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IN VITRO INACTIVATION OF *ENTEROCOCCUS FAECALIS* WITH AN LED DEVICE

ABSTRACT

Non coherent light-emitting diodes (LEDs) are effective in a large variety of clinical indications; however, the bactericidal activity of LEDs is unclear, although the effectiveness of such lights is well known. Currently, no studies have examined the effects of NIR-LED on bacteria. The aims of this study were to verify the antibacterial activity of 880-nm LED irradiation on a bacterial suspension of *E. faecalis* and to compare it with the actions of sodium hypochlorite (NaOCl) and the concurrent use of both treatments. Before we proceeded with the main experiment, we first performed preliminary tests to evaluate the influences of such parameters as the distance of irradiation, the energy density, the irradiation time and the presence of photosensitizers on the antimicrobial effects of LEDs.

After treatment, the colony forming units per milliliter (CFU mL^{-1}) were recorded and the data were submitted to ANOVA and Bonferroni post hoc tests at a level of significance of 5 %.

The results showed that LED irradiation, at the parameters used, is able to significantly decrease *E. faecalis* viability *in vitro*. The total inhibition of *E. faecalis* was obtained throughout concurrent treatment of LED and NaOCl (1%) for 5 minutes.

The same antimicrobial activity was confirmed in all of the experiments ($p < 0.05$), but no statistically significant differences were found by varying such parameters as the distance of irradiation (from 0.5 mm to 10 mm), energy density (from 2.37 to 8.15 mJ/s), irradiation time (from 5 minutes to 20 minutes) or by adding toluidine blue O (TBO).

Keywords: LED, Faecalis, Endodontics, Sodium Hypochlorite, Light Therapy

1. INTRODUCTION

Enterococcus faecalis forms a part of the normal micro-flora of the human body, but relatively low counts are found in the mouth. It is a Gram-positive, non-motile, facultative anaerobic microbe [1]. *E. faecalis* can survive in extreme environmental conditions, such as acid or alkaline pH, high concentrations of salts and heavy metals as well as low nutrient concentrations. It can grow in the range of 10 to 45°C and is resistant to a temperature of 60°C for 30 min [2].

E. faecalis is known to invade dentinal tubules and to bind collagen. It has the ability to survive in root canals as a single body without the support of other bacteria and can resist a wide range of antibiotics and intra-canal drugs. It is highly virulent, producing lytic enzymes, cytolysin, aggregation substances, pheromones, and lipoteichoic acid and is able to alter the host responses and to suppress the actions of lymphocytes [3].

E. faecalis can be found in primary endodontic lesions and is involved in the pathogenesis of secondary endodontic lesions, despite the combined use of root canal irrigants, intra-canal medicaments and mechanical instrumentation during the root canal treatment [4-5].

Among irrigants, sodium hypochlorite is the most commonly used; it is effective against the endodontic flora with some tissue-dissolving properties. However, it is highly toxic, and its efficacy is susceptible to temperature, concentration, and exposure time [6].

These issues and the increasing problem of microbial antibiotic resistance has ignited the interest of researchers into the use of additives and/or alternative antimicrobial treatments [7].

Novel approaches for disinfecting root canals include the use of high-power lasers because the photosensitization of bacterial cells is independent of their antibiotic resistance spectrum [8, 9]. However, lasers function by dose-dependent heat generation and, if incorrectly used, they have the potential to cause damage, such as dentin charring, ankylosis, cementum melting, root resorption, and periradicular necrosis [10].

On the contrary, non-coherent light-emitting diodes (LEDs) are safe, non-thermal, nontoxic and noninvasive, and to date, no side effects have been reported from their use [11].They are complex semiconductors that convert electrical currents into incoherent narrow spectrum light at wavelengths ranging from the ultraviolet (UV) to the visible to the near infrared (NIR). The bactericidal activity of LEDs is unclear, although the effectiveness of such lights is well known [12]. A possible mode of action has been explained in the literature through photon absorption mediated by endogenous (intracellular) sensitizers, such as porphyrins and flavins, or exogenous (extracellular) sensitizers, such as humic compounds [13]. Excited sensitizers transfer energy or electrons to other parts of the cell, causing damage, or to molecular oxygen, producing reactive oxygen species (ROS) that cause photooxidative damage [14]. Depending on whether energy or electrons are transferred to molecular oxygen, ROS, such as singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals, are formed and can damage membrane lipids, proteins, enzymes, or nucleic acids**.**

The effects of LED irradiation are dependent upon the wavelength, power density, quantity (or number) of bacteria, and microbial species [15]. For example the wavelength influences the depth of penetration. Lights that operates at the near infrared spectrum (NIR) are characterized by a greater depth with respect to those characterized by an inferior wavelength [16]. When LED and LASER are used at very low intensities (LLLT), the energy is enough to activate the target cells, but without a rapid or significant increase in tissue temperature [17]. LED-LLLT is effective in a large variety of clinical indications, such as pain attenuation, wound healing, skin rejuvenation, some viral diseases, allergic rhinitis, other allergy-related conditions and so on [18].

