NEAR-INFRARED LEDS PROVIDE PERSISTENT AND INCREASING PROTECTION AGAINST *E. FAECALIS*

Petrini M, Spoto G, Scarano A, D'Arcangelo C, Tripodi D, Di Fermo P, D'Ercole S

Order of Authors:

- Morena Petrini, DDS, PhD, Specialist in Oral Surgery, Department of Medical, Oral and Biotechnological Science, University G. d'Annunzio of Chieti
- Spoto G, Full Professor of Dental Materials, Dental Materials and Medical Chemistry Unit, Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Via dei Vestini 31, 66013 – Italy
- Scarano Antonio, MD, DDS, MS, Full Professor of Oral Surgery, Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Via dei Vestini 31, 66013 – Italy
- D'Arcangelo Camillo, DDS, Full Professor of Restorative Dentistry, Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Via dei Vestini 31, 66013 – Italy
- Domenico Tripodi, DDS, Associate Professor of Paediatric Dentistry, Paediatric Dentistry Unit, Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Via dei Vestini 31, 66013 – Italy
- Di Fermo Paola, BD, Microbiology Unit, Department of Pharmacy, University of Chieti, Via dei Vestini 31, 66013 – Italy
- D'Ercole S, Ph.D, DDS, Microbiology and Paediatric Dentistry Unit, Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Via dei Vestini 31, 66013 – Italy

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Corresponding author address

Giuseppe Spoto, Full Professor of Dental Materials and Medical Chemistry

Department of Medical, Oral and Biotechnological Sciences

University of Chieti - Italy

Address: Via Vestini 31, 66013 Chieti, Italy

Phone: +39 0871 355 4093

Email: materialidentari.uda@gmail.com

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ABSTRACT

In a previous study, we have shown that non-coherent light-emitting diodes (LEDs) are able to significantly decrease *Enterococcus faecalis* viability, *in vitro*. In particular, the concurrent treatment for 5 min with sodium hypochlorite (NaOCl) 1.0% and LED permitted to obtain the total inactivation of the culture.

The primary outcome of this study was to verify if the photoinactivation was only temporary or stable over 1 week. The secondary outcome was to verify if using a minor concentration of NaOCl together with LED irradiation, it was possible to reach the total inactivation of the culture.

Bacterial suspensions were subjected to different protocols. NaOCl samples were treated with 0.5 % sodium hypochlorite for 5 minutes; LED 5 were subjected to 880-nm LED irradiation for 5 minutes and LED+NaOCl were contemporary subjected to LED irradiation and 0.5% sodium hypochlorite for 5 minutes.

Then the colony forming units per milliliter (CFU/mL) was analyzed and recorded at 5 min, 24 h, 48 h and 1 week. The data were submitted to ANOVA and LSD post hoc tests at a level of significance of 0.05. Live dead assay and fluorescent microscope observations were also performed for all group at all timing points.

The results showed that the 3 protocol treatments were able to decrease significantly the bacterial count respect controls, however, after 5 min, 24 h and 48 h the most effective reduction was measured for NaOCl and NaOCl+LED 5. In the first 48 h LED 5 alone reduced significantly the bacterial count respect controls, but it was higher respect the other tested groups. On the contrary, after 1 week we found an interesting result: samples treated with LED were characterized by a decrement of bacterial count, independently from the addition of sodium hypochlorite. Samples treated with NaOCl alone were characterized by a mild increment of bacterial count, although results were still significant respect C+. The secondary outcome was not confirmed: a concentration of 0.5% together with LED irradiation did not permit to reach the total inactivation of the culture.

Keywords: LED; *Enterococcus faecalis*; Endodontics; Photoinactivation; Photobiomodulation

1. INTRODUCTION

Light emitting diodes (LEDs) are complex semiconductors that convert electrical current into incoherent narrow-spectrum light [1]. The wavelength of the photons emitted influences the modality in which cells are trigged and a cascade of events begins. Visible light acts mainly activating intracellular (such as porphyrins and flavins), or extracellular sensitizers (such as humic compounds) [2]. On the contrary, near-infrared (NIR) waveband is adsorbed in the cellular membrane, causing a photophysical response [3, 4].

