Irrigation with Laser-Activated sodium hypochlorite: An antimicrobial alternative in endodontics

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UNIVERSITAT DE BARCELONA

PhD Thesis

IRRIGATION WITH LASER-ACTIVATED SODIUM HYPOCHLORITE: AN ANTIMICROBIAL ALTERNATIVE IN ENDODONTICS

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Que la Tesis Doctoral presentada por **Pablo Betancourt Henríquez** titulada "*Irrigation with Laser-Activated sodium hypochlorite: An antimicrobial alternative in endodontics*" ha sido desarrollada por el autor bajo nuestra supervisión en el Laboratorio de Microbiología Molecular y Antimicrobianos del Campus de Bellvitge.

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L'Hospitalet de Llobregat, 6 de Junio de 2019

“Lo que asumes son tus ventanas en el mundo. Límpialas de vez en cuando, sino la luz no entrará”

Isaac Asimov

“La Ciencia, muchacho, está hecha de errores, pero de errores útiles de cometer, pues poco a poco, conducen a la verdad”

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ACRONYMS AND ABBREVIATIONS

°C:	Celsius degrees
λ:	Wavelength
Δt:	Total exposure time
ΔNaOCl:	The difference in NaOCl concentration before and after exposure time
μm:	Micrometer
μs:	Microsecond
AAP:	Asymptomatic apical periodontitis
Ace:	Collagen-binding protein
Al:	Aluminum
AP:	Apical periodontitis
AS:	Aggregation substance
As:	Arsenic
ATCC:	American type culture collection
BE:	Bile esculin
CA:	Citric acid
CBCT:	Cone beam computed tomography
CFD:	Computational fluid dynamics
Chx:	Clorhexidine
CLSM:	Confocal laser scanning microscopy
Cu:	Copper
Cyl:	Cytolysin
CW:	Continuous wave

DNA:	Deoxyribonucleic acid
Ebp:	Endocarditis and biofilm-associated pili
eDNA:	Extracellular DNA
EDTA:	Ethylenediaminetetraacetic acid
efaA:	Endocarditis antigen A
Eps:	Extracellular polymeric substance
Er:YAG:	Erbium-doped, yttrium, aluminium, garnet
Er,Cr:YSGG:	Erbium, chromium, yttrium, scandium, gallium garnet
Esp:	Enterococcal surface protein
et al.:	And others
f:	Frequency
GelE:	Gelatinase
h:	Hour
HLLT:	High level laser therapy
Hz:	Hertz
Hyl:	Hyaluronidase
IR:	Infrared
kHz:	Kilohertz
KTP:	Potassium, titanyl, phosphate
LAI:	Laser activated irrigation
LLLT:	Low level laser therapy
mJ:	Millijoule

MTAD:	Mixture of tetracycline isomer, acid, and detergent
Min:	Minute
mL:	Millilitre
mm:	Millimeter
NiTi:	Nickel titanium
nm:	Nanometre
PCR:	Polymerase chain reaction
PUI:	Passive ultrasonic irrigation
rRNA:	Ribosomal ribonucleic acid
RR:	Reaction rate
SAP:	Symptomatic apical periodontitis
SEM:	Scanning electron microscopy
SI:	Syringe irrigation
Sp:	Specie
Spp:	Species
TEM:	Transmission electron microscopy
UI:	Ultrasonic irrigation
UV:	Ultra violet
YAP:	Yttrium, alluminium, perovskite
W:	Watt
WL:	Working lenght

ABSTRACT

Bacteria and their sub-products are the main cause of the occurrence and perpetuation of endodontic infection. Environmental factors inside the root canal favor bacterial growth. However, only a few bacterial species are seen as responsible of persistent endodontic infections, among them *Enterococcus faecalis* is the most frequently isolated specie. *E. faecalis* is an opportunistic nosocomial pathogen, which is able to resist the adverse conditions provided inside the root canal, as alkaline pH or long periods of time with low nutrient concentrations. It has several virulence factors, such as aggregation substances, enterococcal surface protein (Esp), "endocarditis and biofilm-associated pili" (ebp) and cytolysin. Moreover, its high resistance to antibacterial agents is enhanced by its ability to form biofilm.

Due to the complex and unpredictable root canal morphology, the complete removal of smear layer and bacterial biofilm is difficult. This is the reason why adequate irrigation is crucial in endodontic therapy. Recently, laser-activated irrigation (LAI) has been introduced as an alternative to achieve a deeper cleaning and disinfection of the root canal system. Its mechanism of action is based on the generation of cavitation bubbles, through the absorption of laser energy by the irrigant. The most used lasers are from the Erbium family, Er, Cr: YSGG (2780nm) and Er: YAG (2980nm).

Sodium hypochlorite (NaOCl) is considered the "gold standard" of endodontic irrigators. It has a broad antibacterial spectrum and is capable of dissolving organic tissue. It is used in a range between 0.5% and 6%, varying its degree of effectiveness. Nevertheless, it is toxic at high concentrations, causing damage to endothelial cells and periodontal ligament cells, which generates an acute inflammatory reaction and pain.

Hence, the aim of this thesis was to explore the bactericidal effect of low concentration of NaOCl activated by Er, Cr: YSGG LAI against *E. faecalis* biofilms in root canals, in order to decipher if it may be similar to the one achieved by high concentrations of NaOCl.

***In vitro* root canal model**

A main objective of the first stage of this thesis was to build a laboratory model to simulate the conditions inside a single-tooth root canal. A modified glass Pasteur pipette was used, which also allowed to observe the cavitational effect of the laser in the irrigant. The Pasteur pipettes were inoculated with *E. faecalis* ATCC 29212 for 24 hours. Bacterial colonization and the subsequent formation of biofilm in the proposed *in vitro* model were demonstrated by atomic force microscopy (AFM).

The second essential point of this part was to determine the antimicrobial capacity of Er,Cr:YSGG laser against *E.faecalis*. In addition, passive ultrasonic irrigation (PUI) was also tested. Several irrigants were used: 0.5% NaOCl, 5% NaOCl and saline. Laser-activated irrigation (LAI) demonstrated higher antimicrobial activity than passive ultrasonic irrigation.

The final stage of this first part consisted in the analysis and measurement of the nano-roughness by AFM of the cells treated and its comparison with that of untreated cells.

Extracted Teeth

This part was focused on the endodontic preparation of extracted human teeth. The root canals were instrumented by a crown-down / step-back technique using conventional sequence of 0.02 taper files up to the master # 55. The teeth were irrigated with 2.5% NaOCl and ethylene-diamine-tetra-acetic acid (EDTA) was used to remove the smear layer. Finally, the apical foramen was sealed with a double layer of bonding agent and autoclaved at 121ºC for 17 minutes. The teeth were inoculated with *E. faecalis* ATCC 29212 for 10 days.

The following stage dealt with the study of the antibacterial action of low concentration of NaOCl activated by Er,Cr: YSGG laser in extracted human teeth. The antimicrobial effectiveness of laser-activated irrigation was compared with passive ultrasonic irrigation activation and conventional manual irrigation. Er,Cr:YSGG laser and 0.5% NaOCl showed a considerable synergistic action.

Finally, the last part of this work consisted on the microscopic visualization of the samples, to complement the results of the microbiological count. The scanning electron microscopy (SEM) was used to determine the degree of effectiveness of bacterial biofilm and smear layer removal both on the dentin surface and inside the dentinal tubules. Additionally, the CLSM was used to visualize the proportion of alive and dead bacteria after treatment.

The results obtained in this thesis showed that LAI is an alternative therapeutic option for infections caused by *E. faecalis* inside of root canal system.

RESUMEN

Las bacterias y sus subproductos son la principal causa de la infección endodóntica y su perpetuación. Ciertos factores ambientales en el canal radicular favorecen el crecimiento bacteriano. Sin embargo, sólo algunas especies bacterianas son consideradas responsables de causar infecciones endodónticas persistentes, entre ellas *Enterococcus faecalis* es la especie más frecuentemente aislada. *E. faecalis* es un patógeno nosocomial oportunista, capaz de resistir condiciones adversas dentro del canal radicular, como pH alcalino o largos períodos de tiempo a bajas concentraciones de nutrientes. Presenta diversos factores de virulencia, como sustancia de agregación, proteínas de la superficie enterocócica (Esp), endocarditis y pili asociado a biopelículas (ebp) y citolisina. No obstante, su alta resistencia a los agentes antibacterianos viene incrementada por la capacidad para formar biofilms.

Debido a la compleja e impredecible morfología de los canales radiculares, la completa eliminación del barro dentinario y biofilm es difícil de alcanzar. Esta es la razón por la cual una irrigación adecuada es crucial en la terapia endodóntica. Recientemente, el riego activado por láser (LAI) se ha propuesto como una alternativa para lograr una limpieza y desinfección más profunda del sistema de canales radiculares. Su mecanismo de acción se basa en la generación de burbujas de cavitación, a través de la absorción de energía láser por parte del irrigante. Los láseres más utilizados pertenecen a la familia Erbium, Er, Cr: YSGG (2780nm) y Er: YAG (2980nm).

El hipoclorito de sodio (NaOCl) es considerado el irrigante de elección entre los irrigantes endodónticos. Tiene un amplio espectro antibacteriano y es capaz de disolver tejido orgánico. Se utiliza en un rango entre 0.5% y 6%, variando en su grado de efectividad. Sin embargo, en altas

concentraciones puede ser altamente tóxico, causando daño a las células endoteliales y las células del ligamento periodontal, lo que genera una reacción inflamatoria aguda y dolor.

Por lo tanto, el objetivo de esta tesis fue explorar si el efecto bactericida de una baja concentración de NaOCl activado por el láser Er, Cr: YSGG, puede ser similar al alcanzado por altas concentraciones de NaOCl contra biofilms de *E. faecalis* en canales radiculares.

Modelo de canal radicular *in vitro*.

El objetivo principal de la primera etapa de esta tesis fue construir un modelo de laboratorio para simular las condiciones al interior de un canal radicular. Se utilizó una pipeta Pasteur de vidrio modificada, que también permitió observar el efecto cavitacional del láser en el irrigante. Las pipetas Pasteur fueron inoculadas con *E. faecalis* ATCC 29212 durante 24 horas. La formación de biofilm en el modelo *in vitro* propuesto fue demostrado mediante microscopía de fuerza atómica.

El segundo punto esencial de esta parte fue determinar la capacidad antimicrobiana del láser Er, Cr: YSGG contra *E. faecalis*. Además, también se probó la acción de la irrigación ultrasónica pasiva. Se utilizaron diversos irrigantes: NaOCl al 0,5%, NaOCl al 5% y solución salina. La irrigación activada por láser demostró una mayor actividad antimicrobiana que la irrigación ultrasónica pasiva.

La etapa final de esta primera parte consistió en el análisis y la medición de la nano rugosidad mediante microscopía de fuerza atómica de las células tratadas y su comparación con la de las células no tratadas.

Dientes extraídos

Esta parte se centró en la preparación endodóntica de dientes humanos extraídos. Los canales radiculares se instrumentaron mediante la técnica *crown-down/step-back* utilizando una secuencia convencional de limas conicidad 0.02 hasta la lima maestra n° 55. Los dientes se irrigaron con NaOCl al 2.5% y se utilizó ácido etilen-diamino-tetra-acético (EDTA) para eliminar el barro dentinario. Finalmente, el foramen apical se selló con una doble capa de adhesivo y se autoclavó a 121ºC durante 17 minutos. Los dientes fueron inoculados con *E. faecalis* ATCC 29212 durante 10 días.

La siguiente etapa se centró en el estudio de la acción antibacteriana de baja concentración de NaOCl activada por el láser Er, Cr: YSGG en dientes humanos extraídos. La efectividad antimicrobiana de la irrigación activada por láser se comparó con la activación ultrasónica pasiva y la irrigación manual convencional. El láser Er, Cr: YSGG y 0,5% de NaOCl mostraron una acción sinérgica considerable.

Finalmente, para complementar los resultados microbiológicos, la última parte se centró en la visualización microscópica de las muestras. El microscopio electrónico de barrido se utilizó para determinar el grado de efectividad en la remoción de biofilms bacterianos y de barro dentinario, tanto en la superficie de la dentina como en el interior de los túbulos dentinarios. Además, se utilizó el microscopio confocal laser de barrido para visualizar la proporción de bacterias vivas y muertas después del tratamiento.

Los resultados obtenidos en esta tesis muestran que la irrigación activada por láser es una alternativa terapéutica para las infecciones causadas por *E. faecalis* dentro del sistema de canales radiculares.

1. INTRODUCTION

1. INTRODUCTION

1.1. Enterococci

1.1.1 Highlights

The members of the genus *Enterococcus* are Gram-positive, facultative anaerobic cocci, catalase-negative, resilient by nature and able to survive a wide array of adverse conditions and can persist in the environment for long periods of time.[1] Enterococcal cells are spherical or ovoid, occurring in singly, in pairs, or as short chains. Endospores are not formed and some species can be motile by scanty flagella. Enterococci are present in the human intestinal lumen, human female genital tracts and the oral cavity in lesser numbers and under most circumstances cause no harm to their hosts. Also, it can also be found in extraenteric habitats, such as soil, beach sand and ambient waters although regarded as contaminants of enteric origin. Enterococci can survive in very harsh environments including temperatures ranging from 10ºC to 60ºC and a pH over 9.6.[2] Also, they resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation. The energy is obtained by the fermentation of a wide variety of substrates including carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and many keto acids.[3] Despite their fermentative metabolism we have defined Enterococci as facultative anaerobic, this is due to the fact that in very particular conditions they may perform respiration. This is the case of several enterococcal species which may express an electron transport chain that enables them to respire. These aerobic respiration has been characterized in *E. faecalis* and strictly depends upon the presence of heme grup in the medium. In the absence of heme, respiration is blocked.

1.1.2 Epidemiology

Enterococci (formerly *S. faecalis* and *S. faecium*) were seen as commensals since its discovery 115 years ago. Nevertheless, in the last years the classification of this genus has been deeply modified and enterococci included in a new genus *Enterococcus*, separated from *Streptococcus*. The two most relevant species *S. faecalis* and *S. faecium* were moved to the new genus.[4] At the last decade, Enterococci have been considered as one of the most common nosocomial pathogens, with a mortality rate close to 61% in medically compromised patients.[5] In 2005, the Health Protection Agency reported 7066 cases of bacteremia caused by *Enterococcus* species in the UK, which meant an increase of 8% compared to 2004; 28% of the cases showed antibiotic resistance.[6] In 2017, the Spanish Society of Preventive Medicine, Public Health and Hygiene (SEMPSPH) reported through the study of Prevalence of Nosocomial Infections in Spain (EPINE) that nosocomial infections caused by Gram-positives was 34.62%. Enterococci was the cause of 11.39% of the total infections, only surpassed by *Escherichia coli* with 15.78%.[7] There are several reasons why *Enterococcus* genus is a relevant nosocomial pathological agent such as its great ability to colonize since it has a great metabolic versatility, but also an unusual resistance to inhospitable conditions. Despite they are unable to form spores they may resist long periods of dryness persisting long in dried environments. Moreover, *Enterococcus* are tolerant to extreme pH values, oxidative stress, and high osmotic pressures; finally, they exhibit intrinsic multiresistance, including total resistance to cephalosporins as well as to heavy metals. It should be also emphasized that these bacteria have a great ability to easy acquisition of new resistance genes.

It has been reported in Spanish hospitals that resistance to aminoglycosides already varies between 25% and 30%.[8] Due to the dramatic increase in

antibiotic resistance of the genus *Enterococcus* in the entire world, it is mandatory to understand its etiology, pathology and virulence mechanisms.

