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Reference


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

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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 μM), 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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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 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
Repeated exposures to blue light-activated eosin Y have been shown to influence the response to antibacterial treatment. The bacterial mode of growth was also repeat a-PDT or intermittent exposures on E. faecalis biofilms. Then, 200 μL of bacterial suspension was transferred to black opaque culture dishes and vortexed for 2 min to allow the detachment of the bacteria. Into 15 mL tubes containing 2 mL of NaCl 0.9% solution, the HA disks were transferred. Viable bacteria were incubated for 15 min in the dark before light activation/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
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% (±7%) 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% (±7%) of cells remained live after treatment. At concentrations of 40 and 80 μM, bacterial viability remained similar with respectively 40% (±12%) and 61% (±15%) 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
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**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/cm²) 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...
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 log₁₀ 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 M) and cumulative concentrations applied to biofilms e.g., four times 10 M or 20 M 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
Repeated exposures to blue light-activated 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 (1O2) by energy transfer (type II mechanism), than reactive oxygen species produced by electron transfer (type I mechanism) [38]. Singlet oxygen (1O2) 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 photosensitzers 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)4 greatly enhances the photodynamic activity of eosin Y, and that eosin-(KLAKLAK)4 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.

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