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ORIGINAL ARTICLE

Effectiveness of low concentration of sodium hypochlorite activated by Er,Cr:YSGG laser against *Enterococcus faecalis* biofilm

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Abstract

Bacteria living in biofilms exhibit altered growth phenotypes, while the biofilm provides benefits, the foremost of which is a certain protection against both immune system and killing effect by antimicrobials. Laser-activated irrigation (LAI) and passive ultrasonic irrigation (PUI) have been proposed as alternative methods for cleaning and disinfecting the root canal, as an adjuvant to conventional chemo-mechanical preparation in order to improve debridement and disinfection. Nevertheless, the potential antibacterial effect of LAI using 0.5% of sodium hypochlorite (NaOCl) has received little attention. Glass Pasteur pipettes were used to mimic single-tooth root canal and to build *Enterococcus faecalis* biofilm**.** Several irrigants and treatments were assayed for 60 s including (I) Saline, (II) NaOCl 0.5%, (III) NaOCl 5%, (IV) Er,Cr:YSGG, (V) Saline + LAI, (VI) NaOCl 0.5% + LAI, (VII) Saline + PUI, and (VIII) NaOCl 0.5% + PUI. Bacterial reduction was measured by counting the colony-forming units (CFUs). Additionally, AFM visualization and measurement of nano-roughness parameters were used to evaluate LAI effect on bacteria. NaOCl 5% unpowered and NaOCl 0.5% + LAI were capable of eliminating all bacteria, whereas non-activated saline solution and NaOCl 0.5% failed to eliminate *E. faecalis*. Lower efficiencies were achieved by PUI. Surface analysis by AFM revealed apparent alterations in NaOCl + LAI-treated cells. The Er,Cr:YSGG laser-activated irrigation (LAI) increased the bactericidal efficiency of 0.5% NaOCl against *E. faecalis* biofilm.

Keywords Er,Cr:YSGG laser · *Enterococcus faecalis* · Root canal disinfection · Laser-activated irrigation · Cavitation · Sodium hypochlorite

Introduction

The main goal in endodontics is the eradication of bacteria from the root canal system [1], since it has been well established that residual microorganisms play a key role in the development and perpetuation of endodontic infections [2]. Despite the fact that frequently endodontic infections are polymicrobial, environmental conditions in the root canal seem to favor some species, being *Enterococcus faecalis* the most frequently encountered when endodontic treatment fails. Nevertheless, *E. faecalis* constitutes only a minute proportion of the healthy oral microbiota [3, 4]. *E. faecalis* is characterized by its ability to withstand theoretically adverse conditions encountered in the root canal, including alkaline conditions

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and lack of nutrients for extended periods of time. This can be partly due to its ability to form biofilm [3]. Biofilms are structured communities of bacteria embedded in a self-produced polymeric matrix and adhered to a surface or an interface [5]. Bacteria living in biofilms exhibit altered growth phenotypes, while the biofilm provides benefits, foremost of which is a certain protection from the host's immune system and the killing effect by antimicrobials [6].

The existence of accessory canals, anastomoses, fins, oval extensions, and apical ramifications generate a complex three-dimensional network, making the complete removal of debris and bacteria extremely difficult when using conventional methods. Furthermore, bacteria reaching the root canal system may invade dentinal tubules resulting in the establishment of persistent infections [7]. Thus, an appropriate delivery and penetration of irrigating solutions into the root canal system is crucial for efficient debridement and disinfection, mostly to impact those areas that cannot be cleaned with mechanical instrumentation [8].

Due to its antimicrobial properties, sodium hypochlorite (NaOCl) has long been considered the primary disinfectant irrigating solution in endodontic procedures. It is used at concentrations ranging from 0.5 to 6% with varying degrees of effectiveness. Because hypochlorite is non-selective, it can also damage human cells, dentine, or periodontal tissues [9]. In this context, there is still controversy regarding which concentration of the solution offers the most safety for the patient's tissues and also renders the highest efficacy in killing microorganisms. Studies have determined that reducing the concentration of NaOCl limits cytotoxicity of the irrigant, however also reduces its bactericidal properties [10].