1.1.3 Pathogenesis

Enterococcus are ubiquitous and potentially pathogenic. They are able to acquire an increased resistance or phenotypic tolerance to many disinfectants or physical agents.[9] They can cause a wide variety of infections in humans, including urinary tract infections, bacteremia, endocarditis, meningitis, oral and wound infections.[10] Also, many cases of biofilm-associated infections of artificial medical devices have been attributed to enterococci.[11] Furthermore, the emergence of multi-drug resistant isolates has complicated the treatment of these infections. Since the 1980s the antibiotic resistance of *Enterococcus* has been increasing and the spectrum enlarged; such is the case of the emergence of vancomycin-resistant enterococci.[12] Diverse are the virulence factors of this genus, among which include hemolysins, aggregation substances, bacteriocins, proteases, agglutinins and Hyaluronidase(*hyl*).[13,14] In addition, cell wall carbohydrates or fibronectin binding sites, which favor adherence to host tissues, may enhance pathogenicity.[15]

The two species *E. faecalis* and *E. faecium*, with the former being predominant, have gained significance in recent decades as leading opportunistic pathogens causing nosocomial infections. Among Enterococcal infections, both species account approximately 90% of cases. Other species such as *E. gallinarum, E. raffinossus, E. casseliflavus* and *E. avium* are isolated to a lesser extent.[1] Also, the Enterococci are of extreme relevancy in endodontic infections. Despite they make up a small proportion of the initial microbiota, which is formed mostly by Gram-negative species, it has been established that *E. faecalis* is the most commonly involved microorganism in asymptomatic persistent endodontic infections.[16]

1.2 *Enterococcus faecalis*

1.2.1 Epidemiology

E. faecalis is an opportunistic pathogen, causing nosocomial infections and responsible for most enterococcal infections in humans,[10] in 2017, ranked fifth among microorganisms most frequently isolated (6.87%) in cases of nosocomial infections (of a total of 61.673 patients in Spain).[7] *E. faecalis* is frequently found in the human intestine and female genital tract , but it may temporarily be found in the oral cavity. The study of *E.faecalis* in dentistry increased in recent decades, since the bacterium was recognized as the most commonly species encountered in the root canals with endodontic failure.[17] Using conventional sampling and culture techniques, *E. faecalis* was recovered from infected root canal samples in 30% of cases in Sweden [18] and United States,[19] and more than 50% of cases in Brazil[20] and Lithuania.[21]

Although *E. faecalis* is present at a low percentage in primary infections, the probability of being found in failed cases with apical periodontitis increases nine times.[22] It was reported that the prevalence of *E. faecalis* in secondary endodontic infections was 33%[23] and from 24 to 77% in persistent infections.[22,24] As a consequence, inflammatory reactions, tissue destruction and the development of abscesses of lymphadenitis and cellulitis are established. [17,25]

1.2.2 Pathogenesis

E. faecalis is the main causative agent of endodontic failure and is involved in the appearance of systemic diseases such as surgical wound infections, urinary tract infection, and may progress to bacterial endocarditis and bacteremia.[20,26] Also, it has been associated with pathogenic oral manifestations such as mucosal lesions

in immunocompromised patients,[27] periodontitis disease[28] and root canal infections.[19]

The bacterium expresses several virulence factors, such as lytic enzymes, cytolysin (*Cyl*), aggregation substance (*AS*), endocarditis antigen A (e*faA*), gelatinase (*GelE*), enterococcal surface protein (*Esp*), collagen-binding protein (Ace), endocarditis and biofilm-associated pili (ebp), bile salt hydrolase and capsule production.[22,29] These factors provide improved capabilities in the adhesion and colonization of the root canal surface, allowing it to compete with other bacteria and alter host responses.

E. faecalis exhibits generalized genetic polymorphisms. It has several proteins that facilitate its binding to dentin, such as serine protease, gelatinase (*gelE*) and collagen-binding protein (*Ace*). Gelatinase (*gelE*) is an extracellular metalloprotease, able to hydrolyze gelatin, collagen and hemoglobin, which has also been reported to contribute to bacterial adherence and biofilm formation.[30] Furthermore, it has been seen that Enterococcal surface protein (*Esp*) has been found to further adherence and colonization of cells and abiotic surfaces.[11] Aggregation substance (AS) has also been reported to increase adherence and invasion of eukaryotic cells [31] as well as to promote biofilm formation.[32] It seems that e*faA* contributes to the adhesion of *E. faecalis* to heart cells in endocarditis.[33] On the other hand, Cytolysin (*cyl*) that is a ß-hemolysin may act as a potent bacteriocin that may kill not only erythrocytes but also other prokaryotes, providing nutrients that may favor enterococci growth thus exacerbating enterococcal infections in humans.[34]

E. faecalis has the capacity to withstand long periods of starvation until the appearance of an adequate nutritional supply, which makes possible its recovery. It has been seen that serum of the periodontal ligament and alveolar bone, in addition to being a nutritional source, also helps it to bind with

collagen type I. In addition, some strains of enterococci have the ability to perform a horizontal gene transfer of many resistance genes, contributing to the pathogenicity and ability to cause disease.[35]

Finally, it has the ability to form communities organized in biofilms, which can be relevant for bacterial resistance and persistence after intracanal procedures (Figure 1). The slow metabolic activity rate of microorganisms in biofilms as well as the extracellular matrix of the biofilm can impede the effectiveness of many antimicrobial agents.[36]

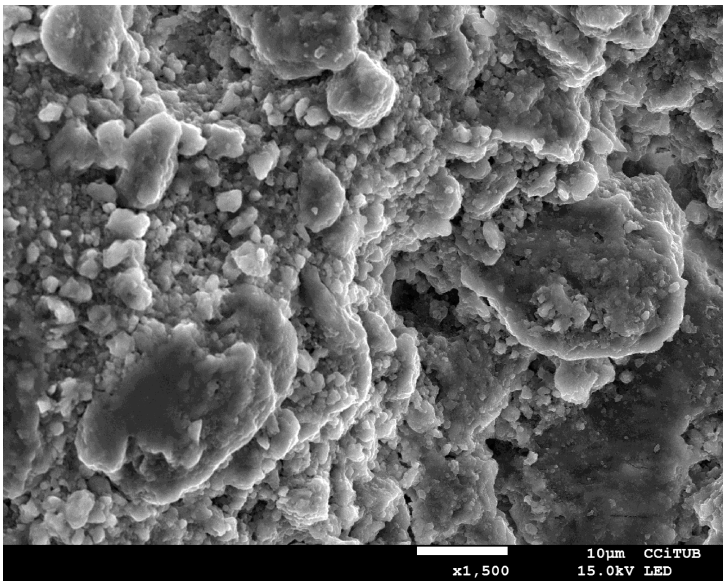

Figure 1. SEM micrograph of 10-days-old *E. faecalis* biofilm on dentin surface after 10 days of inoculation. Magnification X1.500; SEM, scanning electron microscopy.

1.2.3 Identification

Tryptic soy agar, trypticase soy-5% sheep blood agar, brain hearth infusion-5% sheep blood agar, or any agar base containing 5% animal blood may support the growth of enterococci.

E. faecalis grows at 35ºC to 37ºC and do not require high levels of CO_2, although some strains grow better at 5% CO_2. Most strains produce a cell wall-associated glycerol teichoic acid that is identified as Lance-field`s serologic group D antigen. Pfizer selective enterococcus, Bile Esculin (BE) azide, and some other commercially prepared media containing azide are excellent for primary isolation.[37] Based on acid formation from mannitol and sorbose and on hydrolysis of arginine, the enterococcal species can be identified by phenotypic tests. *E. faecalis* produces acid from mannitol and hydrolyze arginine but fail to form acid from sorbose. It also tests positive for sorbitol, 0.04% tellurite and sucrose.[37] Molecular methods such as DNA-DNA hybridization and sequencing of the 16S rRNA genes have also been used for identification.[38]

1.3 Endodontic infection

1.3.1 Oral microbiota

The oral cavity has been considered to possess the second most complex microbiota in human body, only behind the colon.[39] The oral microbiome is highly diverse, including bacteria, fungi, viruses, archaea and protozoa. The oral ecosystem is very intricate because it has several significantly different niches, including saliva, soft tissue surfaces of the oral mucosa and tongue, and hard tissue surfaces of teeth.[40] Although the buccal and palatal mucosa are areas with low microbial diversity, the tongue is highly papillated and therefore harbors more diverse microbiota, including anaerobes.[41] In contrast, the teeth enable large masses of microbes to accumulate as biofilms known as plaque.

Obligate anaerobes, such as genus *Porphyromonas*, *Fusobacterium*, *Prevotella* and *Treponema*, primarily reside in gingival crevices or periodontal pockets where the environment is anaerobic.[42]

It is estimated that approximately 700 species are present in the oral cavity, and most of them are indigenous. Among them, 54% have been cultivated and named, 14% are cultivated but unnamed, and 32% are known only as uncultivated phylotypes.[43] Of the bacterial species that have been cultivated, approximately ten have been recognized to have pathogenic potential, most of which are Gram-negative anaerobic bacteria, located mainly in subgingival pockets such as *P. gingivalis*, *T. denticola*, *F. nucleatum* and *Prevotella* sp. [42] Accumulation of these microbial populations within the dysbiotic community induces inflammation and destruction of oral tissue.

Different facultative anaerobic and aerotorelant anaerobic bacteria, such as *S. mutans*, *Lactobacillus* spp, *Actinomyces* spp, in combination with the acids from enzymatic catabolism, have been reported as the major causes of dental oral diseases such as decay, necrosis and periapical lesions.[44, 45]

Early data recognized the association of streptococci, Gram- positive facultative anaerobe bacterium, such as *S. mutans* and *S. sobrinus*, with the initial phase of human dental caries because their acidogenic and aciduric properties permitted them to create a low-pH environment in dental plaque after the ingestion of sugars.[46] In addition, Lactobacilli and certain acid tolerant non-mutans streptococci can be considered virulent with respect to dental caries.[47]

1.3.2 Endodontic pathogens

From an ecological perspective, the root canal can be considered a highly controlled environment and divided into three (coronal - medium - apical) more or less well-defined segments (niches). The main limiting factors that influence bacterial colonization inside the root canal are the availability of oxygen and nutrients.[48] The environmental conditions in the root canals are different from

those of the oral cavity in terms of availability and type of nutrients, oxygen pressure and pH, often creating a harsh environment for bacterial colonization.[49] In the apical part of the root canal system, oxygen is significantly reduced, while the nutritional supplement comes mainly from the periapical tissues.

The bacterial species most frequently recovered from a root canal associated with symptoms will assume the role of main endodontic pathogen. In an infected root canals system, up to more than twelve microbial species may be found, including both Gram-positive and Gram-negative. Since in an infected root canal the nutrients are mainly peptides and amino acids, the growth of anaerobic proteolytic species is favored.[50] The microbial species isolated most frequently in primary root canal infections are Gram-negative and belong to the genera *Prevotella*, *Phorphyromonas* and *Fusobacterium.*[51, 52] In contrast, the microbiota present in persistent infections is characterized by the predominant presence of Gram-positive microorganisms, facultative and obligate anaerobes,[53] such as *E. faecalis, Propionibacterium propionicum* and *Streptococcus* spp, as well as members of the heterogeneous family Enterobacteriaceae, wich are facultative non-spore-forming, rod-like, Gram-negative bacteria. Other species are isolated more rarely from the root canals include opportunistic pathogens, such a *Pseudomonas aeruginosa* and even yeasts (*Candida* spp).

Bacteria from the oral cavity, may contaminate the root canal during treatment owing to inadequate aseptic control, [54] or invade the root-filling via coronal leakage after root-canal treatment.[55] It has not been well defined whether the bacteria present in a canal of an endodontically treated tooth remain after the first treatment (persistent infection) or are rather a consequence of bacterial reinfection (secondary infection). In the last decades, there has been a marked interest in studying the role of secondary infection, product of coronal filtration

in teeth treated endodontically.[56] However, the incidence of post-treatment disease is significantly higher in cases that showed lesions of preoperative apical periodontitis, indicating that persistent infection is the main cause of endodontic failure.[57] The microorganisms that produce it, certainly have the ability to survive under hardly adverse conditions, such as lack of nutrients, oxygen limitation, as well as high pH values.

1.3.3 Pathways of endodontic infection

It has been shown that the presence of bacteria is a determining factor in the initiation and perpetuation of infection in the root canal system.[58] There are several ways trough which microorganisms can reach the pulp. The most common route of contamination is dental caries, inducing successive inflammatory responses in the pulp tissue. Other secondary routes are: exposed dentinal tubules; direct pulpal exposure; restorative procedures; lateral canals of teeth with periodontal involvement; and entry into the systemic circulation, known as anachoresis.[59, 60]

1.3.4 Apical periodontitits

Apical periodontitits (AP) is a prevalent infectious disease worldwide and it increases with age. [61] It is mainly a consequence of root canal infection, characterized by inflammation and bone destruction of periradicular tissues.[62] Although its etiology is bacterial, fungi, archaea and viruses have been found in association with AP.

Apical periodontitits it is the most prevalent inflammatory lesions of the alveolar bone related to teeth. Several epidemiological studies have associated the appearance of AP with failed endodontic treatments.[63, 64] A range between

50-75% of pericapical lesions have shown a total resolution of the post-endodontic therapy. [63, 65]

Depending on the bacterial load in the root canal and the colonization period, there may be symptomatic apical periodontitis (SAP) or asymptomatic apical periodontitis (AAP). SAP is characterized by multiple adverse effects, including pain, loss of bone support, and even loss of the tooth. Severe pain to percussion and/or palpation is highly indicative of degenerating pulp. Radiographic images, may be normal or periapical radiolucency may be seen depending upon the stage of the disease. AAP does not present clinical symptoms (no pain on percussion or palpation) and appears as an apical radiolucency to the radiographic examination. In AAP the destruction of the bone is due to both the bacterial infection and the immune defense mechanism of the host. [59]

Histologically, AP is classified as an abscess, granuloma or radicular cyst.[66] Periapical abscess reflects a formation of pus as a consequence of a shift in cellular dynamics in response to an acute infection, whereas periapical granuloma consists on granulation tissue with inflammatory cells, fibroblasts and well-developed fibrous capsule. The granuloma can eventually become a radicular cyst when the epithelial rests of Malassez, located in the periodontal tissue, proliferates due to a stimulation of the immune response.[62]

1.4 Microbial Biofilms

1.4.1 General aspects

According to the National Institutes of Health, biofilms are involved in more than 60% of the microbial infections in the body.[67] In 1975, Marshall defined

the biofilm as "very fine extracellular polymer fibrils".[68] In 2002, Donlan & Costerton redefined the concept to "a growth mode of bacteria, bonded irreversibly to a substrate or to an interface or to each other, immersed in a self-produced extracellular polymeric substance (EPS)". [36]

It has been pointed out that bacteria living in biofilms are phenotypically different from planktonic ones, at least in growth rates and gene transcription**.** Indeed, the bacterial removal from a biofilm is approximately 1000 times more difficult than in planktonic state.[69] A biofilm can contain approximately 15% of cells and 85% of extracellular matrix. This matrix contains host factors, extracellular DNA (eDNA) and exopolysaccharides, which form channels through which water, enzymes, nutrients, and waste circulate.[70] There, the cells establish relationships and dependencies: they live, cooperate and communicate through chemical signals (quorum sensing), which regulate the expression of genes located in different parts of the community, as for example a tissue in a multicellular organism.

Biofilms are relevant for several environmental processes, and also the elemental cause of persistent infections in numerous parts of the human body, suchlike the teeth, urinary tract, cystic fibrosis lungs, bones, [71] or medical devices, for instance heart valves and urinary, venous and arterial catheters.[11] Biofilms show great resistance not only to the action of antibiotics, but also to host defense mechanisms. It has been reported in chronic infections, such as *P. aeruginosa* bronchopulmonary infection in patients with cystic fibrosis, that bacteria persist despite an intact immune defense of the host and frequent antibiotic treatment. Probably, the coating of the bacterial cells of the biofilm by the EPS could make them less susceptible to phagocytosis. [58] Further, it has been demonstrated the relationship between the bacterial resistance versus the action of the humoral immune system. [72]

Several factors have been suggested to explain the extraordinary resistance of bacterial biofilms to the action of antibiotics: [73] i) the reduction of metabolism and the growth rates shown by biofilm bacteria, particularly those found inside the biofilm, could make them inherently less susceptible to antibiotics; ii) the EPS biofilm matrix can act as an adsorbent or reagent, thus reducing the amount of agent available to interact with the biofilm. Additionally, EPS offers protection against diverse environmental stresses, suchlike alkaline pH, dryness, high concentrations of salts or lack of nutrients for long periods [69] iii) and biofilm cells are physiologically distinct from planktonic bacteria and express specific protection factors, alike as efflux multidrug pumps and regulons stress response. [36]

The formation of biofilm starts when bacteria attach to a surface or to each other and form aggregates. Five main stages are involved in biofilm development: i) transport of individual microbes to the surface or each other, ii) initial attachment of the microbes to the surface or each other, iii) formation of microcolonies, iv) maduration of the biofilm, v) dispersal of the biofilm. It is relevant to note that dispersal enables biofilms to spread to other locations where new biofilms can be formed. [58] In the Fig. 2 it can be observed in detail the entire development of the biofilm life cicle.