Currently, no studies have been published about the effects of NIR-LEDs used at low intensities (LLLT) on bacteria *in vitro.*

The aim of this study was to assess the antibacterial activity of LED irradiation and sodium hypochlorite (NaOCl) alone and then to evaluate the effects of the concurrent use of both treatments on *E. faecalis*. Before proceeding with the main experiment, we first performed preliminary tests to verify the influence the parameters, such as the distance of irradiation (d), the energy density, the irradiation time and the presence of photosensitizers on LED activity.

2. MATERIALS AND METHODS

2.1 Light source and irradiation parameters

A NIR-LED device characterized by an 880 nm-wavelength was used as the light source (Tr-Lux, Errevi, Italy).

The hand-piece was constituted by 6 LEDs (12-mm diameter) disposed in two lines (Fig 1 A). To simplify the comprehension of the methods used, we will refer throughout the text to the energy output (mJ/s) emitted by a single led. In all of the experiments, the LED hand-piece was mounted perpendicularly to the wells through the use of a particular polystyrene box to maintain a constant distance of irradiation (d), as shown in Fig 1B. Irradiation was performed under a laminar flow hood in the dark under aseptic conditions in all of the experiments.

2.2 Bacteria and culture conditions

Enterococcus faecalis ATCC 29212 was cultured at 37°C for 24 h in Brain Heart Infusion (BHI) broth. The bacterial suspension was evaluated using a spectrophotometer (Agilent Technologies 8453 UV, Santa Clara, USA) to assure an optical density of 0.5 McFarland corresponding to 108 Colony Forming Units (CFU)/mL. *E. faecalis* solution was prepared for 25-well (dimension: 20*20 mm) flat-bottom plates with lids separately for several experiments (test).

Aliquots of 1 mL were dispensed in triplicate into micro-titer plates for each treatment group, and all of the tests included a positive control (C+) and a negative one (C-).

2.3 Test 1

Two groups, characterized by a different distance of irradiation (d), were tested: LED 5 (d=10 mm) and LED 5C (d=0.5 mm). Both groups were irradiated for 5 minutes at an energy output of 2.37 mJ/s.

2.4 Test 2

Two groups, irradiated by a different energy output (e), were tested: prog A (e=2.37 mJ/s) and prog B (e=8.15 mJ/s). Both groups were irradiated for 5 minutes at a constant distance d= 10 mm.

2.5 Test 3

Two groups were tested and characterized by a different irradiation time (t) IR 5 (t=10 min), IR 10 (t=10 min) and IR 20 (t=20 min). Among these groups, another subcategory was characterized by the addition of toluidine blue before the LED irradiation (IR+TBO). Toluidine blue O (TBO) powder (Diapath S.p.A. - Italy) was prepared in deionized water and subsequently dissolved at concentration 25 µM. All irradiations were performed for 5 minutes at d=10 mmat, a measured energy output of 2.37 mJ/s.

2.6 Test 4

Three different test groups were distinguished: LED 5 irradiated for 5 minutes, NaOCl incubated 5 min in 1% sodium hypochlorite solution and LED 5+ NaOCl incubated with sodium hypochloride and simultaneously irradiated.

The irradiation was performed for 5 min at a measured energy output of 2.37 mJ/s. Sodium hypochlorite (NaOCl) for endodontic use (Niclor 5%, Ogna, Italy) was diluted to give accurate concentrations of 1%. NaOCl was neutralized after testing using sodium thiosulphate (Na2S2O) (Sigma) at 3.86% for the neutralization [19]. Dulbecco's phosphate buffered saline 0.00095 M (DPBS) (Lonza, Belgium) at pH 7.1 was used for serial dilutions and to equalize the levels of all of the wells.

2.7 Bacterial analysis

At the end of each treatment, the samples were checked, serially diluted in DPBS and analyzed with a Gram stain and by colony morphology in MacConkey plates without crystal violet (DifcoTM, Becton, Dickinson and Company; Sparks USA); then, the samples were incubated at 37°C for 24 h to confirm the purity of the microorganism which had been inoculated. The number of colony forming units per milliliter (CFU/ml) was then determined.

As a positive control (C+), three wells (for each experiment) were used with only a nutrient solution and *E. faecalis*. They showed bacterial growth with conventional microbial culturing techniques and confirmed the viability of the microorganisms throughout the experiment. As a negative control (C-), wells were used with only sterile nutrient solution. This was confirmed by the transparency of the solution and conventional microbial culturing techniques.