Laser-activated irrigation (LAI) has been proposed as an alternative method for cleaning and disinfecting the root canal, as an adjuvant to conventional chemo-mechanical preparation, in order to improve debridement and disinfection [11]. It has been reported that LAI enhances smear layer removal [12], has a bactericidal effect [13–15] and increases debris removal from the apical third of the root canal system [8]. LAI is based on the high absorption by water, of the energy of erbium laser energy (Er,Cr:YSGG: 2780 nm—Er:YAG: 2940 nm). Blanken et al. [16] demonstrated that the use of Er,Cr:YSGG laser produces immediate fluid movement into the root canal, leading to a vaporization and formation of large vapor bubbles. These vapor bubbles expand until irradiation ends, and then implode. Implosion leads to an underpressure and the subsequent sucking of fluid into the canal, generating a cavitation effect [8]. At this moment, pressure waves, which first move at supersonic speed and then later at sonic speed (shock and acoustic waves, respectively), are generated, causing shear forces. Thus, in fact, the laser acts as a fluid pump. Formation of laser-induced vapor bubbles and secondary cavitation highly depend on the characteristics of the laser, such as the wavelength, energy density, pulse width, and the geometry of the laser tip.

Passive ultrasonic irrigation (PUI) is based on inducing acoustic microstreaming and cavitation in the intracanal irrigant, which may enhance the removal of endodontic biofilms [17]. It has been seen that the mechanical aspect and dissolution properties of the irrigant are improved when activated by PUI or LAI, especially when NaOCl is used [17]. Nevertheless, the potential antibacterial effect of LAI using low concentrations of NaOCl has received little attention.

The aim of this study was to compare the antimicrobial efficacy of Er,Cr:YSGG laser-activated irrigation and passive ultrasonic irrigation of sodium hypochlorite 0.5% against *E. faecalis* biofilm by using an in vitro artificial "root canal" model infection to experiment procedures.

Materials and methods

Bacterial strain and culture conditions

E. faecalis, American Type Culture Collection (ATCC) 29,212, was maintained by weekly subculturing in Trypticase Soy Agar (TSA) plates (Scharlau, Barcelona, Spain). For experiments, it was cultured in 40 ml of Tryptic Soy Broth (TSB) medium (Scharlau, Barcelona, Spain) inoculating a single colony grown on TSA at 37 °C. After 24 h incubation, liquid culture was diluted 100 times in fresh TSB medium, adjusted spectrophotometrically (Unicam UV-2 at 600 nm) to $OD_{600} = 0.018$ (i.e., 3.4×10^7 colony-forming units CFUs/ml) and used.

In vitro "root canal" model and bacteriological evaluation

Glass Pasteur pipettes were used to replicate single-tooth root canal and to obtain the biofilm (Hirschmann Laborgeräte, Eberstadt, Germany) (Fig.1). Model dimensions were 7 cm in length with 6.95 mm in diameter at the top end and 1.1 mm inner diameter. The upper end of the pipette acted as a cylindrical irrigant reservoir. Each pipette was filled with 100 μl of bacterial suspension. The bel-bulb pipettor was adapted to the upper end of the pipette, and the irrigant was drawn up into the root canal model. The extremity was sealed with sterile adhesive (Blu-Tack, Bostik, Barcelona, Spain). The "inoculated pipettes" were incubated at 37 °C for 24 h to allow colonization and adhesion of *E. faecalis* to the inner walls. This allows to "infect" the extremity of the pipette.

A gentle washing of the inner part was performed with 1 ml of Ringer ¼ solution, to remove the non-adhered microbes and liquids, and thus leaving only the bacteria adhered to the glass. To count the remaining bacteria before and after treatment, the last 3 cm of the tip was recovered by grasping a clamp. End points were then dropped into sterile tubes with 5 ml of sterile Ringer ¼ solution and treated in an ultrasonic water bath (Ultrasonic Cleaner, Raypa, Barcelona, Spain) for

3 min at 1.5 W to suspend bacteria. Number of colony-forming units per square centimeter (CFUs/cm^2) was determined by using a bank of serial 10-fold dilutions ranging from 10^{-1} to 10^{-6} of the recovered bacterial suspension and incubated in TSA plates for 24 h at 37 °C. Positive and negative controls were included. All experiments were performed in triplicate on at least three occasions.