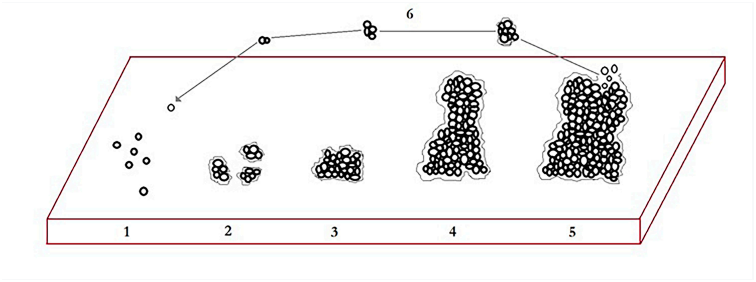

Figure 2. Five stages of biofilm formation. (1) Planktonic bacteria, (2) Initial attachment, (3) Irreversible attachment, (4) Maturation, (5) Dispersion, (6) Cycle repeats. Image adapted from Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov. 2003;2:114-22. [73]

1.4.2 Clinical relevance of *E. faecalis* biofilm

The clinical relevance of infections caused by *E. faecalis* can be attributed mostly to inherent antimicrobial resistance, ability to adapt to harsh environmental changes, and its growth in root canal walls as biofilm. Microbial biofilms account for over 80% of microbial infections in the body [58] and are considered as a primary cause of apical periodontitis in teeth with infected root canal spaces. [74] Apical periodontitis is a prevalent dental pathology that involves an inflammatory reaction and destruction of tissues around the apex of a tooth-root. [75]

The biofilm formation inside the root canals begins after the first invasion of the pulp chamber by oral planktonic organisms and after some degradation of the organic tissue. [76] At this point, the inflammatory lesion frontage that moves successively toward the apex will provide the fluid vehicle for the invading planktonic organisms; thus, these can multiply and continue attaching to the root canal walls. It has been seen that bacteria detached from the walls of the root canal can form a mass *per se* in the inflammatory lesion.[77] Therefore, the inflammatory lesion can act as a fluid source for bacteria to reach and colonize inaccessible sites in the root canal system. Out of the reach of the antimicrobial action, bacterial adhesive substances will help the formation of the biofilm, while proteins derived from the host will allow their nutrition and survival. [78]

The formation of bacterial biofilms inside the root canals has been confirmed by several studies in extracted teeth with periapical lesions. Through transmission electron microscopy (TEM) it has been feasible to visualize dense aggregates of cocci and roads embedded in an extracellular matrix along the dentinal wall of the root canal.[79] When sections of the teeth were examined by scanning electron microscopy (SEM), microcolonies of cocci, rods and

filaments forming bacterial biofilms along the canal were also observed. [80] The introduction of the concept of biofilm to endodontic microbiology has been crucial for the understanding of root canal infections, especially those of persistent type, since the microorganisms growing in biofilms are protected and better prepared against adverse changes and antimicrobial agents effect than planktonics. [58] Additional protection is provided by physiological changes of the bacteria once attached to the surface.[81] Bacterial phenotypic changes result in biofilms becoming up to 1000 times more resistant to antimicrobial therapy than planktonic cells.[69] In fact, oral biofilms are more resistant to chlorhexidine, amine fluoride, amoxicillin, metronidazole and doxycycline than the same bacteria in planktonic state. [82, 83]

Throughout the different stages of the development of the biofilm, bacteria are in different physiological states, being able to find in the base of biofilm dead or lysed cells, while in the superficial layers they can be in a state of active growth. However, it is argued that most remain in the stationary growth phase. [84] The fact that the bacterial cells maintain low metabolic activity inside the biofilm (stationary phase) contributes to perpetuate the chronic apical periodontitis for long periods of time, being one of the leading causes of persistent infection. [78]

1.5 Endodontic therapy

1.5.1 Microbiological goals

Endodontic treatment has different objectives according to the clinical diagnosis. In irreversibly inflamed pulps, the treatment has a prophylactic objective because generally the vital pulp does not present infection. However, in cases of necrotic pulp or AP, where the infection is already established, the therapeutic actions are aimed at eliminating the intracanal infection. [53]

For any bacterial species, a minimum bacterial population concentration or load

is needed to cause disease. Subsequent tissue damage, derived from the bacterial action itself in cooperation with the host defense mechanisms in response to infection. [85] The ideal purpose of endodontic therapy is to disinfect the root canal up to sterility, that would be, to eliminate all microorganisms present in the entire root canal system. However, this is extremely difficult due (at least in great part) to the anatomical complexity of tooth. This difficulty is particularly clear when using conventional therapeutic techniques. Therefore, the achievable goal is to reduce the number of bacteria as much as possible and in any case up to a level lower than the one needed to produce the disease. [53]

It is important to point out that the bacteria, being enclosed within the root canal system, are unavailable for host defenses and for the action of systemic antibiotics, so they must be controlled mainly by endodontic therapy. The use of mechanical instruments and intracanal medication is of great relevance to achieve successful disinfection, especially in the main canal, where the largest amount of bacteria is located. However, the cleaning and disinfection of areas of difficult access depend on the choice and action of the irrigant solution.

Despite various endodontic irrigators have been proposed over the years, up to now, sodium hypochlorite (NaOCl) is the most commonly used. [86] However, several studies have shown that its use accompanied by biomechanical therapy is not sufficient to leave root canals free of cultivable bacteria. In fact, between 40% and 60% of root canal that after being treated by NaOCl, tested positive for the presence of bacteria. [87, 88] An alternative proposed to NaOCl was the use of chlorhexidine, however, it has been shown that its antibacterial action is lower than NaOCl. [89]

Since the residual bacteria can directly affect the outcome of endodontic therapy, the use of intracanal medication between sessions is an action frequently used by clinicians. The objective is to produce a pH change inside

the root canal system, thus promoting an unfavorable environment for bacterial growth and development. Calcium hydroxide is the most used intracanal medication, and its use is supported by several studies that have concluded that its use as a coadjuvant of chemo-mechanical therapy makes the success of endodontic therapy more predictable. [87, 88]

The final phase of endodontic therapy consists in filling the root canal with the use of a thermoplastic material, such as gutta-percha, accompanied by sealant. This seeks to fill the empty space and "entombment" the possible residual microorganisms on the dentin. [90] The consequence of entrapping bacteria is that they remain in the dentinal walls of the main canal or inside the dentinal tubules where nutrients are not available. On the contrary, residual bacteria located in most apical areas of the root, such as apical deltas or lateral canals, receive nutrients from periradicular tissues, which allow them to perpetuate periradicular inflammation and delay healing. It has been shown that the filling itself has a limited effect on the bactericidal action. [91] Therefore, all efforts to eliminate bacterial cells should be applied in the phases prior to root filling.

1.5.2 Endodontic irrigation solutions

Endodontic irrigating solutions are used in order to remove remnants of soft tissue, kill bacteria and dissolve the smear layer. Their effectiveness depends on the temperature, concentration, exposure time and pH. [92] They are also used as lubricant for dentinal walls. The irrigants that are currently used during cleaning and shaping can be divided into antibacterial and decalcifying agents or their combinations. They include NaOCl, chlorhexidine (CHX), ethylene-diamine-tetracetic acid (EDTA) and a mixture of tetracycline and acid and a detergent (MTAD). [93] The NaOCl was the irrigant selected to carry out the experiments of the present thesis, therefore it will be deepened in its composition and mechanism of action.

Sodium Hypochlorite

Sodium Hypochlorite, the main irrigator in endodontic therapy since it was recorded in 1919 is an effective antimicrobial and up to now the only irrigator with capacity to dissolve organic tissues.[86] In addition, it can also act as antimicrobial agent against viruses and fungi. [94]

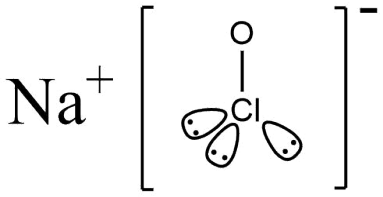

Figure 3. Sodium hypochlorite molecule.

The antibacterial and tissue digestion action of NaOCl is based on the alteration of cellular metabolism as well as the fatty acids and lipids chemical degradation of the cell membrane. Chemical reactions such as saponification, amino acid neutralization and chlorination have been described as part of the mechanism of action. [95] In alkaline solutions, when pure NaOCl is dissolved in water, the following reaction takes place: [92]

$$NaOCl \rightarrow Na^{+} + OCl^{-}$$

$$OCl^{-} + H_2O \leftrightarrow HOCl + OH^{-}$$

The free available chlorine consists of the hypochlorite ion (OCl^-) and the hypochlorous acid (HOCl). The pH variation breaks the chemical equilibrium and determines if the predominant action will be bactericidal effect or tissue-dissolving capacity. If the pH is alkaline (pH> 7), OCl^- prevails, which has a

powerful oxidative effect, and therefore the action will be predominantly tissue-dissolving capacity. Dissolution of organic tissue can be verified in the saponification reaction when NaOCl degrades fatty acids and lipids resulting in soap and glycerol. [95] In acidic solutions (3 <pH <7), HOCl prevails, which has a powerful bactericidal effect since it is a smaller uncharged molecule, which allows penetrate more easily the bacterial membrane, causing a protein degradation.[96] In addition, hydroxyl ions act on bacterial essential enzymatic sites promoting irreversible inactivation.[95] When HOCl comes into contact with organic tissue, it releases chlorine which, combined with the amino protein group, forms chloramines (a chloramination reaction), which interferes with cellular metabolism. Further, HOCl and hypochlorite ions OCl^- lead to amino acid degradation and hydrolysis. [95]

Chlorhexidine (CHX)

Chlorexidine is widely used in endodontic disinfection since it has excellent antimicrobial activity and low toxicity.[97] CHX is bacteriostatic at low concentrations and bactericidal at high concentrations.[98] The concentration frequently used in endodontic therapy is 2%, since it has been seen that reaches its maximum bactericidal effect at the end of the mechanical preparation of the root canal. One of the most important properties of CHX is substantivity, that is the prolonged antibacterial effect. White *et al.*[99] reported that the effect of 2% CHX persisted up to 72 h. CHX is active against Gram-positive and Gram-negative bacteria, bacterial spores, lipophilic viruses, yeast, fungi, and dermatophytes. [100] One of its main limitations is the inability to dissolve organic substances and necrotic tissues present in the root canal system.

Ethylene-diamine-tetraacetic Acid (EDTA)

The use of chelating agents, such as EDTA, citric acid (CA) and tetracycline, as

auxiliary solutions during root canal treatment is recommended. EDTA is the most commonly used chelating agent in endodontics and is used as a 17% neutralized solution. [93] The solution reacts with the calcium ions in the dentin and forms soluble calcium chelates, which helps the subsequent removal of the smear layer inside the root canal. Decalcification is a self-limiting process that eventually stops due to the loss of action of the chelator. However, longer exposures can cause excessive removal of both peritubular and intratubular dentin. Irrigation with 17% EDTA for 60 seconds followed by a final rinse with NaOCl is the most commonly recommended method to remove smear layer. [101] EDTA has a non-significant antimicrobial effect. [102]

Mixture of Tetracycline Isomer, Acid, and Detergent (MTAD)

It is a combination of 3% doxycycline, 4.25% CA, and detergent (Tween-80). It was introduced as a chelator to improve the smear layer removal, and as an alternative to EDTA. The main difference is that MTAD, besides presenting a chelating function, also has antibacterial properties.[103]Since it lacks solvent properties, its use after NaOCl at the end of chemomechanical preparation is recommended. [104]

1.5.3 Conventional syringe irrigation

Syringe irrigation (SI) is the most commonly used method to deliver the irrigant into the root canal system, either alone or alternating with other systems. Generally, the irrigant is administered by a needle/cannula connected to a syringe, positioning the tip preferably closest to the working length (WL). [105] The needles are designed to dispense irrigant through their most distal ends (closed-ended) whereas others are designed to deliver an irrigant laterally (side-vented). The side vented design was proposed to improve the hydrodynamic activation and reduce the chance of apical extrusion. The flow through the

needle is generated by applying a pressure on the attached syringe.[106] Advantages of irrigation with syringe is the control of volume that is flushed through the canal, the control of the depth of needle penetration within the canal and manual pressure exerted by the operator. [107]

1.5.4 Limitations of conventional endodontic therapy

Root canal morphology

The goal of a root canal treatment is to prevent or cure apical periodontitis. Therefore, microorganisms that have colonized the root canal system must be removed to promote healing. Unfortunately, due to the existence of accessory canals, anastomoses, fins, oval extensions, as well as apical ramifications, it is generated a complex three- dimensional network, making the complete removal of debris and bacteria extremely difficult when using conventional methods.[108] In addition, the surface of the root canal formed of dentine is porous and forms tubules with an average diameter of 0.6 to 3.2 μm and a length of 1 to 2 mm, which provides a refuge for microorganisms. [108]

Conventional instrumentation technique

Various undesirable apical preparation outcomes such as apical transport, weakening the root structure and promoting apical cracks have been described.[109] Peters *et al.*[110] showed through Cone Beam Computed Tomography (CBCT) that rotatory mechanized instrumentation left ≥ 35% of untreated root canal surface area, impeding complete biofilm disruption.

The smear layer, by definition, is the mixture of dentin residues, pulp tissue remnants, odontoblastic processes and microorganisms (if present). It is strongly attached to the root canal wall and it has been seen that it can penetrate up 40 μm into the dentinal canals.[111] Dentin debris are dentin chips, tissue

remnants, and particles attached to the root canal wall or present in the root canal. Both produce two major drawbacks, i) protect the biofilm from the action of antimicrobials and ii) inactivate the antimicrobial action of drugs and endodontic irrigants.[112] Usually, after the first endodontic file is used, the dentine wall is covered with a smear layer. The risk is that the residual microorganisms will strongly bind to the smear layer and being protected from an eventual antimicrobial therapy. Due to this situation, which normally occurs in daily practice, irrigation plays a crucial role in the disinfection of the root canals system.

Syringe irrigation

Several authors refer the use of the syringe as a simple procedure and provide guidelines to make irrigation more effectively by decreasing the likelyhood of extrusion. However, there is still uncertainty about the action of irrigant in narrow canals and in areas of difficult access, such as apical area, isthmuses, lateral canals and dentinal tubules. Initial efforts in demonstrating the flow of the irrigant were based on macroscopic observations, through the use of dyes,[113] or through the radiographic observation of radiopaque solutions.[114] The development of new technologies enabled studies through real-time images of bioluminescent bacteria,[115] steremicroscope and digital imaging,[107] and lately through Computational Fluid Dynamics study (CFD). [105] This may decipher the effect of irrigant flow rate on the flow pattern within a prepared root canal, during final irrigation with a syringe and needle using a CFD model. They concluded that irrigant replacement was limited to 1-1.5 mm apical to the needle tip, so to ensure the exchange of fluids, the tip of the needle should be positioned at 1mm of the working length (WL). However, due to the anatomical complexity, especially of teeth with pronounced curves, this is not always feasible. On the other hand, obviously the flow produced by the syringe may not reach deep zones. Therefore, the flow is conditioned by both chemical

and mechanical factors.

The side-venting needles have poor apical penetration, but concentrates the flow against the walls of the canal, producing high local velocity gradients, increasing shear stresses and greater biofilm disruption. However, this is restricted to the wide and straight areas of the canal, where the irrigation needle has easy access. [116] Various needle sizes are available, but most commonly used are the 27 and 30 gauge needles (respectively 0.4 and 0.3 mm outer diameter). Needles are made from stainless steel, NiTi, or flexible material like polyimide.

Some studies have suggested that the increase in the taper of the apical preparation improves the distribution of the irrigating solution to the root apex. [117, 118] Unfortunately, this can weaken the dental structure, compromising the subsequent rehabilitation.