Clinical uses of LED devices range from pain attenuation, wound healing, skin rejuvenation, treatment of viral diseases, allergic rhinitis to other allergy-related conditions and so on [3, 4]. However, also an antimicrobial activity has been described, and, for this reason, these devices have been considered a promising option against the antibiotic resistance challenge [5,6]. It is important to highlight that therapeutic action of LED devices are effective also with very low levels of energy and for this reason, in the recent years, the term photobiomodulation therapy (PBMT) has been introduced in order to comprehend both low-level laser therapy (LLLT) that LEDs treatment. PBMT involves the use of visible or near-infrared light (NIR), to cause physiological changes and therapeutic benefits, without thermal damages [7–9]. However, the effects of PBMT depends on several parameters like wavelength, power intensity, frequency, duration and the distance of the irradiation.

Enterococcus faecalis is a gram-positive bacterium, part of the commensal flora of the intestine that is particularly known for its high resistance and ability to survive in extreme environmental conditions such as acid or alkaline pH, a high concentration of salts and of heavy metals and a low concentration of nutrients [10,11].

It is very resistant to many antibiotics and antimicrobials, so, it is frequently the cause of nosocomial infections. In dentistry, this bacterium is particularly important for secondary endodontic lesions although the extensive use of root canal irrigants, intra-canal medicaments, mechanical instrumentation during the root canal treatment and the multitude of novel methods for the endodontic fillings [12–15]. Therefore, advanced disinfection methods are required to obtain the total eradication of microorganisms present in the endodontic system and eliminate the pulpal and the periapical inflammation [14].

NaOCl irrigants, at different concentrations, are considered the gold standard in endodontics, even so, they display some degree of toxicity levels toward the host tissues [16]. The antibacterial action of NaOCl depends on the concentration of undissociated hypochlorous acid ($HClO^-$) in solution. $HClO^-$ generates an oxidative action on sulfhydryl groups of bacterial enzymes. As essential enzymes are inhibited, important metabolic reactions are disrupted, resulting in the killing of the bacterial cells [17]. However, the research of novel, efficient and non-toxic alternatives is advisable.

In previous studies we have shown the ability of *in vitro* LED irradiation to significantly reduce the bacterial count of *E. faecalis* and *Pseudomonas aeruginosa*, and we have hypothesized the use of this device in

dentistry, alone or in combination with chemical antimicrobials such as sodium hypochlorite and chlorhexidine [5,6]. Increasing the efficacy of traditional irrigants it could be possible to use reduced concentrations, in order to decrease costs and the risk of toxicity.

An important issue that needs to be solved is the stability of the antimicrobial activity promoted by light treatment: most of the literature evaluated the bacterial count after a relatively short time respect light exposure. However, some authors have claimed that although the light therapies were able to remove completely microorganisms from irradiated sites, bacteria were able to regrowth and reestablish a new infection after only 24 h [18].

So, it is fundamental to be sure of the stability of the antimicrobial activity of LED devices, in order to permit the long-term success of the endodontic treatment. The aim of this study is to monitor *E. faecalis* along a relatively long time of 1 week, after the exposure to LED light.

2. MATERIALS AND METHODS

Enterococcus faecalis ATCC 29212 was grown in Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h. The bacterial suspension was determined using a spectrophotometer (Agilent Technologies 8453 UV, Santa Clara, USA) adjusting the concentration to 9.270 log10 Colony Forming Units, log10(CFU/mL). *E. faecalis* solution was prepared for 25-well (dimension: 20*20 mm) flat-bottom plates with lids separately for several experiments (test), Fig. 1. Aliquots of 1 mL were dispensed in triplicate into microtiter plates for each treatment group, and all of the tests included a positive control (C+) and a negative one (C-). As a negative control (C-), wells were used with the only sterile nutrient solution. This was confirmed by the transparency of the solution and conventional microbial culturing techniques. As a positive control (C+), three wells (for each experiment) were used with only *E. faecalis*: they showed bacterial growth with conventional microbial culturing techniques and confirmed the viability of the microorganisms throughout the experiment.