Experimental procedures

Several irrigants and treatments were tested for 60 s: (I) Saline, (II) NaOCl 0.5%, (III) NaOCl 5%, (IV) Er,Cr:YSGG, (V) Saline + LAI, (VI) NaOCl 0.5% + LAI, (VII) Saline + PUI, and (VIII) NaOCl 0.5% + PUI. Hypochlorite solutions were freshly prepared for each experiment by diluting with Milli-Q water stock solutions reaching a final pH of 10.

Experiments were carried out by incubating the biofilm with the unpowered irrigant at room temperature, during 60 s; the bel-bulb pipettor was adapted to the upper end of the pipette, and the irrigant was drawn up into the artificial model.

Before applying the laser or ultrasonic systems, the extremity of the model device was first securely sealed with sterile adhesive (Blu-Tack; Bostik, Barcelona, Spain) in order to prevent flowthrough of the irrigant across the apex, as well as to promote the flushing action and provide a closed-end system causing a vapor lock effect [18]. The irrigant reached 4 cm above the closed end, ensuring that the cylindrical irrigant reservoir was filled.

Laser-activated irrigation

LAI protocol was achieved by using Er,Cr:YSGG pulsed laser (Waterlase iPlus; BIOLASE technology, Irvine, CA, USA) at a wavelength of 2780 nm. Laser operating parameters were 1-W power, 10-Hz repetition rate, 100 mJ per pulse energy, and 140-μs pulse duration for all the groups where lasers were used. The co-axial water spray feature of the Gold handpiece (BIOLASE technology, Irvine, CA, USA) was turned off during treatment. A RFT 2 tip (Endolase, BIOLASE Technology, Inc.; 200 μm in diameter, length 21 mm, calibration factor of > 0.55) was used. This is a conical tip with an angle at the end of about 50°, designed for the endodontic treatment. The real power was 0.55 W at 10 Hz, 55 mJ per pulse. Tips were autoclaved before use. The tip was placed into the cylindrical reservoir only (Fig.1) and activated with short movement (2–3 mm) up and down. This procedure was the same when the laser was used both alone and with irrigant. No irrigation solution was added during the laser irradiation cycles (60 s).

Passive ultrasonic irrigation

This was performed by using an ultrasonic device (Newtron® P5 XS, Satelec Acteon, Merignac, France). A non-cutting ultrasonic tip (Irrisafe; Acteon, Merignac, France), stainless steel 25/.00, 25 mm in length, mounted in a handpiece unit (Newtron Slim B.LED, Satelec Acteon, Merignac, France) was inserted only into the cylindrical irrigant reservoir, avoiding contact with the walls. The tip was placed for each pipette with short moves (2–3 mm) up and down and was directed to the extremity of the model device, with a frequency of 30 kHz in the endomode (medium power) following the manufacturer's instructions. No additional irrigation was performed during PUI cycles (60 s).

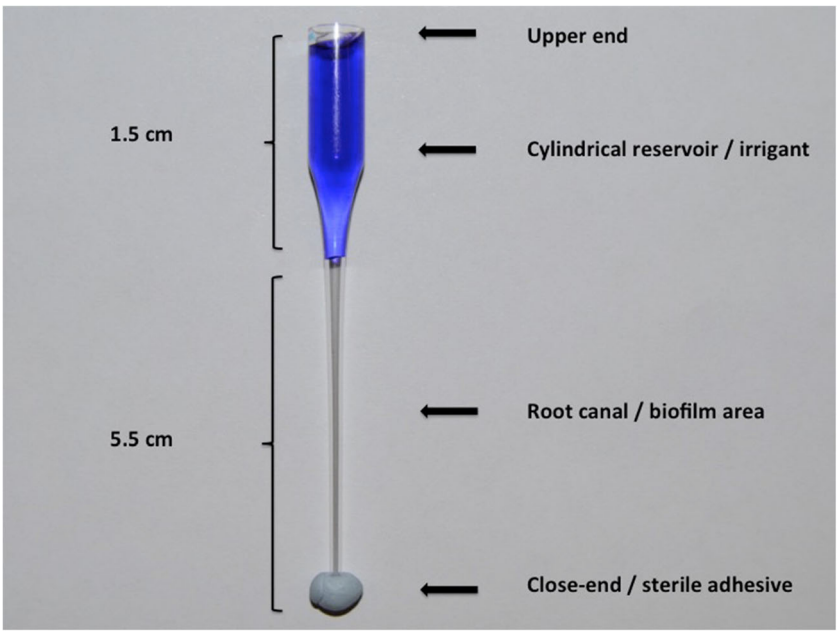

Fig. 1 Artificial root canal model infection. The irrigant is represented by violet blue staining. By suction, the inoculum or irrigant is carried into the pipette. The liquid does not drop due to the surface tension produced by the sterile adhesive action