Toxicity of NaOCl

Despite the antibacterial effect of NaOCl, chemical properties that induce bacterial death degrade or interfere with the integrity of host cells. Hypochlorite effects on proteins, produces nitrogen, formaldehyde and acetaldehyde in a short time. As a consequence peptide links are broken resulting in dissolution of the proteins. During the process, hydrogen of the amino groups (-HN-) is replaced by chlorine (-NCl-) thereby forming chloramine, which is highly cytotoxic. [119]

Hypochlorite has a nonspecific action and it has been seen that the damaging is concentration-dependent.[120] When concentration decreases, bactericidal capacity decreases proportionally. This means that when safety is gained, bactericidal efficacy is lost. Prolonged involuntary contact with host cells can cause pain and inflammation.[121] Most complications of the use of NaOCl appear to be the result of its accidental injection beyond the root apex which can cause violent

tissue reactions characterized by pain, swelling, hemorrhage, and in some cases facilitates secondary infection and paresthesia.[122] Mainly this can happen by excessive pressure in the release of the irrigant, or by the existence of immature apices, root resorption and apical perforations. Other complications have also been reported during irrigation, when NaOCl coming into contact with mucous membranes, skin and eyes, resulting in hemolysis, skin ulceration and necrosis.[121]

1.5.5 Activated irrigant flow in root canals

Agitation / activation procedures seem to be a good alternative to improve the irrigant delivery throughout the root canal system and particularly for deep regions.

The objectives of the irrigant flow used during irrigation of the root canal seeks to create fluid dynamics with the following characteristics: [123]

- A close contact along the entire canal with the walls, in order to eliminate biofilms and serve as a lubricant for biomechanical instrumentation.
- Ensure a correct mixture of the irrigant along the entire root canal system, maintaining an effective concentration.
- Ensure a pressure against the walls of the root canal resulting in shear forces, which allows the removal of biofilm, smear layer and debris.
- Avoid periapical extrusion of the irrigant during activation, preventing damage to the surrounding tissues to the root.

Two phases can be identified during the irrigation procedure: a flow phase, where the irrigant is delivered and flows in and out of the root canal. The second phase is characterized by a resting state of the irrigant in the root canal.

The mechanism of energy transmission determines the specific flow characteristics of the irrigation systems and consequently their efficacy and safety. To improve the efficiency of the irrigant flow, several systems have been developed to activate / agitate the irrigator with various energy sources. [124]

1.5.6 Irrigant agitation systems

Negative Pressure Irrigation

This system uses a microcannula that is placed in the middle part of the root canal or close to the working length. [125] The flow is directed towards from the pulp chamber using a larger needle. True apical negative pressure only occurs when the cannula is utilized to aspirate irrigants from the apical constriction of the root canal. This technique is therefore considered safer than positive pressure SI, due to the absence of a flow directed towards the foramen.

Sonic activation

The oscillations of the instruments agitate the irrigant inside the root canal in order to enhance mixing and cleaning of the irrigant by fluid flow. Sonic activation employs instruments that are driven into vibration at one end (at the hand- piece). The other (free) end of the instrument is inserted near the working length of the root canal. Sonic devices operate at audible frequencies (below 20 kHz, typically 100 Hz for the current devices). [126] The energy transmitted from a file generate an acoustic stream of the irrigant solution. The sonic energy also generates significantly higher amplitude or greater back-and-forth tip movement. When the movement of the sonic file is constrained, the sideway oscillation disappears, therefore, contact between the tip and the root walls should be avoided. [123]

Ultrasonic activation

Energy is transmitted from a file or smooth oscillating wire to the irrigant by means of ultrasonic waves that induce two physical phenomena: stream and cavitation of the irrigant solution. The files are designed to oscillate at ultrasonic frequencies of 25–30 kHz, which are beyond the limit of human auditory perception (>20 kHz). Non-cutting instruments are available which can safely be used in the root canal. Two types of ultrasonic irrigation have been described in the literature. The first type is a combination of simultaneous ultrasonic instrumentation and irrigation (UI). The second type, often referred to as passive ultrasonic irrigation (PUI), operates without simultaneous instrumentation.[127] The instruments can be applied up to 1-2 mm from working length or at the beginning of a strong curvature in order to prevent heavy wall contact. [128] For optimal cleaning efficacy of oval extensions, isthmuses, and lateral canals of which the position is known, the instrument should be directed to oscillate towards these areas when possible. [126] Once again, heavy contact of the file with the root canal walls should be avoided.

Laser-activation irrigation (LAI)

Over the past decade, the use of laser energy to induce cavitation and acoustic streaming of intracanal irrigants has been investigated and several clinical protocols have been developed for fluid agitation using lasers.[129] LAI is based on the high absorption of laser energy by water. Absorption generates photoacoustic and photomechanical effects as steam and air bubbles are created in the irrigant. A forced collapse of bubbles causes implosions that impact on surfaces, causing shear forces, surface deformation and removal of surface material. [130] Most of the works on LAI have used lasers operating in the middle infrared region, where the absorption of water is strongest, such as the Er:YAG laser (2940 nm wavelength) and the Er,Cr:YSGG laser (2780 nm wavelength).

[131] The details of the operation of the LAI will be discussed in the "Laser in endodontics" chapter.

1.6 Laser

1.6.1 Historical considerations of Laser [132]

The 1917 seminal publication "Zür Quanten Theorie der Stralung" by Einstein contained the elements of the conceptual basis for stimulated emission of radiant energy. Eventually became the basis of modern laser physics. Einstein based his work on the theories of the field of quantum mechanics, formulated by the Danish physicist Bohr at the beginning of the 20th century. In 1955, Gordon et al, were the first to demonstrate the stimulated emission of microwaves within the electromagnetic spectrum. In 1958, the american physicists Arthur Schawlow and Charles Townes described in an article published in the magazine "Optical Review" the operating principles of the laser. They revealed that it was possible to stimulate emission of radiant energy in the form of photons in the infrared and visible or optical portions of the spectrum, which rapidly led to development of the laser. Maiman in 1960, introduced the acronym "LASER" (Light Amplification by Stimulated Emission of Radiation) and created the first operational laser, a ruby laser that emitted a brilliant red beam of light. This laser emanated pulses of light radiation of 0.69 microns of one millisecond duration or less within the visible portion of the electromagnetic spectrum. In 1963 the ruby laser was employed in the treatment of pigmented dermatologic lesions and for photocoagulation of the retina.

The first application of laser in dentistry was carried out by the physicist Leon Goldman in 1965, who was the first to use the ruby laser in dental tissues. In the recent years, an improved understanding of light-tissue interactions, new

technologies for delivering laser light to the tissue, have transformed lasers into versatile and valuable instruments.

1.6.2 Basis components

To understand the emission and generation of the laser beam, it is convenient to know some basics elements of laser (Figure 4): [133]

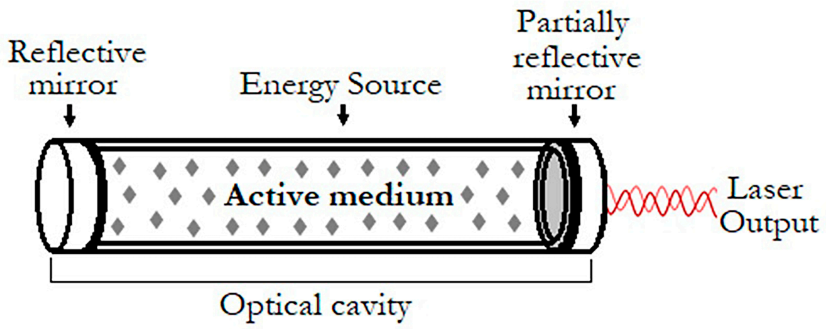

Figure 4. The fundamental components of laser. The optical cavity of the laser that contains the active medium, bounded by two perfectly parallel mirrors. Image adapted from Catone GA, Alline CC. Laser applications in oral and maxillofacial surgery. 1997. [132]

Active medium

It can be solid, gas, liquid or semiconductor (diode). Not all substances can be used to generate the laser beam, since they must have specific optical, mechanical, atomic and molecular characteristics. For example, in the CO_2 laser the active medium is a gas (carbon dioxide) or in the Er,Cr: YSGG laser are crystals of yttrium, scandium, gallium, and garnet, doped with atoms of erbium and chromium. An external source of energy is able to invert the atomic population of the active medium, supplying electrons for the energy transition from one orbit to another, which allows the emission of laser photons. This is

the basic element that will give the name to the laser.

Optical cavity

It is a component which contains the active medium and is constituted by a resonance cavity that has two concave mirrors at each extremes. One of them is totally reflective, while the other is only partly reflective. In this second mirror there is an area through which the beam of laser light will flow towards the conduction system. The photons coming from the excitation of the active medium (stimulation), are reflected inside the optical cavity, and pass through the active medium many times, amplifying its energy before leaving through the partially reflecting mirror. Light traveling in others directions is lost as heat (Figure 5).

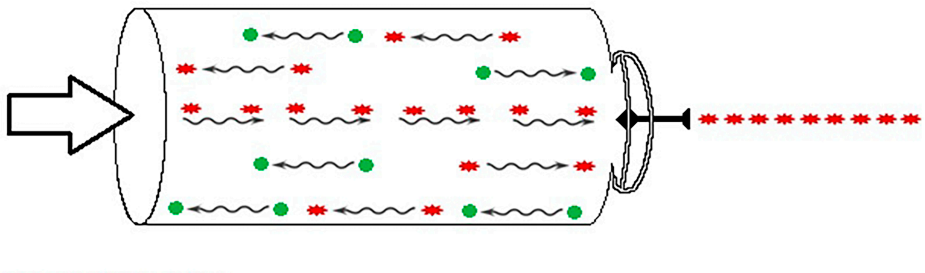

Figure 5. Scheme of the optical cavity. The partially reflective mirror permits a small amount of incident light to be released. Image adapted from Catone GA, Alline CC. Laser applications in oral and maxillofacial surgery.1997. [132]

Energy Source (or Pumping Source)

Many different sources of energy including electric discharge generator, light flash or another laser may be used. The pumping system excites the atoms of the active medium, generating the "inversion" of the population into the

resonance cavity (Figure 6). This condition is necessary to generate laser light. Radiant photons move longitudinally along the axis of the laser chamber and stimulate other proximal excited atoms to also emit additional identical photons that will travel with the same directionally as the other stimulated photons within the laser chamber. This is the "cascade phenomenon". The effect of cascade amplification is represented by the letter A of the laser acronym (LASER). The beam of photons that comes out through the non-reflective area of the resonance cavity is those that will form the laser light.

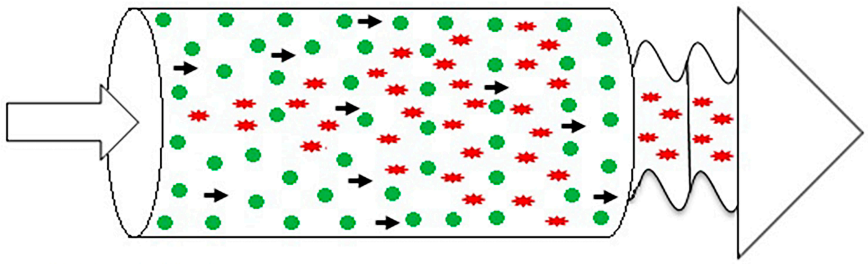

Figure 6. Cascade Phenomenon. Stimulation of photons (amplification) in axial direction. Image adapted from: Catone GA, Alline CC. Laser applications in oral and maxillofacial surgery.1997.[132]

Controller (or microprocessor) and cooling system

There is a microprocessor that control and verifies the characteristics of the production of the laser energy, the mode of emission (continuous wave, interrupted or mechanically pulsed), the frequency of pulsation of the repetition (pulses per second or frequency of repetition of the pulse) and the duration of the single pulse. A cooling system is the responsible for dissipating the heat produced by the pumping system.

Delivery system

The laser light is conducted to the target tissue through a driving mechanism, which varies with the laser that is used. Fiber optic is a flexible conduction system, with diameters ranging from 10-20 μm to 200-1000 μm. Due to its high fragility, it is wrapped in a protective cover. Lasers with wavelengths in the far infrared, such as CO_2, cannot be conducted through optic fiber, so an articulated hollow arm, composed of different mirrors, is used to reflect the laser beam to the target tissues.

Handpiece and tips

Laser systems use angular or straight hand pieces for the delivery of light, to which a tip is attached. Some handpieces have a terminal tip (close-contact handpiece) that works close to the tissue and/or within a root canal. Other handpieces are hollow handpieces, which allow the passage of fiber up to the extremity (near-infrared laser). Other systems does not have any terminal tip, but it has a reflecting mirror which works at a distance from the target tissue (tipless or non-contact or far-contact handpiece).

1.6.3 The Physics of Lasers

In nature, the electromagnetic spectrum of light is composed by visible and non-visible radiations. Laser is a form of electromagnetic energy, which, like other forms of energy such as light, radio waves, microwaves, x-rays, or gamma rays, is transmitted by waves. In these waves, three basic parameters are used to describe radiation: amplitude, frequency and wavelength. The amplitude is defined as the maximum distance that exists between a crest and the origin of a wave. It is the amount of energy that a certain wave has; the greater amplitude,

the greater amount of energy. Wavelength (λ) is the distance between any two points equivalent to one wave and defines the color of the light. Frequency (f) is defined as the complete number of waves that pass during one second measured in cycles per second (hertz (Hz)).

Visible and Invisible spectrum of light

Human eye does not recognize electromagnetic radiation beyond the violet zone of the spectrum (0.4 μm) or beyond the red zone (0.7 μm). The border is difficult to determine, because it depends on several factors, although it has been established between 380-400 nm and 700-800 nm.

Beyond the violet area, with a wavelength less than 0.4 μm (400 nm), there is a zone called ultra violet, located between 0.4 μm and 0.01 μm. Further to the left, with a decreasing wavelength, there are the X-rays extended to a wavelength of about <0.3 μm. Finally, located in the most extreme left area, there are the gamma rays (Figure 7).

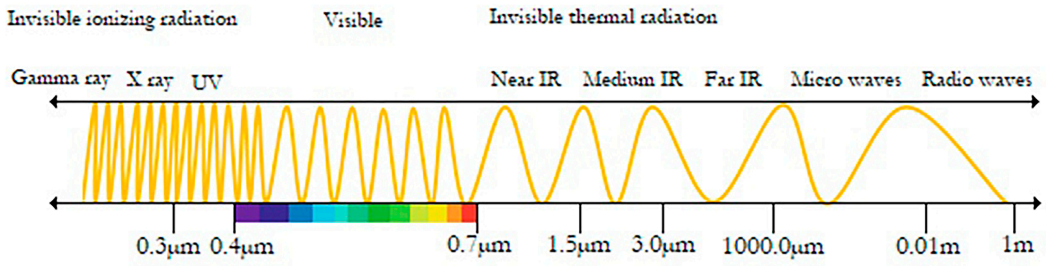

Figure 7. Spectrum of electromagnetic waves. UV: ultra violet; IR: infrared. Image adapted from Olivi G, De Moore R, DiVito E. Scientific background and clinical applications. 2016. [123]

1.6.4 Classification

Lasers are classified according to: a) wavelength, b) active medium, c) emission power and d) the way of emitting the energetic light.

a) According to their position in the electromagnetic spectrum (Figure 8).

Figure 8. Lasers are represented on the electromagnetic spectrum depending on their specific wavelength. Image adapted from: Olivi G, De Moore R, DiVito E. Scientific background and clinical applications. 2016. [123]

b) According to their active medium they can be grouped in:

- **Gases:** CO_2, Argon, Excimer, Helium-Neon, Cu vapor, Krypton.
- **Liquids:** Dyes.
- **Solids:** Nd: YAG, Nd: YAP, Er: YAG, Er, Cr: YSGG,alexandrite, Ruby, KTP.
- **Semiconductors or diode:** (AsGa, AsAlGa).

c) Depending on the emission power and its power density:

- **High power:** These are called surgical lasers. They act on the tissues by means of the thermal effect producing ablation.
- **Low power:** Also called therapeutic lasers. Its indication is non-invasive, since they do not produce tissue biostimulation effect.
- **High level laser therapy (HLLT):** primarily for surgical use.
- **Low level laser therapy (LLLT):** non-invasive therapeutic indications

d) According to its way of emitting the energetic light:

- **Continuous (CW):** The amount of energy obtained is constant over time.
- **Pulsed:** It produces less heat that spreads in the target tissue, the effect being purer. The amount of energy emitted in each pulse is less than the continuous mode.
- **Q-Switched:** produces very intense peaks of short duration, therefore the effect is very accurate with little heat dispersion.