Three different TEST groups were distinguished: “LED 5” irradiated for 5 minutes, “NaOCl” incubated 5 min in 0.5% sodium hypochlorite solution and “LED 5 + NaOCl” incubated with sodium hypochlorite and simultaneously irradiated. Sodium hypochlorite (NaOCl) for endodontic use (Niclor 5%, Ogna, Italy) was diluted to give accurate concentrations of 0.5%. 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. A NIR-LED device characterized by an 880 nm-wavelength was used as the light source (TR LUX, ERREVI GROUP, Bergamo, Italy), as previously described [6]. Irradiation was performed maintaining the

hand-piece perpendicularly to the well, at a constant distance of 10 mm under a laminar flow hood in the dark under aseptic conditions. The LED irradiation was performed for 5 min at a measured energy output of 2.37 mJ/s.

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 and the number of colonies forming units per milliliter (CFU/ml) was then determined at different time points: 5 min, 24 h, 48 h and 1 week, as shown in Fig. 1.

LIVE/DEAD assay

The cells viability was evaluated with a BacLight LIVE/DEAD Viability Kit (Molecular Probes, Invitrogen detection technologies, USA). SYTO 9 stains viable cells with a green fluorescent signal, and propidium iodide stains cells with impaired membrane activity red.

Three independent experiments on multiwell plates were performed in triplicate as described above.

After treatments, 1 mL of the bacterial culture was concentrated by centrifugation at 10,000 × g for 10–15 minutes. Then 250 µL of BacLight LIVE/DEAD Viability Kit has been added to the bacterial pellet, mixed thoroughly and incubated at room temperature in the dark. After 15 minutes, 10 µL of the stained bacterial suspension were trap between a slide and an 18 mm square coverslip.

Then the images were observed at fluorescent Leica 4000 DM microscopy (Leica Microsystems, Milan, Italy) were recorded at an emission wavelength of 500 nm for SYTO 9 (green fluorescence) and of 635 nm for propidium iodide (red fluorescence) and more fields of view randomly were examined.

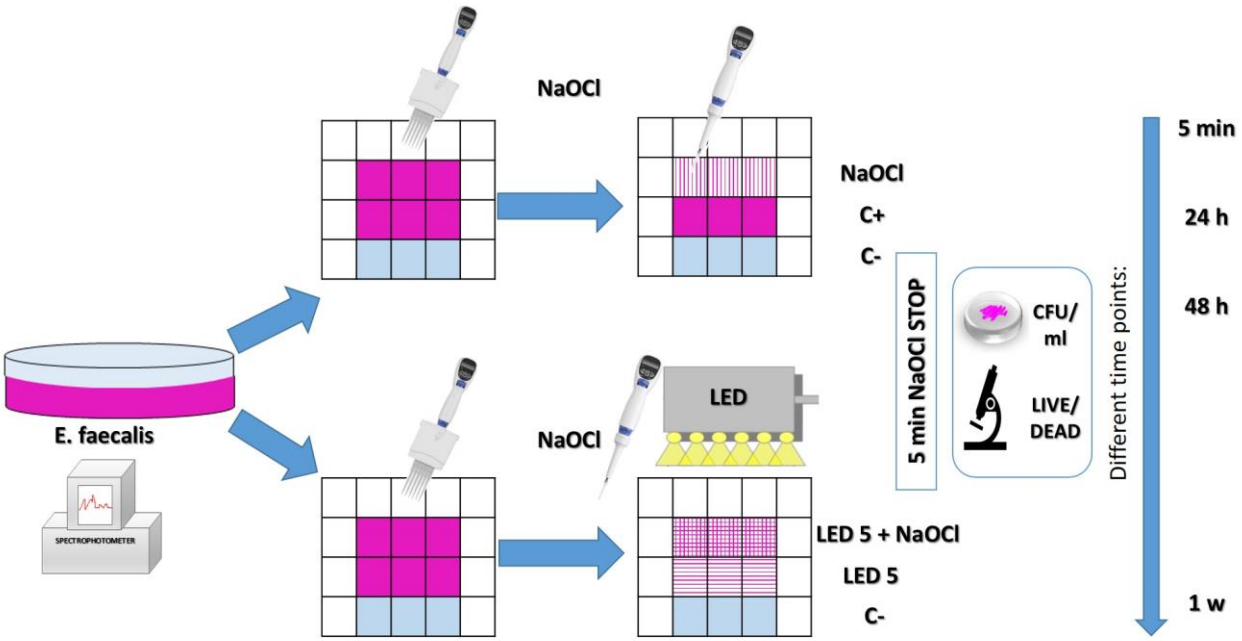

Figure 1 Experimental overview and workflow.