2.8 Statistical analysis

The results obtained were tabulated, expressed in log10 (CFU/mL) and subjected to statistical analysis. The means and standard deviations of the colony forming units were calculated and compared by one-way ANOVA using a statistical software program, Stat View 4.0 software (Abacus Concepts, Berkeley, CA, USA). Multiple comparisons were corrected using Bonferroni post-hoc tests.

3. RESULTS

3.1 Test 1

All of the experimental wells were inoculated with bacterial suspensions of *E. faecalis* that showed an optical density of 0.5 McFarland, corresponding to 8.73 (± 0.15) log10CFU/mL. The bacterial count (log10CFU/mL) after the treatment was 7.63 (±0.48) and 7.97 (±0.42) for LED 5 and LED 5C, respectively. Statistically significant differences were found between the control (C+) and both irradiated groups: LED 5 ($p=0.0047$) and LED 5C ($p= 0.0121$) No statistically significant differences were found between the two test groups.

3.2 Test 2

The bacterial suspension inoculated in all of the experimental wells showed an optical density corresponding to the concentrations of 8.75 ± 0.33 log10CFU/mL, as determined using a positive control by the culture method. The bacterial counts (log10CFU/mL) after treatment were 8.05 (± 0.05) and 7.98(± 0.03) for program A and program B, respectively. The LED irradiation was characterized by a reduction that was statistically significant compared to the C+: program A (p=0.0005) and program B (p=0.001). No statistically significant differences were found between the two programs.

3.3 Test 3

The bacterial suspension inoculated in all of the experimental wells showed an optical density corresponding to 9.140 ± 0.170 log10CFU/mL, as determined by the culture method. At the end of the LED irradiation, both treatment groups, independently from the irradiation time, were characterized by a significant reduction in mean bacterial counts (log10CFU/mL): 7.120 ± 0.055 (p= 0.001), 7.180 ± 0.052 (p= 0.001) and (7.220 ± 0.054 (p= 0.001) as characterized LED 5, 10 and 20, respectively. No statistically significant differences were measured when adding toluidine blue O (TBO) into the *E. faecalis* bacterial suspension before the LED irradiation.

3.4 Test 4

All of the experimental wells were inoculated with *E. faecalis* bacterial suspensions that showed an optical density corresponding to 8.740 ± 0.270 log10CFU/mL. LED treatment for 5 min showed a statistically significant reduction (7.840 ± 0.34 log10CFU/mL) compared to the control group (C+) (p = 0,005). Treatment with 1% NaOCl caused a significant reduction in bacterial counts to 3.930 ± 0.210 log10CFU/mL (p = 0.001). The matched actions of LED and 1% NaOCl per 5 min totally inhibited the microbial growth.

4. DISCUSSION

In this study, we tested the *in vitro* inactivation of *E. faecalis* with a NIR-LED used at low intensities.

We selected *E. faecalis* ATCC 29212 because it has been extensively used by many authors as a representative control strain for clinical and laboratory experiments to assess the quality of commercially prepared microbiological culture media, test the susceptibility of pathogens to antibiotics, identify commercialized bacterial species (API, Vitek, etc.), and microbiologically test endodontics. *E. faecalis* strains are now regarded as opportunistic pathogens that share resistance to clinically relevant antibiotics and represent a serious public health problem that not only involves dentistry because they are also major causes of hospital-acquired infections [20, 21]. The aim of test 1 was to verify if decreasing the distance of irradiation from 10 to 0.5 mm impacted the effectiveness of LED antibacterial activity on *E. faecalis.* The results suggested that varying the distance of irradiation inside this range did not significantly affect the mean CFUs/ml; on the contrary, there were statistically significant differences with respect to the controls (C+) for all of treatment groups. These results are in accordance with the literature that has shown that NIR lights have great penetration ability, and for this reason, they may exert antimicrobial activity at a distance of 10 mm without reducing their effectiveness [22].

The aim of test 2 was to verify whether increasing the energy output from 2.37 mJ/s (prog A) to 8.15 mJ/s (progr B) affects the antibacterial activity of LEDs. The results showed no significant differences on the mean bacterial survival rates between the two irradiated groups; on the contrary, there were statistically significant differences with respect to the controls (C+). These results suggest that small differences in energy output, at this particular wavelength, are enough to obtain the antibacterial effect of LEDs and that

increasing this parameter affords no additive effects. These results encourage the use of LEDs at this particular wavelength to obtain an antibacterial activity. However, as a note, Maclean et al. observed no measurable destruction of *E. faecalis* using a different LED device with a wavelength of 405 nm at an energy density of 72 J/cm^2 (10 mW/cm2 for 2 hours) [23].

The first two preliminary experiments permitted us to verify the effectiveness of NIR-LED by reducing the viability of *E. faecalis*, so we the proceeded to tests 3 and 4.