1.7 Laser in endodontics

1.7.1 Highlights

Laser technology was introduced into endodontics with the goal of improving the cleaning ability of the root canal system obtained with traditional procedures, as well as ultrasound and chemical irrigation. Its conventional use is to introduce the fiber of the laser into the root canal up to the working length and activate it successively while retracted from the root canal. The near-infrared wavelengths show a deep penetration into the dentine, causing obvious thermal changes in the dentin surface, with areas of recrystallization and occlusion of the dentinal tubules by smear layer fusion. Medium-infrared wavelengths are well absorbed by water and spread their energy superficially over the canal surface. They have the ability to produce a thermal and ablative effect, which allows vaporizing the smear layer, increases dentinal permeability and exposes the dentinal tubules, improving the cleaning of the root canal. In order to reach complex anatomical zones within the root canal system, wavelengths that show high transmission although hydroxyapatite and water are required.

1.7.2 Laser-Tissue Interaction in endodontics

The incidence of laser light on the surface of a tissue alters its physical and chemical properties, causing modifications in the organic and inorganic components. The laser light absorbed by the tissue can be reflected, transmitted, diffused and absorbed (Figure 9).

Reflection: Is a property that occurs due to the low absorption of the laser beam by the target tissue, rejecting the light completely. Due to the possible reflection in the tissues it is mandatory to wear protective glasses during the use of the laser.

Transmission: It is the passage of laser light through the tissue without producing any physical or biological effect on it.

Diffusion (Scattering): Phenomenon through which energy diffuses more deeply and irregularly in the tissue. This gives the possibility of performing treatments at a distance from the target. This property is responsible for the effects of disinfection and decontamination of some wavelengths such as visible red and near infrared.

Absorption: High affinity between the target tissue and the emitted light, which makes possible the transfer of energy from the laser beam to the tissue. It is responsible for the greatest endodontic therapeutic action of the laser through photomechanical energy, photochemical energy or photoacoustic energy, depending on the wavelength and type of laser used. It has been seen that the energy of the erbium laser family (Er: YAG 2940 nm - Er, Cr: YSGG 2780 nm) can be absorbed by the water present in the dentine, which helps to clean the channel by means of ablation.[134] In addition, its wavelength coincides with the

maximum point of absorption of the irrigating solution, achieving a synergistic effect. This mechanism will be described.

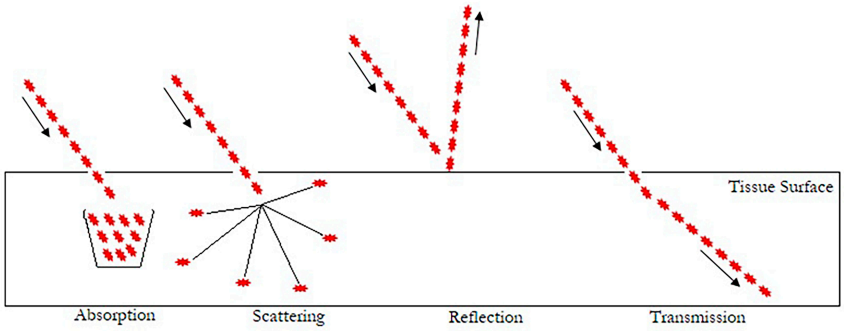

Figure 9. Mechanisms of interaction between laser light and target tissue. Transmission, absorption, diffusion and reflection. Image adaptad from: Lalwani AK. Current diagnosis & treatment in Otolaryngology-Head and neck surgery. 2008. [135]

Once absorbed, the laser light can cause photothermal, photochemical and photothermal-photomechanical-acoustic effects. The photothermal effect is carried out when the dentine wall is irradiated directly. All wavelengths cause this effect. The photochemical effect occurs due to the activation of photosensitive substances, responsible for producing the antibacterial effect, by laser of visible wavelength (635-675 nm) and near infrared (810 nm). An example of this effect is photoactivated disinfection (PAD). Finally, the photothermal and photomechanical-acoustic effect is generated by LAI, due to the high absorption in the irrigator of the medium-infrared laser (2,780nm - 2,940nm), which generates a great agitation in the irrigant, with the formation and implosion of air bubbles, a phenomenon known as "cavitation".

1.7.3 Erbium, Chromium YSGG laser

The Er,Cr:YSGG is a high power laser that contains a solid active medium, a crystal of yttrium, scandium, gallium and garnet, doped with atoms of erbium and chromium. Erbium is a metal of the group of rare heart elements (Er, atomic number of 68). Chromium is a steely-grey, lustrous, hard and brittle transition metal (Cr, atomic number of 24). The radiation is emitted, with a wavelengths of 2,780 nm in the medium-infrared spectrum. The laser energy is delivered to a terminal handpiece through optic fiber.

The system emits light in a pulsed mode, with a pulse duration that ranges between 140 and 200 μs and a repetition frequency of 20 Hz, which is constant. The power output can vary between 0.0W and 6W, with the possibility of making successive increments of 0.25W.

The wavelength is the one having maximum absorption by water, making it its principal objective chromophore. This explains its action in the dentin, especially in the intertubular region, where there is more volume of water. The Er,Cr: YSGG laser has an ablative action and vaporization on the smear layer in the root walls due to its photothermal effect. However, this action is superficial (up to 250-300 μm), which is up to where the energy is absorbed. On the other hand, the high absorption of Er, Cr: YSGG laser energy in the water allows the activation and agitation of irrigating solutions (NaOCl, EDTA) and three-dimensional streaming through the LAI, mechanism that allows to act indirectly against bacteria and improve the cleaning of the root canal system.

1.7.4 Laser-Activated Irrigation (LAI)

LAI is based on the collapse shock waves and the high-speed streaming of fluid, which are caused by rapid expansion and implosion of laser-induced bubbles,[136] phenomenon known as "cavitation". The energized irrigating

solution becomes more reactive, which could flow into the complex three-dimensional network of the root canal system, improving its degree of cleaning and disinfection (Figure 10).

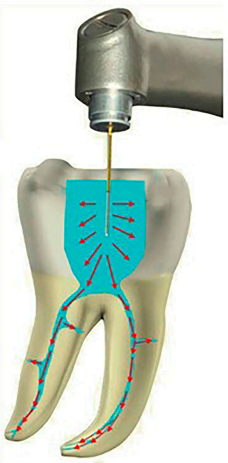

Figure 10. LAI inside of root canal system. Image provided by Dr. Olivi. J Laser Dent 2013;21:58-71. [137]

Due to the specific affinity of water with medium infrared lasers, specifically the erbium laser family Er: YAG 2940 nm and Er, Cr: YSGG 2780 nm, both wavelengths are currently the most used.

1.7.5 Cavitation: laser-induced bubbles formation and collapse

Cavitation is defined as "the formation of an empty space (bubble) and fast collapse of a bubble in a liquid". [138] The theoretical bases of cavitation in a endodontic solution are described below. Once the laser energy is absorbed by the solution, there is an instantaneous superheat to the boiling point of water (100° C), resulting in the formation of an initial cavitation vapor bubble, which expands from the tip. While the emission of the laser beam lasts, it goes through the bubble evaporating the water in front of the bubble, allowing it to continue increasing in volume. This phenomenon was described by Leeuwen *et*

al.[139] and was called the "Moses effect". Once the emission of the laser stops the bubble begins to collapse. The shrinkage generates pressure waves that first displace at supersonic speed (shock waves) and later at sonic speed (acoustic waves), [136] creating shearing forces along the root canal. [130] Additionally, a high-speed liquid jet is formed during the collapse of the bubble. [140] As a result of an abrupt and extensive change in pressure, once the collapse of the main steam bubble is over, new smaller cavitation bubbles are observed. This phenomenon is called "rebound". [141]Also these secondary cavitation bubbles may collapse, forming even smaller bubbles, which can disappear repeatedly in decreasing numbers. Figure 11 shows the formation, expansion and implosion of the bubble induced by laser in free aqueous solution and Figure 12 in a root canal model. [136]

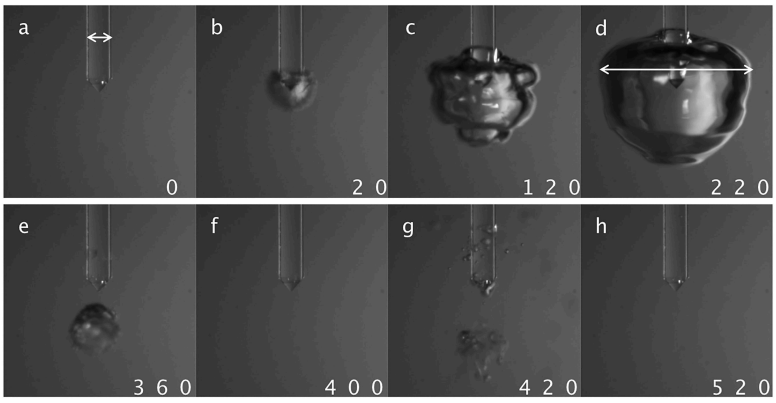

Figure 11. Representative laser-induced bubbles in free water (50 mJ, 1 pps). Numbers represent the time (microseconds) from the start of the laser pulse. (D) Largest vapor bubble, (E) implosion, (G) secondary cavitation bubbles. Image provided by Dr. Matsumoto. J Endod 2011;37:839–843). [136]

The forces released by the implosion of the cavitation bubbles are greater within the root canal than in an open space.[136] This is because the bubble maintains a very high pressure, because it is limited ahead by the water that surrounds it, behind by the fiber of the laser and on the sides by the root canal.

These physical phenomena may improve the removal of debris - smear layer and bacterial biofilms.

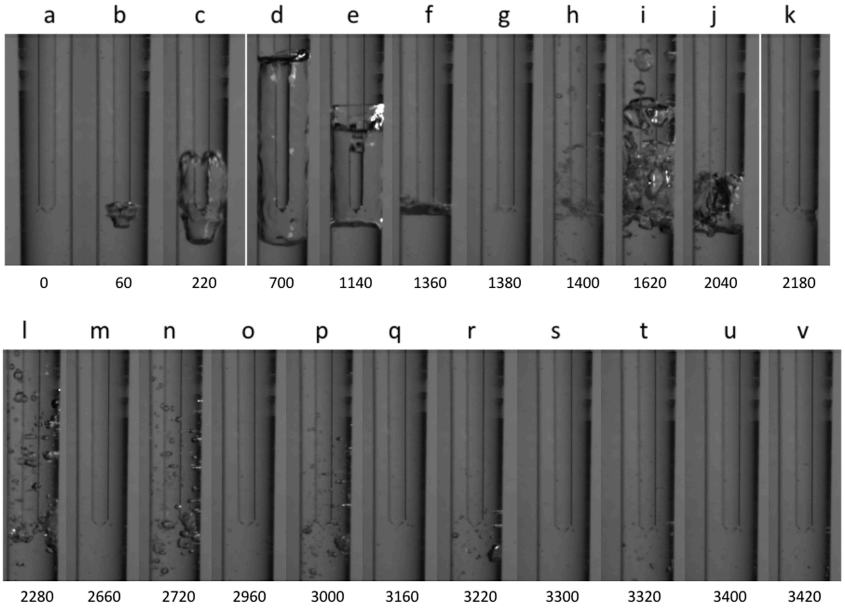

Figure 12. Representative laser-induced bubbles in root canal model (50 mJ, 1 pps), at different times the laser pulse started. (B–D) Vertical expansion of vapor bubble, (D) largest vapor bubble, (E–G) implosion, (I, L, N, P, R) repeatedly emerging secondary cavitation bubbles. (Image provided by Dr. Matsumoto. J Endod 2011;37:839-843). [136]

1.7.6 Laser-activated irrigation: Activation and resting phase of Sodium Hypochlorite

Inside the system of root canals, NaOCl reacts with organic matter, such as pulp tissue, microorganisms and organic compounds that are part of the dentinal radicular wall.[142] The result of this reaction is the loss of available chlorine (Δ [NaOCl]), which will result in a decrease in its therapeutic efficacy. The average rate of chlorine consumption is defined as the reaction rate (RR)

and can be determined by the quotient between the difference in NaOCl concentration before and after exposure time (Δ[NaOCl]) and the total exposure time (Δt) (RR = Δ[NaOCl]/ Δt). [92]

The laser activation is a strong factor that modulates the reaction rate of NaOCl. There are two mechanisms through which the flow of molecules occurs inside a liquid: diffusion and convection. Diffusion is the random and passive movement of molecules into the liquid. It is a slow movement that depends on the temperature and concentration gradients. Convection is the transport of particles through activation or movement of the fluid. This mechanism is faster and more efficient. The molecules of the irrigants activated by laser inside of the root canal are mobilized by means of convection sustained by acoustic microstreaming. The increased speed movement of the molecules by laser activation improves the effectiveness of NaOCl increasing the contact of free chlorine with organic matter or bacterial biofilms inside. On the other hand, the increase in the kinetics of the fluid increases its temperature, which may favor its reactivity and bactericidal effect.[143] Macedo *et al.* [92] observed that during a 3 min interval between the cycles activation the available chlorine consumption increased significantly. Therefore, the inclusion of a rest phase makes the NaOCl react even more.

2. HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

Three partial hypothesis are presented:

- Er, Cr: YSSG laser-activated irrigation is effective in killing *E. faecalis* biofilm.
- Laser-activated irrigation improves the antibacterial effect of low concentrations of NaOCl in *E. faecalis* when growing in biofilm.
- Laser-activated irrigation improves the antibacterial effectiveness of 0.5% NaOCl against *E. faecalis* biofilms.

form the tripod of a general hypothesis of this thesis that is "optimization of root canals disinfection may be achieved by laser-activated irrigation"

2.2 JUSTIFICATION OF THE STUDY AND OBJECTIVES

E. faecalis is frequently isolated from root canals with failed endodontic treatment. Inflammatory reactions can lead to the destruction of the pericapical tissue, abscesses, lymphadenitis or cellulitis. Multidrug-resistant phenotypes as well as those having high capacity to form biofilm are frequent. The bacterium presents several virulence factors that favor root canal colonization and contribute to remain for long starvation periods. The intrinsic morphological complexity of the root canal system offers additional protection to bacterial colonizers, making it very difficult to eradicate them through conventional therapies. All of these sentences resume the reasons why we have developed this work.

Laser activated-irrigation (LAI) is gaining increasing attention as an alternative to conventional endodontic therapy. It is based on the generation and implosion of cavitation bubbles in the irrigator. The release of energy generates shear forces that contribute to achieve a deeper cleaning and disinfection in the root canal system. Moreover, it was crucial to investigate if there is a synergistic effect between LAI and NaOCl that would allow to reduce concentration and therefore reduce the toxicity. The main purpose was to explore the antimicrobial action of the LAI technique at low concentrations of sodium hypochlorite against *E. faecalis* biofilm in the context of teeth with endodontic persistent infections.

MAIN OBJECTIVE

To quantitatively determine the bactericidal effect of 0.5% NaOCl activated by Er, Cr: YSGG laser-activated irrigation against *E. faecalis* biofilms in root canals.

SECONDARY OBJECTIVES

A) In *in vitro* model

a) To build a model of *in vitro* "root canal"(with *E. faecalis* biofilm).

b) To compare the antimicrobial efficacy of NaOCl 0.5% activated by Er,Cr:YSGG laser-activated irrigation (LAI) and passive ultrasonic irrigation (PUI) in such a model.

c) To determine the bactericidal effect of activated serum by Er, Cr: YSGG laser activated irrigation (LAI) and passive ultrasonic irrigation (PUI).

d) Visualization, through the atomic force microscopy (AFM) of bacterial surface alterations after laser and ultrasonic activation

B) In extracted teeth

a) Comparison of antimicrobial efficacy of 0.5% NaOCl activated by Er,Cr:YSGG laser activated-irrigation and passive ultrasonic irrigation against *E. faecalis* biofilm.

b) Comparison of the bactericidal action of serum by passive ultrasonic activation PUI vs laser activated-irrigation (LAI).

c) Scanning electron microscopy (SEM) visualization of removal of smear layer and *E. faecalis* biofilm from root canal after Er,Cr:YSGG laser activated-irrigation.

d) Confocal laser scanning microscopy (CLSM) determination of alive and dead bacteria inside the root canal after Er,Cr:YSGG laser activated-irrigation treatment.

3. PAPERS

3.1 PAPER 1

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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).