2.1 Statistical analysis

The results obtained were tabulated and subjected to statistical analysis using SPSS for Windows version 21 (IBM SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) and the LSD test was used to compare the parameters analyzed in the study. The significance threshold was set at 0.05.

3. RESULTS

All of the experimental wells were inoculated with *E. faecalis* bacterial suspensions that showed an optical density corresponding to 9.270 log10CFU/mL. Mean values of *E. faecalis* CFU/mL expressed in log10 (+/- standard deviation) at different time points are shown in Tab. 1 and Fig. 2.

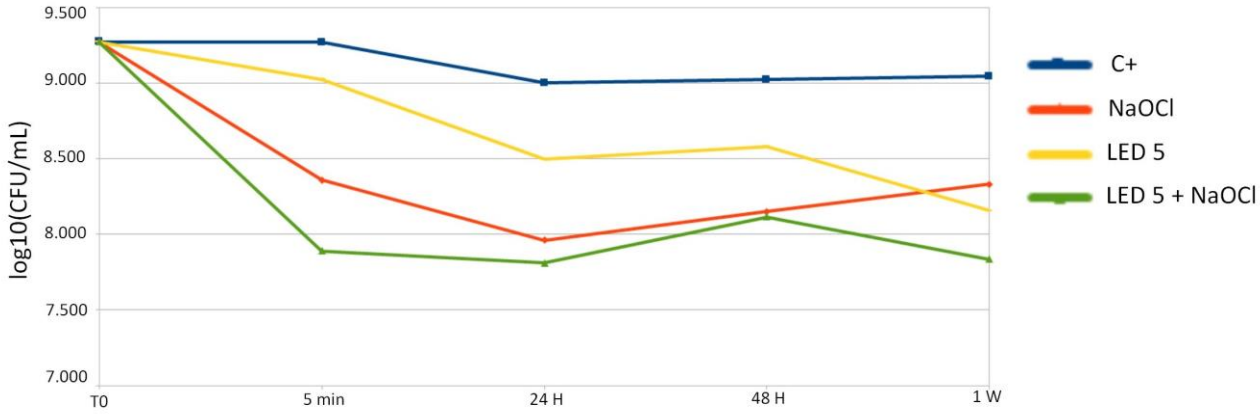

Figure 2 The effects of different protocols on the bacterial count of E. faecalis: bacterial count expressed in log10(CFU/mL) after the different treatment and at different time points.

LOG$_{10}$(CFU/ML)	***5 MIN***	***ST. DEV***	***24 H***	***ST. DEV***	***48 H***	***ST. DEV***	***1 W***	***ST. DEV***
C+	9.269	8.355	9.001	8.604	9.023	8.791	9.043	8.780
NAOCL	8.358	8.085	7.960	7.470	8.151	7.827	8.332	8.318
LED 5	9.024	7.695	8.496	8.297	8.580	8.441	8.156	8.286
LED 5 + NAOCL	7.890	7.693	7.809	7.076	8.114	8.180	7.835	7.947

Table 1 Mean values (+/- standard deviations) of *E. faecalis* colony forming units (log10) at different time points (5 minutes, 24 hours, 48 hours and 1 week). Before starting the treatments, all groups were characterized by the same amount of bacteria, 9.270 (log10 CFU/mL).

Intra-group correlations (Tab. 2) showed that C+ remained constant for all time points, except a mild deflection of CFU at 24 h. On the contrary, other TEST groups treated with NaOCl, LED 5 and LED 5+NaOCl showed statistically significant differences between T0 and all measured time points. It means that all treatments were effective in reducing significantly *E. faecalis, in vitro*. It is important to highlight that LED 5 continues to decrease bacterial count also after the end of the treatment, indeed there are statistically significant differences between mean values at 5 min and other time points. Otherwise, both groups treated with NaOCl are characterized by a mild increase of bacterial count between 24 and 48 h, although no significant differences have been found.