Table 1 Bacteria recovered from *E. faecalis*-infected canal models after different treatments. *CFUs*, colony-forming units; *LAI*, laser-activated irrigation; *PUI*, passive ultrasonic irrigation; *IQR*, interquartile range. *Untreated biofilm

Group	Median CFUs/cm^2 recovered	IQR	Exposure time (s)
Control*	5.31×10^5	1.71×10^5	60
Saline	9.60×10^4	5.80×10^4	60
NaOCl 0.5%	7.70×10^3	5.17×10^3	60
NaOCl 5%	< 10	< 10	60
Er,Cr:YSGG	1.38×10^5	8.41×10^4	60
Saline + LAI	7.00×10^3	3.38×10^3	60
NaOCl 0.5% + LAI	< 10	< 10	60
Saline + PUI	4.55×10^4	2.60×10^4	60
NaOCl 0.5% + PUI	5.21×10^4	6.70×10^3	60

Atomic force microscope

AFM is a widely used tool for exploring mechanism of action and surface alterations produced by new drugs or novel treatments. Samples were imaged in air using an atomic force microscope (AFM) XE-70 (Park Systems, South Korea). Images were collected in non-contact mode using pyramidal-shaped silicon cantilevers with a spring constant of ± 40 Nm^{-1} and a resonance frequency of ± 300 kHz. Topography, amplitude, and phase images were generated at a scan rate of 0.4 Hz and scan size of 5 × 5 μm, from which mean length and width of individual cells as well as surface roughness were measured and analyzed using the XEI software (Park Systems, South Korea). An average of 100 cells in each sample was analyzed to ascertain the effect of different treatments on surface morphology of bacterial cells. Experiments were carried out in triplicate.

Statistical analysis

Statistical analysis was carried out with Stata14 (StataCorp®, College Station, USA). Data were logarithmically transformed. Bactericidal effects were expressed as a bactericidal index (BI) according to Rooney et al. [19], that is, the difference between the logarithm of the bacterial counts of the control and the treatment groups. Normality of scale variables was explored using the Shapiro-Wilk test and through visual analysis of the P-P plot and box plot. Where normality was rejected, both the interquartile range (IQR) and median were calculated. Statistical analysis to compare CFU values using the non-parametric Kruskal-Wallis and post hoc Bonferroni's tests for multiple comparisons were carried out. Level of significance was set at $p < 0.05$.

Results

Bacterial population recovered from biofilms after 24 h was $5.31 \times 10^5 \pm 1.71 \times 10^5$ CFUs/cm^2. Median and interquartile range of colony-forming units recovered after each treatment group are shown in Table 1. The bactericidal index, represented in Table 2 and Fig. 2 (as a box plot), was used as the main parameter to define effectiveness. The Shapiro-Wilk test showed that the distribution was not normal ($p < 0.05$), and the non-parametric Kruskal-Wallis test confirmed significant differences between different groups ($p < 0.05$).

It should be highlighted that the use of Er,Cr:YSGG laser without irrigation showed a weak bactericidal effect on the *E. faecalis* biofilm. Furthermore, NaOCl 5% unpowered and NaOCl 0.5% + LAI were the most effective treatments. Both

Table 2 Multiple independent variables on the bactericidal index. Statistically significant differences were set at $P < 0.05$ (shown in italics). *LAI*, laser-activated irrigation; *PUI*, passive ultrasonic irrigation

Parameter	NaOCl 5%	NaOCl 0.5% + LAI	Saline + LAI	NaOCl 0.5%	Saline + PUI	NaOCl 0.5% + PUI	Saline
NaOCl 0.5% + LAI	1.000						
Saline + LAI	*0.028*	*0.028*					
NaOCl 0.5%	*0.002*	*0.002*	1.000				
Saline + PUI	*< 0.001*	*< 0.001*	*0.001*	*0.013*			
NaOCl 0.5% + PUI	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	1.000		
Saline	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	0.902	1.000	
Er,Cr:YSGG	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	0.078	0.465	1.000