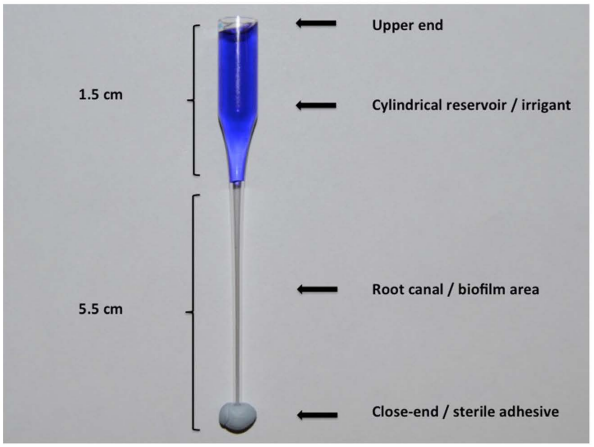

Fig. 1 Artificial root canal model infection. The irrigant is represented by violet blue staining. By suction, the inoculum or irrigant is carried into the pipette. The liquid does not drop due to the surface tension produced by the sterile adhesive action

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

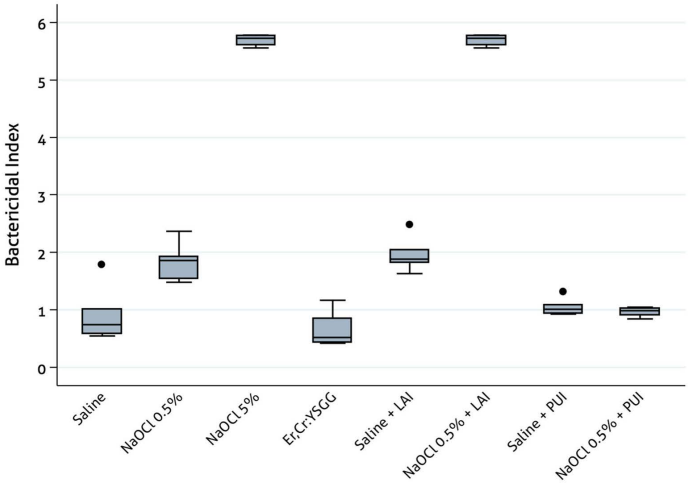

Fig. 2 Box plot of bactericidal index by the different treatments tested after 60 s

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) ($p < 0.0001$).

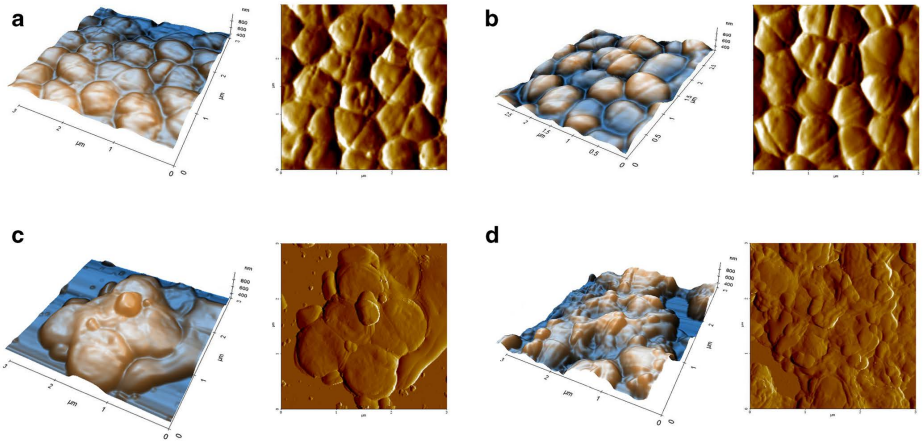

Fig. 3 3D topography and error signal images respectively of *E. faecalis* biofilm using atomic force microscope (AFM), visualized immediately after treatment. **a** Untreated biofilm, **b** Saline + LAI for 60 s, **c** NaOCl 5% unpowered, **d** NaOCl 0.5% + LAI for 60 s

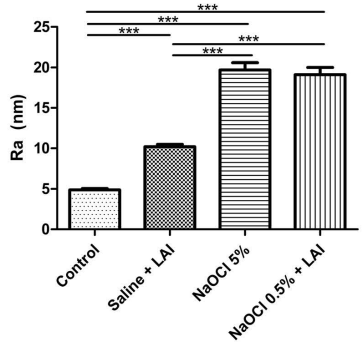

Fig. 4 Roughness average (Ra) values of *E. faecalis* cells exposed to different treatments after 60 s obtained from AFM analysis. Data were obtained from AFM images of samples in non-contact mode and processed by XEI software (Park Systems, South Korea). Means ± standard errors of the means are presented. 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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3.2 PAPER 2

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Perspective

May be Laser a key for endodontics?

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Photonics, understood as the science devoted to light generation, its detection, and manipulation is gaining every day positions in the ranking of tools to improve human health and wellbeing. However, this was not from the beginning. Laser was defined as "a solution looking for a problem." The laser was only recognized as useful tool in medicine several decades after the discovery. In part the growth of laser applications comes from military research. Applications of laser exceed nowadays the military field and have acquired a waste variety of applications including

medicine and dentistry. Bacterial persistence within the root canal system is the main cause of apparition and persistence of endodontic infection and subsequent endodontic failure. 1 *Enterococcus faecalis*, a gram-positive coccoid, anaerobic-aerotolerant, is the most prevalent microorganism isolated in cases of endodontic failure, due to its ability to survive in adverse environments ad endodontium characterized by lack of nutrients, alkalinity and dryness. The antiseptic irrigating solutions that are delivered conventionally with end-vented or side-vented needles lack a turbulent flow, limiting the ability to reach complex areas, such as istmus or lateral canals. It has been demonstrate that endodontic instruments leave 35% or more of untreated dentine surfaces.2 This leads to a decrease of percentage of success considerably, especially in persistent infections.

Recently, the use of laser-activated irrigation (LAI) has been proposed as an adjuvant to conventional chemo-mechanical therapy to improve cleaning and disinfection. Erbium lasers (Er, Cr: YSGG 2780nm - Er: YAG 2940nm) are the most commonly used due to the high affinity of their wavelength with water. The absorption of the laser energy generates an instantaneous superheat, causing cavitation vapor bubbles inside the fluid, which expand and implode, generating shock waves and high speed streaming of fluid.3 The generated pressure waves move first at a supersonic speed (shockwaves) and then at a sonic speed (acoustic waves), able to remove bacterial biofilms and smear layer from complex anatomical areas. Morphological injuries in the membrane of bacterial cells have been demonstrated through atomic force microscopy after LAI.4 One of the main advantages of LAI is that the laser fiber is placed at the entrance of the root canal during the entire activation, decreasing the possibility of extrusion of the irrigant and minimizing the thermal side effects.

Sodium hypochlorite (NaOCl) is the "gold standard" of endodontic solutions based on its drastic bactericidal capability, and its effective pulpal tissue solvent effect.5 It is used in a range between 0.5 and 6% being its toxicity concentration-dependant. Since NaOCl is not selective, dentine and periodontal ligament cells may be damaged, leading to inflammation and pain. Lately, several studies have shown the increase of the bactericidal effectiveness of NaOCl after being

activated by laser; mostly these research has been done at high concentrations of NaOCl6 .

A challenge in endodontics is to find alternatives to reduce the toxicity of NaOCl without losing the antibacterial activity. Thus, the study of eventual synergistic effects between laser and low concentrations of NaOCl becomes a field of great interest. In a recently published study, our group (Betancourt *et al.*,4) demonstrated through an in vitro model a significant increase of the effectiveness of 0.5% NaOCl when activated by Er,Cr:YSGG laser. Similarly Jaramillo *et al.*,7 reported that the activation of buffered 0.5%NaOCl improved its antibacterial capacity against 4 weeks-old biofilm of E.faecalis in extracted teeth. Nevertheless, not all contributions are in agreement. Christo *et al.*,8 failed in demonstrating improvement of 0.5% NaOCl in identical biofilms. This disagreement may be a consequence of difference in the laser powers used.

The use of erbium lasers to activate irrigating solutions inside the root canal have opened a new field in endodontics. Activation systems seems to be a good alternative to improve the irrigant delivery through the root canal system, above all, to the areas where the instruments cannot reach.

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CERTIFICADO DE PUBLICACIÓN DE MANUSCRITO.

Se certifica que el **PROF. DR. MIGUEL VIÑAS y DR. PABLO BETANCOURT DDS, MSc:** Han enviado el siguientes Manuscritos a nuestro Journal:

"May be Laser a key for endodontics?"; Posee revisión por pares revisores, y siendo resuelto como aceptado para la publicación 8(5), de Journal of Oral Research en Septiembre-Octubre 2019.

Se extiende el presente certificado para fines curriculares.

Atentamente,

Celia A. Lima, PhD
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3.3 PAPER 3

Photobiomodulation, Photomedicine, and Laser Surgery

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Er,Cr:YSGG Laser-activated irrigation and Passive ultrasonic irrigation: comparison of two strategies for root canal disinfection

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Er,Cr:YSGG Laser-activated irrigation and Passive ultrasonic irrigation: comparison of two strategies for root canal disinfection.

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Abstract

Objective: To compare the antibacterial effectiveness of 0.5% NaOCl activated by the Er,Cr:YSGG laser activated irrigation (LAI) and Passive ultrasonic irrigation (PUI) against a 10-day-old intracanal *E. faecalis* biofilm.

Background: LAI and PUI are regarded as alternative methods to release the irrigant in the inner regions of the root canal system achieving enhanced cleaning ability. Nevertheless, little evidence regarding the activation of low concentrations of NaOCl has been reported.

Methods: Seventy-two single-rooted teeth were instrumented, inoculated (*E. faecalis* ATCC 29212) and incubated for 10 days to allow biofilm formation. Specimens were randomly divided into six groups (n=12 each): (I) 0.5%NaOCl + Er,Cr:YSGG LAI (II) saline + Er,Cr:YSGG LAI (III) 0.5%NaOCl + PUI (IV) saline + PUI (V) positive control (no treatment) (VI) negative control (no bacteria). The activation time was distributed as follows: 30 seconds of activation, followed by a rest phase of 30 seconds and ending with 30 seconds of activation. The number of bacterial survivors was determined by plate counting.

Results: Both irrigation regimens LAI and PUI reduced the number of CFU. Moreover, LAI + 0.5% NaOCl and the rest of groups significantly differ ($p < 0.001$ for all comparisons).

Conclusion: Er,Cr:YSGG LAI proved to be more effective than PUI in enhancing the antimicrobial activity of 0.5% NaOCl against 10-day-old intracanal *E. faecalis* biofilms.

Key words: laser-activated irrigation, passive ultrasonic irrigation, sodium hypochlorite, *Enterococcus faecalis*, endodontics.

Introduction

Bacteria and their subproducts are the main cause of the progression and perpetuation of pulpal and periradicular diseases.[1] The root canal environment favors polymicrobial growth; however, a small number of bacteria are responsible for the perpetuation of persistent periapical infections, among which the most frequently isolated is *Enterococcus faecalis*.[2] *E. faecalis* can survive in adverse conditions like alkaline medium or nutrient deprivation for extended periods of time. The bacterium express several virulence factors, such as aggregation substances, enterococcal surface protein (Esp), endocarditis and biofilm-associated pili (ebp) and cytolysin.[3] It has been postulated that its high resistance to various antibacterial agents is, at least in part, due to its ability to for biofilms.[1] Biofilms consist in attached bacteria included in a self-produced matrix mostly polysaccharides and adhered to a surface or an interface. [4]

The ability to clean, debride and disinfect the root canal system is limited due to a complex three-dimensional network formed by oval extensions, accessory canals, anastomoses, apical ramifications etc. It has been shown that endodontic instruments do not prepare the entire root canal surface. [5] Although conventional syringe irrigation (SI) is widely used, it has been shown that the irrigant does not reach more than 2mm from the tip of the needle once it has been released, which means that the irrigant often do not reach the apical region of the canal.[6] This facilitates the persistence of biofilm and the survival of a significant number of viable bacteria, even when the biomechanical instrumentation is considered finished. Even more, *E. faecalis* and *Porphyromonas gingivalis* species can invade dentinal tubules up to 500 µm, and act as an etiological factor of persistent periradicular pathology. [7] Thus, adequate penetration of antimicrobial irrigation solution is crucial to achieve efficient debridement and disinfection, especially in untreated areas of the root canal system.

Sodium hypochlorite (NaOCl), the most commonly used endodontic irrigant, is used in concentrations between 0.5% and 6%. Characteristics such as the solubility of the tissue and the proteolytic effects on microorganisms make it a powerful disinfectant.[8] NaOCl is not selective, at high concentrations can damage dentin, periodontal tissues and human cells. [9] On the other hand, reduction in the concentration of NaOCl will reduce the cytotoxicity of the irrigator, and its bactericidal properties as well. In view of this, the study of therapeutic alternatives that enhance the antimicrobial activity of NaOCl at low concentrations is pertinent.

Laser-activated irrigation (LAI) using erbium lasers (Er:YAG: 2980 nm - Er,Cr:YSGG: 2780 nm) has been proposed as an alternative method to release the irrigant more deeply increasing cleaning ability inside the root canal system. [10,11] LAI is based on the formation, expansion and subsequent collapse

of vapor bubbles caused by the pulsed laser, due to the induction of specific cavitation phenomena and acoustic transmission. [11,12] The explosion generates pressure waves / shock waves, which act as shear forces. [10] It has been reported that LAI has an efficient bactericidal effect while improves the elimination of the smear layer, even from the apical third of the root. [13,14,15]

Another way to activate irrigant solutions in root canals is the use of passive ultrasonic irrigation (PUI). PUI uses an ultrasonically activated file to energize the irrigant solution in the canal and create acoustic streaming, which may result in a better removal of biofilms. [16] It has been observed that activating NaOCl by PUI eliminated more residues and smear layer compared to syringe irrigation.[17]

In general it has been reported that the cleaning and bactericidal efficiency of NaOCl improves when activated by LAI or PUI; however, to the best of our knowledge, there is little evidence regarding the activation of low concentrations of NaOCl. The aim of this *ex vivo* study was to evaluate the antibacterial effectiveness of 0.5% NaOCl activated by the Er,Cr:YSGG LAI and PUI against a 10-day-old intracanal *E. faecalis* biofilm.

Materials and methods

Sample preparation

The study protocol was approved by the Clinical Research and Ethics Committee of the University of Barcelona (#2016-23). A total of 72 single-rooted human teeth extracted by therapeutic indication were used. The specimens were subjected to a cleaning on the external part of the root by ultrasonic endodontic tips (Endo ProUltra Zirconium Satelec, Dentsply Maillefer, Ballaigues, Switzerland) and a Gracey 7/8 curette (Hu-Friedy, Chicago, USA) to remove remnants of periodontal ligament and root surface calculus. The specimens were stored in 10% formalin solution at 4ºC until use. Teeth were decoronated under the cementoenamel junction and the length of each root was adjusted to 14 mm.[18] A # 016 cylindrical diamond bur (Komet, Rock Hill, SC) was used to create a 5mm coronal reservoir at the root canal entrance. The apical permeability and single canal confirmation were verified with a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland). The working length (WL) was determined to be 1mm less since the #15 K-File was visible through the apical foramen.

Root canal treatment

The root canals were instrumented by a crown-down/step-back technique using the conventional sequence of 0.02 taper files up to the master #55 K-File (Dentsply Maillefer®, Ballaigues, Switzerland). Each instrument was irrigated in between treatments with 2.5% NaOCl using a syringe and a 30-gauge side-vented needle (Becton Dickinson, Madrid, Spain). To remove the smear layer, the root canals were rinsed with 1 mL 17% ethylenediaminetetraacetic acid (EDTA) (Denta Flux, Madrid, Spain) for 3 min followed by 1 mL 2.5% NaOCl and 1 mL of saline solution. The apical foramen and whole root surface was covered with a double layer of bonding agent (0_2 Nail Polish,

Depend Cosmetic AB, Halmstad, Sweden) to prevent extrusion of the irrigant and to simulate clinical conditions.[18] The dental roots were stored in Eppendorf tubes and autoclaved at 121ºC for 17 minutes.