TREATMENT	TIME POINT		Sign.
C+	T0	5 MIN	0.823
		24 h*	0.040
		48 h	0.053
		1 w	0.068
	5 MIN	24 h	0.082
		48 h	0.105
		1 w	0.132
	24 h	48 h	0.883
		1 w	0.775
	48 h	1 w	0.889

TREATMENT	TIME POINT		Sign.
LED 5	T0	5 MIN*	<0.001
		24 h*	<0.001
		48 h*	<0.001
		1 w*	<0.001
	5 MIN	24 h*	<0.001
		48 h*	<0.001
		1 w*	<0.001
	24 h	48 h	0.645
		1 w	0.249
	48 h	1 w	0.058

NaOCl	T0	5 MIN*	<0.001
		24 h*	<0.001
		48 h*	<0.001
		1 w*	<0.001
	5 MIN	24 h	0.204
		48 h	0.582
		1 w	0.158
	24 h	48 h	0.456
		1 w*	0.025
	48 h	1 w	0.075

LED 5 + NaOCl	T0	5 MIN*	<0.001
		24 h*	<0.001
		48 h*	<0.001
		1 w*	<0.001
	5 MIN	24 h	0.825
		48 h	0.376
		1 w	0.876
	24 h	48 h	0.221
		1 w	0.921
	48 h	1 w	0.248

Table 2 Intra-group correlation at different time points. *p<0.05 statistically significant differences

Extra-group correlations (Tab. 3) showed that at 5 minutes all TEST groups were significantly decreased respect C+, but LED 5 were characterized by a higher bacterial count respect NaOCl and LED 5+NaOCl ($p<0.001$). The same trend was maintained at 24 h. At 48 h and at 1 w the level of NaOCl was characterized by a slight increase, however, the bacterial level was still significantly lower respect C+ ($p<0.001$ and $p=0.010$, respectively). On the contrary, LED 5 between 48 h and 1 w continued to be characterized by a decrease of the bacterial count and no statistically significant differences have been found between LED 5 and NaOCl and LED 5+ NaOCl at 48 h and 1 w.

LSD	TREATMENT		Sign.
5 min	C+	NaOCl *	<0.001
		LED 5 *	<0.001
		LED 5 + NaOCl *	<0.001
	NaOCl	LED 5 *	<0.001
		LED 5 + NaOCl	0.079
	LED 5	LED 5 + NaOCl*	<0.001
24 h	C+	NaOCl*	<0.001
		LED 5*	<0.001
		LED 5 + NaOCl*	<0.001
	NaOCl	LED 5*	<0.001
		LED 5 + NaOCl	0.079
	LED 5	LED 5 + NaOCl*	<0.001

LSD	TREATMENT		Sign.
48 h	C+	NaOCl*	<0.001
		LED 5*	0.009
		LED 5+NaOCl*	<0.001
	NaOCl	LED 5	0.318
		LED 5+NaOCl	0.925
	LED 5	LED 5+NaOCl	0.282
1 w	C +	NaOCl*	0,010
		LED 5*	<0.001
		LED 5+NaOCl*	<0.001
	NaOCl	LED 5	0.204
		LED 5+NaOCl	0.115
	LED 5	LED 5+NaOCl	0.630

Table 3 Extra-group correlation at different time points. *p<0.05 statistically significant differences.

Fig. 3 shows the live dead images of the analyzed treatment protocols at different time points. C+ (first column) remains constant in vitality at all time points. The more significant effect of LED 5 is visible after 1 week from irradiation, with the evidence of an aggregate of dead cells (fourth row, second column). NaOCl shows an evident killing effect (third column) at all time points observed. The combination of LED 5 + NaOCl results in a marked killing effect on the population of E. faecalis in all measurements made with a progressive aggregation of dead cells.