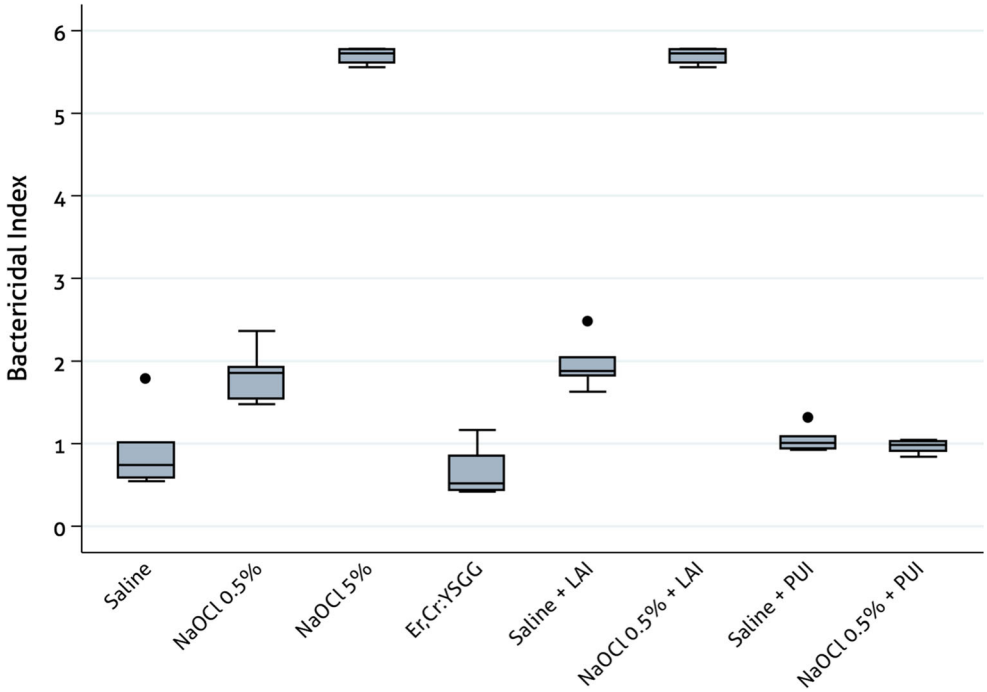

Fig. 2 Box plot of bactericidal index by the different treatments tested after 60 s

treatments were capable to eliminate all bacteria, and there was no statistically significant difference between them ($p > 0.05$). Bacterial counts were significantly lower after treatment with NaOCl 0.5% + LAI than those obtained with non-activated solution ($p < 0.05$). Saline solution and NaOCl 0.5% upon being in contact with the bacterial cells without activation failed to completely eliminate *E. faecalis*. Lower efficiencies were achieved by PUI.

AFM surface analysis revealed alterations in treated cells; topography and error signal images showed differences in cell surfaces after laser exposure compared to *E. faecalis* control cells (Fig. 3); cell turgency and wall integrity were found to be altered, as well as the surface roughness (Ra) parameter, which was increased in treated cells as can be seen in Fig. 4. Top differences were achieved with NaOCl 5% unpowered and NaOCl 0.5% + Er,Cr:YSGG (LAI) ($p < 0.0001$).