Microbiological methods

Bacterial strain used in this work was *E. faecalis* ATCC 29212 (American Type Culture Collection). The bacterium was maintained by subculturing on trypticase soy agar (TSA) plates (Scharlau, Sentmenat Barcelona, Spain) weekly. For experiments single colonies were inoculated into 40 mL of tryptic soy broth (TSB) medium and incubated at 37ºC for 24 hours. The *E. faecalis* culture was diluted 100 times in fresh TSB and then adjusted spectrophotometrically (Unicam UV-2 at 600 nm) to approximately 10^8 cells CFU/ml. Root surfaces were coated with 0.01% (w/v) poly-L-lysine hydrobromide (Sigma-Aldrich, Dorset, UK) to enhance bacterial adhesion and inoculated with 10 µl of bacterial culture using a 30-gauge syringe and needle (Becton Dickinson, Madrid, Spain). The dental roots were placed in Eppendorf tubes and incubated at 37ºC for 10 days. Re-inoculation at days 1, 4 and 7 was performed to ensure the presence of live bacteria during the incubation period.[19] Finally, the inner part of the root canal was gently washed with 1 ml of Ringer's ¼ solution to remove the free-floating microbes and liquids. Bacteria were recovered by using an ultrasonic cleaner (Raypa, Barcelona, Spain) for 3 minutes at maximum power followed by vortex agitation for 45 seconds to suspend them in Ringer ¼. Colony-forming units (CFU) per ml were enumerated by plating tenfold serial dilutions on TSA plates incubated for 24 hours at 37°C. Values were transformed to CFU/mm2.

Experimental procedures

Seventy-two tooth roots were randomly divided into 6 experimental groups (12 specimens each) and treated according to the following protocols: (I) 0.5%NaOCl + Er,Cr:YSGG LAI (II) saline + Er,Cr:YSGG LAI (III) 0.5%NaOCl + PUI (IV) saline + PUI (V) positive control (no treatment) (VI) negative control (no bacteria).

Laser-activated irrigation

LAI was performed using an Er,Cr:YSGG pulsed laser (Waterlase iPlus; BIOLASE technology, Irvine, CA, USA) at 2780 nm wavelength, equipped with a RFT 2 tip (Endolase, BIOLASE Technology, Inc.; 200 µm in diameter, length 21 mm, calibration factor > 0.55). The treatment was at 0.55 wats average power at 10 Hz (60 µsec/pulse); irradiance 0.90 w/cm² yielding an energy density of 55 J/cm². During the laser activation the co-axial water spray from the gold handpiece (BIOLASE technology) was switched off and tip positioned only in the coronal reservoir. The total activation time was 60 sec: 30 seconds activation, followed by a rest phase of 30 seconds and ending with 30 seconds of activation. LAI was always performed within an irrigant-filled canal since solution was added when needed. Finally, 2 mL of 5% sodium thiosulfate was used to neutralize the remaining NaOCl and washed with 1 mL of saline.

Passive ultrasonic irrigation

An ultrasonic device (Newtron® P5 XS, Satelec Acteon, Merignac, France) equipped with a handpiece (Newtron Slim B.LED, Satelec Acteon, Merignac, France) 30 kHz frequency in the endo-mode (medium power)was used for PUI. The whole root canal and pulp chamber were first filled with the irrigating; then, a non-cutting ultrasonic tip (Irrisafe; Acteon, Merignac, France), stainless steel 25/.00, 25 mm in length, was inserted 2 mm short of the working length, with short vertical moves (2–3 mm), contact with the walls was avoided. The total activation time was 60 seconds, as for LAI protocol: 30 seconds initial activation, 30 seconds resting and 30 seconds activation. During the activation procedure, gently irrigation was continued; 5% thiosulfate and saline used as before.

AFM imaging.

AFM measurements were obtained by a XE-70 (Park Systems) at room temperature in noncontact mode using an ACTA silicon cantilever (Applied Nanostructures) with a nominal resonance frequency of 300 kHz and a nominal force constant of 37 N/m. Samples were air dried for imaging. Measurements began by scanning a random area of 30 by 30 µm, which was gradually decreased until surface could be observed in detail. Topography, amplitude, and phase images were recorded simultaneously. The acquired data were converted into topography, amplitude, and phase images and analyzed with XEI software (Park Systems). AFM imaging also allowed cell surface roughness measurement. A roughness average (Ra), meaning the average distance from the roughness profile to the center plane of the profile, was obtained from the acquired topography images.

SEM

A water-cooled diamond cutting blade mounted on a precision cutting machine (Mecatome, Persi, France) was used to cut the specimens longitudinally. The two parts were mounted on the microscope supports by means of a conductive double-sided adhesive disc. Next, they were covered with a fine graphite layer to improve their electrical conductivity (Emitech K950X high vacuum evaporator) and examined in a Jeol J-7100F scanning electron microscope (Tokyo, Japan) at 15.0 kV.

Statistical analysis

After logarithmic transformation of CFU values and bactericidal index (BI) was calculated. BI is defined as the difference between the logarithm of bacterial counts of the control and the treatment groups. [20] Shapiro-Wilk test rejected normality. Both the interquartile range (IQR) and the median were calculated. A statistical analysis was performed to compare the CFU/mm² values using the Kruskal-Wallis nonparametric test and Bonferroni`s . test for multiple comparisons. A level of $p < 0.05$ was considered significant.

Results

Median values and interquartile range of before and after CFU for each irrigation regimen are shown in table 1 and are plotted in figure 1. The BI values are shown in fig 2, this was considered the main parameter to define effectiveness. Both irrigation regimens LAI and PUI reduced the number of CFU; although, reduction was significantly higher for LAI group compared to the other groups ($P < 0.001$ for all comparisons) (Table 2). Effect of treatments may be observed in figure 3 where control 10 days-old biofilm, LAI and PUI treated images of SEM and figure 4 were AFM imaging of treated and untreated *Enterococcus* cells are shown and allow to observe the reduction of bacterial population, the roughness (table 3) and bacterial lysis.

Discussion

Unlike primary endodontic infections, which in nature are polymicrobial being frequently caused by anaerobic gram-negative bacteria, persistent endodontic infections are caused by only a few species, among which the most common is the gram-positive coccus *E. faecalis*. [2] The research done in this field has been performed in biofilms of various ages; while some use 24 hours of incubation, [21] others use 48 hours [22] or even weeks.[23] Gergova *et al.*[24] showed that 48 hours was enough for the tested bacterial strains to build well-formed biofilms. Nevertheless, it should be taken into account that under natural conditions, it is likely that much older biofilms (up to 10 days or more) will be found. Thus, in order to ensure a certain relevancy, we used 10-day-old biofilms.[19,25]

Syringe irrigation (SI) is currently used to release the irrigant into the root canal system. Nevertheless, it does not achieve turbulent fluid dynamics; thus, viable bacteria and the smear layer may remain in inaccessible parts of the root canal. Previous studies reported SI being insufficiently effective in the apical third of narrow root canals.[15] The use of a laser device and ultrasonic systems has been proposed as a complementary tool to improve the dispersion and activation of aqueous solutions.[19]

Saline solution alone has no bactericidal effect; although some bacterial death was observed when activated by LAI or PUI, maybe due to the intense turbulent flow caused by both activation systems.[18]

Both LAI and PUI activated 0.5% NaOCl were used to point out the enhancement of antimicrobial activity. Reduction of 3 logarithms reached with 0.5% NaOCl + LAI is consistent with the observations made by Jaramillo *et al.*, [26] who reported a significant decrease in the bacterial count by applying 0.5% NaOCl and Er:YAG laser. By contrast, Christo *et al.*[18] did not observe modifications of bactericidal activity of 0.5% NaOCl activated by low power (0.5W) Er,Cr:YSGG laser. In our experience teeth treated with 0.5% NaOCl and PUI showed limited bacterial reduction compared to the laser-treated group (1 logarithm of CFU/mm^2). The reason is possibly attributable to the fact that LAI is much more effective than PUI to create cavitation.[14,27] Juric *et al.*[19] reported no significant differences between LAI and PUI; although it should be noted that they used a higher concentration of NaOCl (2.5%). A so high concentration makes chemical antibacterial action much higher than the

one we achieve at 0.5 %. It should be taken into account that in curved root canals the use of PUI may be unsuitable, since the tip has to be positioned at 1 or 2 mm of the working length, and sometimes this is not feasible.[19] Additionally, root canals with pronounced curvature increases the possibility of the tip of the ultrasound coming in contact with the root walls, which may result in decreased amplitude and a reduction of the irrigant's streaming velocity, thus, reducing its action. In view of those factors, the use of the LAI seems to be more efficient by positioning the laser tip only at the pulp chamber, without needing to approach the root apex, so it could work well in both straight and curved root canals.

It has been reported that laser activation within the canal can have undesired effects on the dentine structure, such as cracks, small fissures or carbonization**.** [28] In our experiments, the laser fiber was only positioned at the entrance of the root canal, which decreases the heating of tooth structure or the surrounding tissues (alveolar bone and periodontal ligament). Cameron *et al.*[29] reported that if the irrigant is continually replenished during the activation of PUI, the temperature is maintained in safe ranges and will not cause damage to the tissues surrounding the root.

Some studies have suggested that the increase in the taper of the apical preparation improves the distribution of the irrigating solution to the root apex.[30,31] In the present study, the irrigant was activated in straight canals, prepared with an ISO 55# /0.02 file, which ensured a free oscillation by the ultrasonic tip, which may explain the bactericidal effect obtained. It has recently been reported that the use of an Er:YAG laser + NaOCl laser achieved an effective bacterial reduction in root canals with minimally invasive endodontic preparations.[32] This is relevant and novel, because it shows that the action of LAI, from the most coronal part, would help maintain a large part of the tooth structure intact, which improves the prosthetic prognosis of the tooth.

Several complications have been reported as a result of accidental extrusion of NaOCl into the periapical tissues through the apical foramen, accessory root canals or perforations. Ultrasonic activation causes an acoustic current that results in a rapid movement of the fluid in a circular or vortex motion. By contrast, the laser energy released during LAI causes both lateral and vertical movement, and caution must be exercised during irrigation.[33] Peeters and De Moore[34] showed that the possibility of extrusion of the irrigant increases when the tip of the laser approaches the root apex. We performed laser activation by positioning the laser fiber at the entrance of the root canal, reducing the risk of extrusion. Peeters *et al.*[35] measured *"in vivo"* the degree of extrusion of radiopaque contrast medium in 20 teeth with open apex, using Er,Cr:YSGG LAI (1W, 35 Hz) showing that no extrusion occurred, thus supporting the safety of its use. Lack of evidences of extrusion of the contrast medium in periapical tissues of 300 teeth after using Er,Cr:YSGG LAI (1W and 35Hz) has been also reported.[36] Nevertheless, extreme caution should be exercised when activating irrigator solutions near the apical constriction.

Conclusion

Er, Cr: YSGG LAI improves the antimicrobial efficacy of 0.5% NaOCl against 10-day-old *E. faecalis* biofilm in extracted teeth. This decreases the toxicity and adverse effects of NaOCl without losing its efficacy. Moreover, there was no significant increase in the antimicrobial efficacy of 0.5% NaOCl when activated by PUI.

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Group	Median CFUs/cm²	IQR
Control	$2.05x10^5$	$1.15x10^6$
0.5%NaOCl+LAI	$4.45x10^2$	$5.31x10^2$
Saline+LAI	$4.43x10^4$	$3.83x10^4$
0.5%NaOCl+PUI	$1.89x10^4$	$2.50x10^4$
Saline+PUI	$3.67x10^4$	$5.66x10^4$

Table 1. Bacterial count values of *E.faecalis* after the disinfection protocols. The values are expressed in median and interquartile range (IQR). LAI, laser-activated irrigation; PUI, passive ultrasonic irrigation; NaOCl, Sodium Hypochlorite.

Group	Treatment	0.5%NaOCl+LAI	Saline+LAI	0.5%NaOCl+PUI	Saline+PUI
1	0.5%NaOCl+LAI				
2	Saline+LAI	*<0.001*			
3	0.5%NaOCl+PUI	*<0.001*	0.472		
4	Saline+PUI	*<0.001*	0.996	0.834	

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; NaOCl, Sodium Hypochlorite.

FIGURE LEGENDS

Fig 1. Bacterial counts values of surface area (mm2) of E. faecalis biofilm after irrigation protocols. Data expressed as median and range.

Fig 2. Box plot of Bactericidal index values of E. faecalis biofilm after different irrigation protocols. * Statistically significant difference.

Fig 3. Figure 3. SEM imaging of root Canals: A and B canals with 10 days old biofilm on the walls; C canals after treatment with Laser in absence of NaOCl; D,E and F canals after treatment with NaOCl 5 % plus laser.

Fig 4. Effect of treatment on cell morphology: A control, B *Streptococcus* cells treated with Laser in serum; C Cells treated with NaOCl 0.5 % and D treated with NaOCl 0.5 % and Laser.

Fig 5. Nanoroughness average. Negative control and saline gave values lower than 10 (intact cells) while 5 % NaOCl and the combination of 0.5 % NaOCl with LAI made similar effects of bacterial integrity.

FIG.1

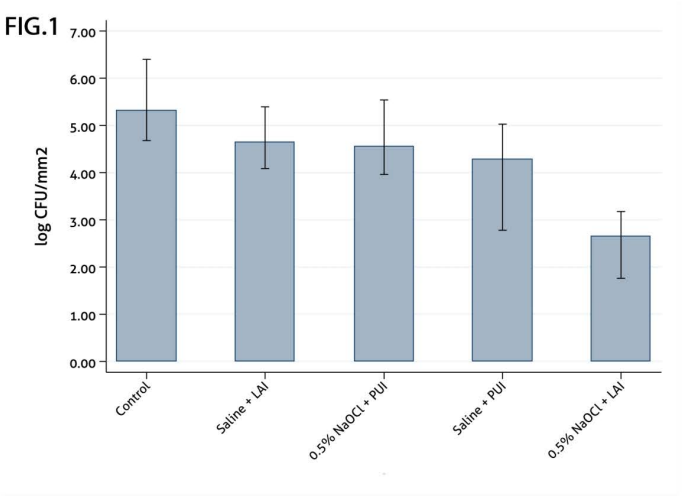

Fig 1. Bacterial counts values of surface area (mm2) of E. faecalis biofilm after irrigation protocols. Data expressed as median and range.

FIG.2

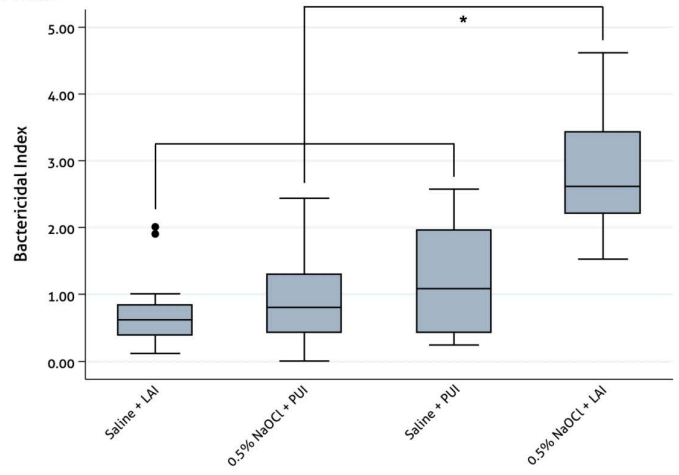

Fig 2. Box plot of Bactericidal index values of E. faecalis biofilm after different irrigation protocols. * Statistically significant difference.

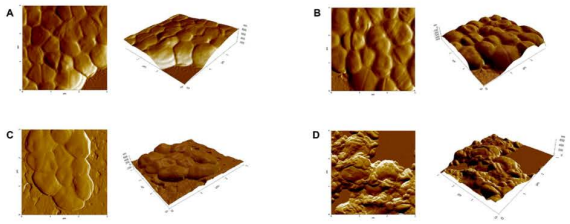

Fig 3. Figure 3. SEM imaging of root Canals: A and B canals with 10 days old biofilm on the walls; C canals after treatment with Laser in absence of NaOCl; D,E and F canals after treatment with NaOCl 5 % plus laser.

338x190mm (96 x 96 DPI)

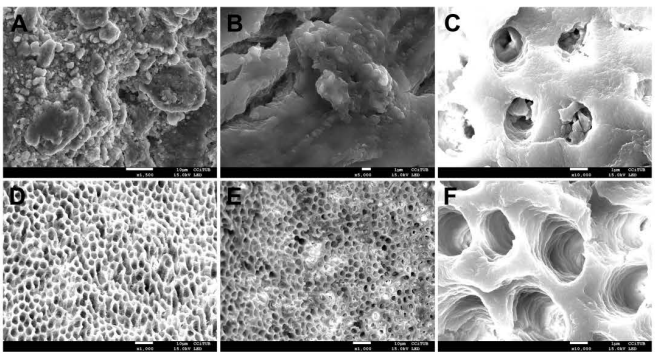

Fig 4. Effect of treatment on cell morphology: A control, B Streptococcus cells treated with Laser in serum; C Cells treated with NaOCl 0.5 % and D treated with NaOCl 0.5 % and Laser.