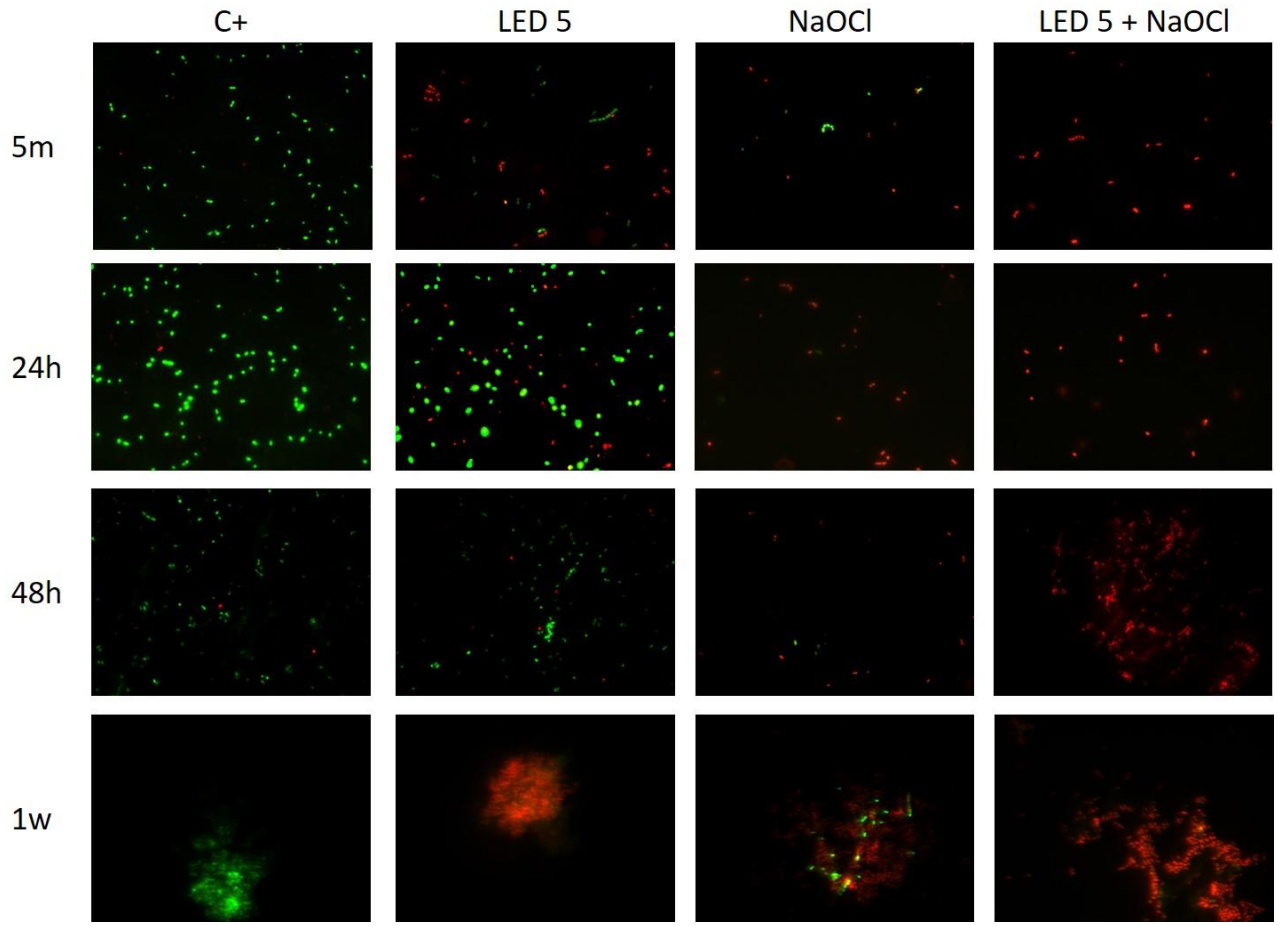

Fig. 3 Representative images of fluorescent microscope observations of live dead assay of all treatment protocols during the time.

4. DISCUSSION

The aim of this study was to verify if the antibacterial effect of LED irradiation on *E. faecalis* that we had previously described, was persistent over time [5, 6]. *E. faecalis* was cultured in Dulbecco's phosphate buffered saline, which allows the maintenance of the viability of the bacterium that was used in this study[20].