The aim of test 3 was to verify whether the irradiation time or the presence of toluidine blue (TBO) influenced the antimicrobial effectiveness of LED. TBO has been reported to be an antifungal and antibacterial drug for the inactivation of yeast and some Gram-positive and Gram-negative bacteria [24]. It has been largely described as a photosensitizer that is able to increase the antimicrobial effects of laser irradiation; however, this additional effect is wavelength-dependent. For example, TBO and diode laser irradiation of wavelengths ranging between 625 and 805 nanometers are effective in eliminating *E. faecalis*, whereas the same treatment with wavelengths ranging between 635 and 660 nm are ineffective [25].

Our results suggest that using an 880-nm LED device, the irradiation time and the presence of TBO had no effects on the viability of *E. faecalis*. The lack of the additional bactericidal effect found in this study by adding toluidine blue to the LED irradiation is not necessarily in contrast with the literature, as the antibacterial actions of TBO are a complex phenomenon and depend upon many parameters, such as the dye concentration, the type of microorganism, the dark incubation time, and the exposure to red laser light [26].

The aim of test 4 was to detect the additive effects of 5 minutes of LED irradiation + 1% NaOCl on *E. faecalis*. LED irradiation and NaOCl alone are able to significantly decrease the viability of *E. faecalis*. The total inhibition of *E. faecalis* was obtained throughout the concurrent treatment with LED and NaOCl (1%) in only 5 minutes. On the contrary, it is known that NaOCl 1% alone requires 10 minutes to totally eliminate *E. faecalis* [19].

Our study gains more importance if we consider that the extensive use of sodium hypochlorite and other antibiotics during endodontic treatments *E. faecalis* is still one of the major causes of secondary endodontic lesions [27]. Our findings suggest that the addition of a 880-nm LED treatment to conventional treatment protocols, without the need for adjunctive use of TBO, provides similar bactericidal effects to conventional treatment alone in a shorter time. The same antimicrobial activity has been described in the literature by means of laser irradiation with higher power intensities, such as 2000 mW for 60 s, vs. the 14.22 mJ for 300 s used in our study, with the probable development of thermal effects [28-29-30]. Another advantage of LEDs are that these systems offer large planar arrays, so that they can irradiate a large area of the body in a hands-free manner compared with the point-by-point application of a laser system [18]. In addition, many different cell types can be simultaneously targeted, also with systemic effects [31-32]. It is now known that the symptoms and signs of endodontic lesions are the results of inflammation due to the bacterial activity and the consequent host response [33-34]. For this reason, the capacity for bio-stimulation and anti-inflammatory activities, as shown in the literature, together with the antimicrobial activity of LED suggested by this study, allow us to hypothesize possible clinical applications of this device as an additive or alternative method to treat acute endodontic abscesses, pulpitis and other endodontic pathologies. However, more *in vitro* and clinical studies are necessary to confirm this hypothesis.

5. CONCLUSIONS

The results of the present in vitro study show that 880-nm LED irradiation reduces *E. faecalis* counts.

Total inactivation of *E. faecalis* was achieved through 5 minutes of concurrent use of NaOCl 1% and LED irradiation.

This study is only the first steps in the development of an optimum protocol treatment for *E. faecalis* infections, considering that there are no previous studies of NIR-LED in this field. For future studies, we are applying the methods used in this study on other bacteria and microorganisms organized as biofilms. If other studies on more specific bacteria confirm these results, LED irradiation and NaOCl 1% could be a viable option for many clinical applications, including the treatment of acute endodontic abscesses.

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Figure captions:

Fig. 1 Schematic illustration of the apparatus utilized to obtain uniform LED irradiation of the samples, maintaining a constant distance

(d) from the LED bulbs to the samples.

Fig. 2 The effects of the distance (d) from the light source on *E. faecalis*: LED 5: d = 1 cm; LED 5C: d= 0.5 mm. *Statistical significance at 0.05 in comparison to the control group.

Fig 3 The effects of different output energies on *E. faecalis:* PROG A: 2.37 mJ/s for each of the 6 lamps; PROG B 8.15 mJ/s for each of the lamps. *Statistical significance at 0.05 in comparison to the control group.

Fig 4 Different irradiation times (IR), with or without the presence of a photosensitizer toluidine blue O (TBO), on *E. faecalis.* C+: controls, IR 5: 5 minutes; IR10:10 minutes; IR 20: 20 minutes of irradiation. *Statistical significance at 0.05 in comparison to control group.

Fig 5 The effects of various treatments on *E. faecalis*: LED 5: 5 minutes of irradiation; NaOCl: 5 minutes of Sodium Hypochlorite 1%; LED+NaOCl=5 minutes of concurrent irradiation and Sodium Hypochlorite 1%. *Statistical significance at 0.05 in comparison to control group.