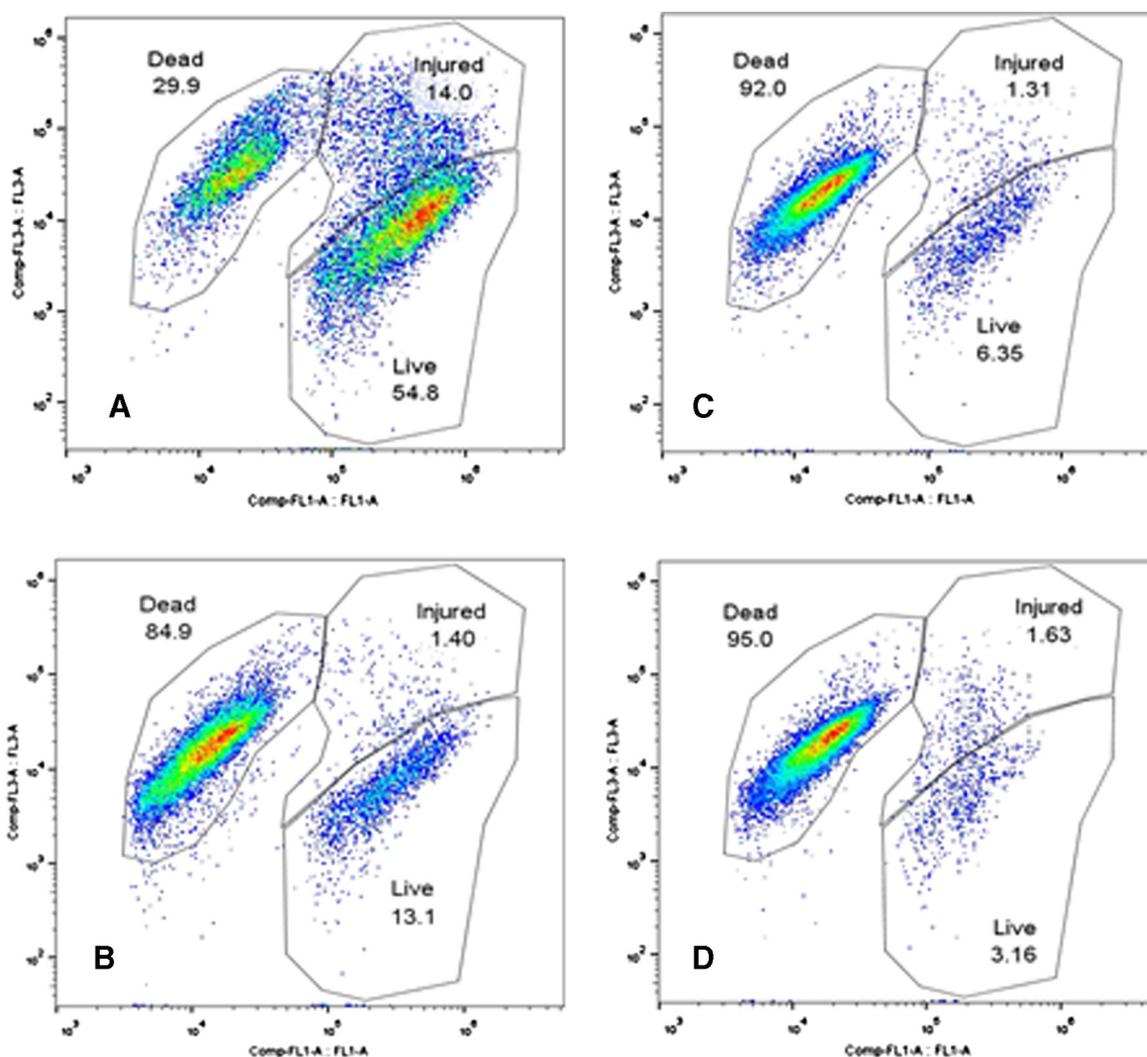
Fig. 5 Flow cytometric dot plots of *Enterococcus faecalis* from the repeated exposure group stained with LIVE/DEAD Baclight bacterial viability kit. FL1-A (BP 530/30 collecting SYTO 9 fluorescence) is plotted against FL3-A (LP $\geq$ 670 collecting PI fluorescence). A—D: *E. faecalis* biofilms exposed one (A), two (B), three (C) and four (D) times to 10 μM of blue-light activated eosin Y.

antimicrobial agents because it easily estimates the number of bacteria able to grow and multiply under defined medium conditions. Further, CFU count allows a standardization of the results which can be expressed in term of $\log_{10}$ reduction. However, it is becoming clear that many pathogens can enter a viable but non-cultivable state (VBNC) when exposed to various chemical or environmental factors. *E. faecalis* was shown to enter the VBNC state in response to low nutrient concentration, osmotic or oxidative stress and low temperature [24,25].