We compared the bacterial count, at different time points, of samples treated with different protocols: 5 minutes of 0.5% NaOCl, 5 minutes of LED irradiation and 5 minutes of contemporary NaOCl and LED irradiation (Fig. 1). Following the indication of several studies, we used NaOCl at a concentration of 0.5% in order to obtain the bactericidal levels with an acceptable level of cytotoxicity [21].

Our results have shown that after 5 min from treatment, all 3 TEST protocols were effective in significantly reducing the bacterial count (Fig. 2, Tab. 1). Live dead staining confirmed that the better antimicrobial activity in all timing points is achieved with LED 5+NaOCl (Fig 3, fourth column). LED irradiation seems to

potentiate the killing effect of NaOCl and a marked antibacterial activity is evident. However, also LED 5 (Fig 3, second column), is characterized by an antibacterial effect, with a progressive aggregation of dead cells that reach the maximum after 1 week from the irradiation.

These results are in accordance with our previous work in with 5 min of LED irradiation was able to significantly reduce *E. faecalis and Pseudomonas aeruginosa, in vitro* [5].

The most effective treatment at 5 min, 24 and 48 h seems to be 0.5% NaOCl and LED 5 + NaOCl, with the latter group characterized by the lower level of bacterial count.

The lower concentration of NaOCl used in this study, 0.5%, respect that adopted in the previous work, 1.0%, did not permit to reach the total inactivation of the bacteria when applied in contemporary with LED irradiation [6].

The antibacterial action of NaOCl is mainly due to the effect of hydroxyl ions and chloramination, that lead to irreversible oxidation of SH groups (sulphydryl group) of bacterial enzymes that are essential for their metabolism [22].

However, the antibacterial action of NaOCl seemed to be influent especially in the first part of the experiment, then its effects seem to be slightly attenuated. Indeed, after 48 h from the treatment, the antibacterial effect of NaOCl seems to decrease, on the contrary, both group irradiated by LED continued to decrease the bacterial count. The antibacterial effect of LED irradiation on *E. faecalis* in the present study was not immediate but developed in a time-dependent manner.

These results are in accordance with our previous work, in which the effect of photoinactivation enhanced by LED treatment was evaluated on *Pseudomonas aeruginosa* [5]. The effect of photoinactivation enhanced by LED was more evident after 24 h; on the contrary, the antibacterial activity of chlorhexidine seemed to be reduced with time [5].

In particular, we have shown that, after a stationary phase between 24 and 48 h, the effect of LED 5 reappears and antibacterial activity increases significantly up to a week. The photoinactivation enhanced by NIR-LED devices is promoted by a photophysical reaction: membrane molecules undergo a vibrational and rotational change in their energy level after that membrane phoacceptors adsorbed photons emitted by LED [3, 4]. This mechanism that activates transports mechanisms, increase cell energy requirement and the mitochondrial ATP production, led to cytoplasmatic and extracellular changes and results with the production of ROS species harmful for bacterial cells, promoting the reduction the bacterial count. Several authors demonstrated that photoinactivation, UVA- or visible light-induced, causes a sporadic and not massive wall destruction of *E. faecalis* cells [23]. According to the authors, this phenomenon indicates that the induced damage was directed to proteins and/or lipids of the cytoplasmic membrane, resulting in the leakage of cellular contents [24, 25].

With regards to the time-dependent effect of LED irradiation, it is thus possible that the main targets of this treatment are various structures and components of bacterial cells.

Therefore, this method could promote oxidative stress, which results in DNA damage and the destruction of cell components whose dysfunction finally leads to cell death.

If the oxidative pressure is too great it could evoke apoptotic death. Several studies showed that prokaryotes undergo death by a mechanism similar to apoptosis known as the thetoxin-antitoxin system [25,26].

In this study, it is thus possible that the stress associated with the production of ROS species also induced apoptotic death of bacteria.

The contemporary action 5 min, LED 5 + NaOCl, incubated with sodium hypochlorite and simultaneously irradiated enhances the antibacterial effect that is kept constant over time.

5. CONCLUSIONS

These results show that the antibacterial effect of LED treatment is persistent after 1 week from irradiation and seems to increase over time, contrary to the effect of NaOCl.

0.5% NaOCl added to LED irradiation permitted to achieve a higher level of photoinactivation in all timing point, but the total inactivation of the bacteria was not reached with these treatment protocols.

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