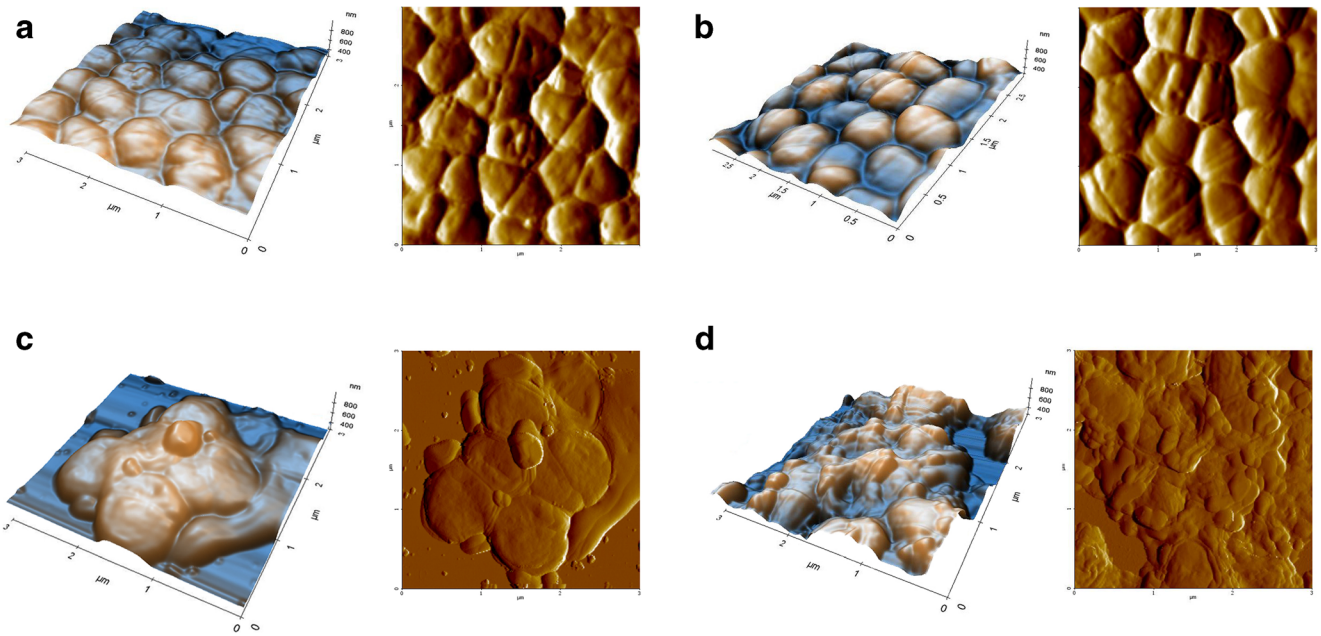

Fig. 3 3D topography and error signal images respectively of *E. faecalis* biofilm using atomic force microscope (AFM), visualized immediately after treatment. **a** Untreated biofilm, **b** Saline + LAI for 60 s, **c** NaOCl 5% unpowered, **d** NaOCl 0.5% + LAI for 60 s

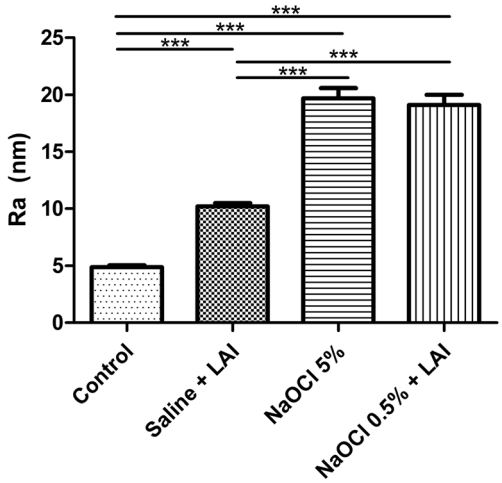

Fig. 4 Roughness average (Ra) values of *E. faecalis* cells exposed to different treatments after 60 s obtained from AFM analysis. Data were obtained from AFM images of samples in non-contact mode and processed by XEI software (Park Systems, South Korea). Means ± standard errors of the means are presented. One-way analysis of variance was used for statistical analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Discussion

E. faecalis is known to be frequently involved in endodontic treatment failures [4]. Many reports are based on experimental work done with planktonic bacteria [20], although endodontic infections are in fact caused by sessile bacteria. Prior research has been carried out with biofilms of various ages (young biofilms and also old biofilms having several days or even weeks of incubation) [14, 21–23]. The present research used 24-h-old biofilms [24]. Further colonization and biofilm formation was confirmed by bacterial count and atomic force microscopy (Fig. 3).