On the contrary, flow cytometry combined with fluorescent markers allow identification of different bacterial physiological states including live cells (SYTO 9 positive), dead cells (PI positive) but also injured cells (double stained) [26]. Further, the large number of bacteria analyzed by flow cytometry increases counting precision and accuracy; according to Nebe-von-Caron, variation between readings is less than 1% when more than 1000 events are used for viability measurements [27]. Flow cytometry has been used to monitor the inactivation of *Escherichia coli* on solid food,

to control water quality after chlorination, to verify the vitality of probiotic lactic acid bacteria exposed to gastric acid and bile salts [28—30]. Previous work from our group also used FCM for measuring viability of several oral pathogens after photo-inactivation [9,14].

In this study, radiant exposure that corresponds to the time-integrated irradiance at the surface of the culture wells was kept equivalent for all groups; biofilms were either exposed to 4 irradiation cycles of 240 s or to a single exposure of 960 s. Also, a comparison between the higher concentrations tested (40—80 μM) and cumulative concentrations applied to biofilms *e.g.*, four times 10 μM or 20 μM was made possible.

Results indicate that blue light-activated eosin Y may be a candidate for inactivating *E. faecalis* biofilms. This is in agreement with previously published papers on *E. faecalis* inactivation by a-PDT showing that planktonic cells can be successfully photo-inactivated whereas the efficacy of the treatment on biofilms was more strain dependent [31]. However, for both the continuous and

intermittent groups, increasing concentrations of eosin Y did not improve antibacterial activity as observed for the 40—80 μM concentrations. As reported by Goulard et al., when high concentrations of photosensitizers are incubated for longer periods of time, they can form aggregates, thereby decreasing treatment efficiency [32]. Also, a longer irradiation time may have accelerated photobleaching of the photosensitizer, which was shown to occur with irradiation times longer than 15 min [33]. Photobleaching is likely to happen when singlet oxygen or other ROS produced upon illumination reacts with the photosensitizer itself thereby reducing its availability for further photosensitization [34]. There is also evidence showing that high radiant exposures may cause oxygen depletion in the culture medium which consequently weakens the therapeutic effect [35]. Undoubtedly, repeated exposures were more effective at inactivating *E. faecalis* biofilms than continuous or intermittent exposures. A significant reduction in bacterial viability was reported after each successive exposure to the photosensitizer/light combination except for the last exposure, which did not add any significant effect. As shown in Fig. 5, biofilms exposed to 10 μM of photo-activated eosin Y exhibited 55% live, 30% 'dead'' and 14% ''injured'' cells after the first exposure to the light (240 s) whereas only 13% of live cells and 85% of dead cells were observed after a second exposure to the light (2 × 240 s). This indicates that the injured population died upon the second exposure to the light which agrees with previously published reports [9,14]. Repeating exposure of biofilms to a-PDT has undoubtedly increased the amount of oxidative stress produced in bacterial cultures. This is in agreement with Metcalf et al. who showed that erythrosine-mediated PDT of *S. mutans* biofilms can be enhanced by repeating light irradiation. The authors have attributed this result to the replenishment of target molecules such as oxygen for the photosensitizer during the dark periods [11].

There are reports showing that eosin Y is a highly soluble dye in water at physiological pH and that this rather hydrophilic photosensitizer does not significantly associate with membranes on its own [36,37]. Other reports indicate that, upon light-irradiation, eosin Y is more likely to produce singlet oxygen ($^1O_2$) by energy transfer (type II mechanism), than reactive oxygen species produced by electron transfer (type I mechanism) [38]. Singlet oxygen ($^1O_2$) production is assumed to be the main mechanism involved in a-PDT. The half-life of singlet oxygen in biological systems is<40 ns, and therefore the radius of the action of singlet oxygen is of the order of 20 nm [39]. Also, the antimicrobial activity of photosensitizers mediated by highly reactive singlet oxygen was shown to have a direct effect on extracellular molecules. Thus, the polysaccharides present in the extracellular polymeric matrix of a bacterial biofilm are also susceptible to photo-damage [40]. Therefore, it is likely that repeating exposures to blue light-activated eosin Y has progressively disrupted the biofilm and ultimately killed all pathogens exposed to oxidative stress.

Although repeating exposures to blue light-activated eosin Y was shown to be successful, other options to increase the efficiency of eosin Y have been recently described. Johnson et al. reported that the peptide (KLAKLAK)₂ greatly enhances the photodynamic activity of eosin Y, and that eosin-(KLAKLAK)₂ can photoinactivate

Gram-positive and Gram-negative strains to a similar extent [41].

Estevão et al. have shown that eosin Y and its ester derivatives in micelles are promising photosensitizers for photodynamic applications because increasing hydrophobicity of the photosensitizer promotes a higher interaction with biological membranes [42]. Finally, the use of blue light-activated eosin Y as a disinfecting agent for endodontic therapy would require the development of thin fiber optics that can deliver light to all recesses of a root canal [17].

Conclusions

The results *of the* current study indicate that blue light-activated eosin Y has the potential to photoinactivate *E. faecalis* biofilms grown on hydroxyapatite disks. Also, repeated exposures to blue light-activated eosin Y were shown to significantly improve efficacy. Further studies seem warranted to optimize the antibacterial activity of blue light-activated eosin Y on major oral pathogens.

Acknowledgments

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