Several endodontic infection models have been proposed to elucidate the perspectives in the use of laser to achieve canal disinfection; this includes human teeth ex vivo [14, 15, 25], infected artificial root canals [26], dentine slices from infected bovine teeth [27], and slices of human root dentin [28]. In all cases, it seems that irrigants cannot reach the distal extremity of canals. We used an original standardized model in order to simulate the conditions within a root canal at the solution-air interface. The extremity of the Pasteur pipette sealed with sterile adhesive mimics those of the root surrounded by bone and periodontal ligament and creates an apical air lock. Furthermore, it limits the forward expansion of the vapor bubble generated by the laser and prevents the expulsion of irrigant out of the canal [29]. It was observed that direct laser irradiation in agar plates or microtubes was effective on *E. faecalis* [21, 30]. However, as previously mentioned, some regions of the root canal systems remain out of contact with the irrigant. In fact, these observations were confirmed since a slight bactericidal effect of Er,Cr:YSGG laser (without irrigant) and PUI was observed.

In the search of a more efficient endodontic treatment, the use of lasers at different wavelengths and ultrasonic systems as a complementary tool to enhance irrigant dispersal and activation has been proposed [13, 15, 29].

By using LAI, it is feasible to reduce undesired thermal effects and damage to the apical area by increasing the distance between the tip and the apex. The laser tip was placed at 5 cm from the closed end of the pipette and kept there for the entire duration of the cycle. Furthermore, the expanding shockwaves contribute to the global photomechanical effect by facilitating the access of the irrigant to the apical third of the canals [31]. Regarding the confines of the microenvironment of the root canal, DiVito et al. [12] suggested that the induced laser pumps would remove smear layer and debris and disrupt microbial biofilms, producing morphological alterations in cell membranes, as has been assessed by atomic force microscopy in this study (Fig. 3).

In our experimental work, similarly to other studies [11, 12], we became aware that the immersion of either the laser tip or ultrasonic tip in a liquid resulted in a shockwave effect; in fact, turbulences of the fluid may be seen immediately after each pulse.

The use of LAI allows overcoming the surface tension which prevents penetration, whereas PUI did not, since the irrigant did not reach the extremity. This can be attributed to the ability of LAI to create cavitation much more effectively than PUI [18]. It is known that the effectiveness of NaOCl strongly depends upon the time of contact biofilm/irrigant.

As expected, saline had no antibacterial effect. Nevertheless, when used as irrigant in LAI, some antibacterial effect was seen; this is due to bacterial death originated from the intense streaming and flushing action created within the irrigant [14], although it failed to significantly remove bacteria.

Radcliffe et al. [10] demonstrated that several *E. faecalis* strains could have a certain tolerance to NaOCl and recommended 30 min of contact with 0.5% NaOCl to achieve complete bacterial removal, while 2 min in the presence of 5.25% NaOCl was enough to achieve disinfection. It should be taken into account that cytotoxicity of NaOCl is dose-dependent. Most studies have tested LAI with high concentrations of NaOCl [23, 29, 32], but little is known about the effectiveness of laser Er,Cr:YSGG-activated irrigant in eliminating bacteria using 0.5% NaOCl concentration.

Here, we demonstrated that NaOCl at 0.5% combined with Er,Cr:YSGG laser may reach a full disinfection, allowing the use of a much less toxic concentration of hypochlorite. Moreover, injuries on bacterial structure have been assessed by AFM. As shown in Fig. 3, cell envelopes were broken and cytoplasmatic contents were leaking out of the cell, in agreement with changes in roughness (Fig. 4).

Similar results were obtained by Jaramillo et al. [15] who concluded that the activation of buffered 0.5% sodium

hypochlorite by Er:YAG laser significantly increased its antimicrobial effectiveness. On the contrary, Christo et al. [14] reported that in a biofilm model using extracted teeth, LAI had limited potential of increasing the antibacterial effect of 0.5% NaOCl.

Conclusion

Here, we propose a laboratory model to mimic single-tooth root canal, in which formation of *E. faecalis* biofilm is feasible. The Er,Cr:YSGG laser-activated irrigation (LAI) increased the bactericidal efficiency of 0.5% NaOCl, allowing it to achieve the same level of effectiveness as 5% NaOCl. Moreover, no significant increase was done when the activation of the irrigant was achieved by PUI. In conclusion, activation by laser improved the bactericidal efficacy of 0.5% NaOCl, which could be of great interest in clinics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent This article does not contain any studies with human participants or animals.

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