105x58mm (600 x 600 DPI)

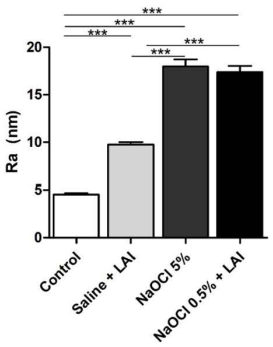

Fig 5. Nanoroughness average. Negative control and saline gave values lower than 10 (intact cells) while 5 % NaOCl and the combination of 0.5 % NaOCl with LAI made similar effects of bacterial integrity.

338x190mm (96 x 96 DPI)

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3.4 PAPER 4

BMC Oral Health

Sodium hypochlorite activated by Er,Cr:YSGG laser on Enterococcus faecalis biofilm root canals.

--Manuscript Draft--

Manuscript Number:	OHEA-D-19-00299
Full Title:	Sodium hypochlorite activated by Er,Cr:YSGG laser on Enterococcus faecalis biofilm root canals.
Article Type:	Research article
Section/Category:	Dental techniques; tools, materials and surgical research
Funding Information:	Chile (Becas doctorado) — Mr Pablo Betancourt CONICYT (5594/2015) — Mr Pablo Betancourt Marato TV3 (BARNAPA) — Prof. Miguel Viñas
Abstract:	The aim was to evaluate the antibacterial effectiveness of sodium hypochlorite (NaOCl) at low concentrations activated by the Er,Cr:YSGG laser-activated irrigation (LAI) against 10-day-old intracanal Enterococcus faecalis biofilm. Biofilms were formed inside the root canals and divided into 7 groups (n13): 0.5%NaOCl + Er,Cr:YSGG; Saline + Er,Cr:YSGG; 0.5%NaOCl + syringe irrigation(SI) ; 2.5%NaOCl + SI; 5%NaOCl + SI; positive and negative controls. Bacterial survivors were counted and specimens visualized under scanning electron and confocal laser scanning microscopy. Treatments with 0.5%NaOCl + Er,Cr:YSGG and 2.5%NaOCl + SI gave a significant reduction in the number of CFU/mm2. Moreover, SEM and CLSM imaging confirmed and reinforced bacteriological data. Thus, Er,Cr:YSGG LAI proved to be able to improve the antibacterial properties of 0.5% NaOCl after 60 seconds of activation, reaching the same level of effectiveness as 2.5% NaOCl. This is regarded as of clinical interest, since working with lower concentrations reduce undesired effects.
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Sodium hypochlorite activated by Er,Cr:YSGG laser on *Enterococcus faecalis* biofilm root canals.

Running title: Low concentration of NaOCl activated by LAI

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Conflict of interest and sources of funding statement

Declaration of interest: None

Ethical approval: The study protocol was approved by the Clinical Research and Ethics Committee of the University of Barcelona (#2016-23).

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Abstract

Background: he appearance and persistence of endodontic infections due to residual biofilm after chemical disinfection promotes secondary bacterial infection. Alternative methods to disinfect operated root Canals are a matter of great interest. The aim was to evaluate the antibacterial effectiveness of sodium hypochlorite (NaOCl) at low concentrations activated by the Er,Cr:YSGG laser-activated irrigation (LAI) against 10-day-old intracanal *Enterococcus faecalis* biofilm.

Methods: Biofilms were formed inside the root canals and divided into 7 groups (n13): 0.5%NaOCl + Er,Cr:YSGG; Saline + Er,Cr:YSGG; 0.5%NaOCl + syringe irrigation(SI) ; 2.5%NaOCl + SI; 5%NaOCl + SI; positive and negative controls. Bacterial survivors were counted and specimens visualized under scanning electron and confocal laser scanning microscopy.

Results: Treatments with 0.5%NaOCl + Er,Cr:YSGG and 2.5%NaOCl + SI gave a significant reduction in the number of CFU/mm2. Moreover, SEM and CLSM imaging confirmed and reinforced bacteriological data. Thus, Er,Cr:YSGG LAI proved to be able to improve the antibacterial properties of 0.5% NaOCl after 60 seconds of activation, reaching the same level of effectiveness as 2.5% NaOCl.

Conclusion: This is regarded as of clinical interest, since working with lower concentrations reduce undesired effects.

Key words: Root canal infection; Streptococcus faecalis; Biofilm; Er,Cr:YSGG laser.

Background

The appearance and persistence of endodontic infections due to residual biofilm after chemical disinfection promotes secondary bacterial infection [1]. Environmental conditions provided in the root canal favor polymicrobial growth; nevertheless, *Enterococcus faecalis* is the most frequently encountered bacterium in secondary infections [2]. *E. faecalis* is an aerotolerant anaerobic Gram-positive coccus, expressing several virulence factors, such as aggregation substances, enterococcal surface protein (Esp), endocarditis and biofilm-associated pili (ebp) and cytolysin [3]. Furthermore, its antimicrobial resistance seems to be strongly linked to its capacity to form biofilms [4]. A biofilm is defined as a growth mode of bacteria, bonded irreversibly to a substrate or to an interface or to each other, immersed in a self-produced extracellular polymeric substance (EPS). It has been pointed out that bacteria living in biofilms are phenotypically different from planktonic ones, at least in growth rates and gene transcription [5]. Theoretically, EPS offers protection against various environmental stresses, such as alkaline pH, dryness, high concentrations of salts or lack of nutrients for long periods. Indeed, the bacterial removal from a biofilm is approximately 1000 times more difficult than in planktonic state [6]. The success of endodontic therapy lies, therefore, in the ability to eradicate bacterial biofilms. The complex and unpredictable nature of the anatomy of the root canal system, comprised of accessory canals, isthmi, side canals and apical deltas, makes the complete removal

of bacterial biofilms difficult. Therefore, adequate irrigation is crucial to disinfecting those areas that may not be cleaned sufficiently by instruments.

Conventional syringe irrigation (SI) is widely accepted. Yet it has been argued that in SI the irrigant may not reach the apical region of the canal [7], subsequently allowing the persistence of biofilm and the survival of a significant number of viable bacteria, even when the apical preparation is considered to be “complete” [8].

Recently, laser-activated irrigation (LAI) has been proposed as an alternative method to achieve cleaning and disinfection of the root canal system [9]. The LAI mechanism of action consists in the generation of cavitation bubbles through the high absorption of the laser energy by water. This is particularly relevant when using Erbium family lasers (Er:YAG: 2980 nm - Er,Cr:YSGG: 2780 nm) [10, 11]. A turbulent flow and the subsequent formation of vapor bubbles in the liquid immediately after the Er,Cr:YSGG (2780 nm) laser activation has been demonstrated by Blanken et al.[12]. Bubbles expand during pulse and then implode generating pressure waves that first displace at supersonic speed (shock waves) and later at sonic speed (acoustic waves). This creates shearing forces along the root canal [13]. This offers a significant advantage over conventional SI, where significant effect take place only in the vicinity of the needle [7]. It has been demonstrated that LAI has bactericidal effect [14], improving the elimination of the dentin smear layer [15], and contributing to the elimination of residue from the apical third of the root [13].

Sodium hypochlorite (NaOCl) is the most widely used endodontic irrigant; it has a broad antibacterial spectrum and dissolves dental pulp tissue [16]. It is used at concentrations ranging between 0.5% and 6% to varying degrees of effectiveness. It has been reported that cell damage is directly proportional to NaOCl concentration [17]. Moreover, prolonged contact causes damages to dentin and periodontal ligament cells, involving acute inflammatory reaction and pain [18]. In a previous work, we reported that Er,Cr:YSGG LAI of 0.5% NaOCl increased the bactericidal effectiveness, on planktonic bacteria and young biofilms, in vitro, reaching the same level of antibacterial effectiveness as 5% NaOCl [19]. This should make feasible the use of NaOCl at lower, and subsequently safer, concentrations. The study was conducted in a laboratory condition by using 24-hour-old biofilms. Here we evaluate the antibacterial effectiveness of 0.5% NaOCl activated by the Er,Cr:YSGG laser against a 10-day-old *E. faecalis* biofilm *ex vivo*, in extracted teeth. Effectiveness was estimated by both bacteriological and microscopy approaches.

Methods

Specimens

The study protocol was approved by the Clinical Research and Ethics Committee of the University of Barcelona (#2016-23). A total ninety-one human single-rooted teeth extracted for therapeutics purposes were collected. To eliminate periodontal ligament remnants and calculus from the root surface, the specimens were subjected to cleaning using endodontic tips (ProUltra Zirconium Nitride,

Dentsply Maillefer, Ballaigues, Switzerland) and a Gracey 7/8 curette (Hu-Friedy, Chicago, USA). The specimens were stored in formalin solution 10% at 4ºC until use.

All teeth were decrowned under the cemento-enamel junction to a standardized length of 14 mm as described by Christo et al. [20]. A coronal reservoir of 5 mm was created with a #016 cylindrical diamond bur (Komet, Rock Hill, SC) at the entrance of the root canal. Apical permeability and single canal confirmation were checked with a K-File #10 (Dentsply Maillefer, Ballaigues, Switzerland). The working length (WL) was determined by reducing 1 mm from the point at which the K-File #10 was visible through the apical foramen. The canals were instrumented using the conventional sequence of 0.02 taper files up to the master K-File #45 (Dentsply Maillefer®, Ballaigues, Switzerland). After the use of each instrument, the root canals were irrigated with 1mL of 2.5% NaOCl using a syringe and a 30-gauge side-vented needle (Becton Dickinson, Madrid, Spain) to the WL. The canals were irrigated with 1mL ethylenediaminetetraacetic acid (EDTA) (Denta Flux, Madrid, Spain) for 1 minute, followed by 1mL of 2.5% NaOCl and 1mL of saline. The apical foramen and the root surface were sealed with a double layer of nail polish (02 Nail Polish, Depend Cosmetic AB, Halmstad, Sweden) to prevent the extrusion of the irrigant through the apex and to provide a closed system [20]. The dental roots were stored in Eppendorf tubes and autoclaved at 121ºC for 17 minutes.

***Enterococcus faecalis* biofilm formation**

E. faecalis ATCC 29212 (American Type Culture Collection) was maintained by weekly subculturing on trypticase soy agar (TSA) plates (Scharlau, Sentmenat Barcelona, Spain). A single colony was inoculated in 40 mL of tryptic soy broth (TSB) medium and incubated at 37ºC. After 24 hours of incubation, the culture was diluted 100 times in fresh TSB, and adjusted spectrophotometrically (Unicam UV-2 at 600 nm) at OD600 = 1.3 (i.e., 7.8 x 108 colony-forming units CFU/mL). Root surfaces were coated with 0.01% (w/v) poly-L-lysine hydrobromide (Sigma-Aldrich, Dorset, UK) to enhance bacterial adhesion and inoculated with 10 µl of bacterial culture using a 30-gauge syringe and needle (Becton Dickinson, Madrid, Spain). The dental roots were placed in Eppendorf tubes and incubated at 37ºC for 10 days. Re-inoculation at days 1, 4 and 7 were performed to ensure the presence of live bacteria during the incubation period [21]. Finally, the inner part of the root canal was gently washed with 1 ml of Ringer's ¼ solution to remove the free-floating microbes and liquids.

Experimental procedures

The teeth were randomly distributed into seven groups (n=13). Each group was submitted to a different treatment: (I) 0.5%NaOCl + Er,Cr:YSGG LAI (II) Saline + Er,Cr:YSGG LAI (III) 0.5%NaOCl + SI (IV) 2.5%NaOCl + SI (V) 5%NaOCl + SI (VI) Positive control (no treatment) (VII) Negative control (no bacteria). Eighteen teeth were then randomly divided in to two subgroups for investigation with CLSM (n= 8) and SEM microscopy (n = 10) techniques.

The SI protocol was done by slowly placing up to 5 mL of the irrigant into the WL and allowing it to act for 60 seconds. Finally, canals were irrigated with 2 mL of sodium thiosulfate 5% to inactivate the remaining NaOCl and washed with 1 mL of saline.

Laser irradiation took place using an Er,Cr:YSGG pulsed laser (Waterlase iPlus; Biolase Technology, Irvine, CA, USA) at a wavelength of 2780 nm. The laser operating parameters were 1W of power, 10Hz of repetition frequency, 100mJ energy per pulse and 140-μs of pulse duration. The coaxial water spray from the Gold Handpiece (Biolase Technology, Irvine, CA, USA) was switched off throughout the treatment. An RFT 2 tip (200 μm in diameter, 21 mm long, calibration factor >0.55, Endolase, Biolase Technology, Inc.) was used. It is a conical tip with a 50º angle, designed for endodontic treatment. The real power was 0.55W at 10Hz, 55mJ per pulse. Autoclaved tips were positioned only in the coronal reservoir during activation. During the laser irradiation cycles, irrigant was added as the coronal reservoir was empty; thus, LAI was permanently carried out in the presence of irrigant. The Er,Cr:YSGG laser was activated for 30 seconds, followed by a rest phase of 30 seconds and ending with 30 seconds of activation (60 seconds of activation in total). Finally, sodium thiosulfate and saline were used as before.

Bacterial count

Bacteria were suspended in Ringer ¼ by using an ultrasonic cleaner (Raypa, Barcelona, Spain) at maximum power followed by vortex agitation for 3 minutes. Colony-forming units (CFU) per ml were enumerated by plating tenfold serial dilutions on TSA plates incubated for 24 hours at 37° C. Values were transformed to CFU/mm2.

Scanning electron microscopy (SEM)

A water-cooled diamond cutting blade mounted on a precision cutting machine (Mecatome, Persi, France) was used to cut the specimens longitudinally. The two parts were mounted on the microscope supports by means of a conductive double-sided adhesive disc. Next, they were covered with a fine graphite layer to improve their electrical conductivity (Emitech K950X high vacuum evaporator) and examined in a Jeol J-7100F scanning electron microscope (Tokyo, Japan) at 15.0 kV. Visualizations were done at 1000X and 10000X to assess the bacterial biofilm and the smear layer in the coronal (10-12 mm from the apex), middle (6-7 mm from the apex) and apical (1-2 mm from the apex) parts.

Confocal laser scanning microscopy (CLSM)

To stain the biofilms, a mixture of SYTO 9 and propidium iodide prepared at a dilution ratio of 1:2 (1.5μL of SYTO 9 and 3 μL of propidium iodide (PI) in 1 mL of Ringer ¼) was applied to the whole biofilm. After 30 min of incubation in the dark at 37°C, the stained biofilms were washed once with Ringer ¼ to remove nonspecific staining. Fluorescence was observed using a Zeiss LSM 880 spectral confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a 488-nm argon laser

and 561-nm diode lasers. The reconstruction of whole teeth was performed with stitched images of different focal planes obtained with 10x magnification objective (0.45 numerical aperture) using the Zen black software (Carl Zeiss, Jena, Germany). The zoom images were obtained with 40x immersion oil objective (1.3 numerical aperture). The image resolution was 1,024×1,024 pixels with both magnifications. ImageJ software (National Institutes of health, Bethesda, MD, USA) and IMARIS software (Bitplane AG, Zurich, Switzerland) were used to obtain LSM images.

Statistical analysis

Statistical analysis was performed with Stata14 (StataCorp®, College Station, USA). Data were transformed logarithmically. The bactericidal effects were expressed as a bactericidal index (BI); i.e., the difference between the logarithm of the bacterial counts of the control and the treatment groups. The normality of the scale variables was explored using the Shapiro-Wilk test and the visual analysis of the P-P graph and the box plot. When normality was rejected, both the interquartile range (IQR) and the median were calculated. A statistical analysis was performed to compare the UFC/mm2 values using the Kruskal-Wallis nonparametric test and Bonferroni's post hoc test for multiple comparisons. The level of significance was set at $p < 0.05$.

Results

Bacterial elimination

Bacterial counts and IQR values are shown in Figure 1. 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 ($p<0.05$). The bactericidal index values are shown in Table 1 and Figure 1. In groups treated with 0.5%NaOCl + LAI and 2.5%NaOCl + SI there was a significant reduction in the number of CFU/mm2 ($p<0.001$). Moreover, reduction of CFU was significantly greater for 5% NaOCl + SI group ($p<0.001$). Lower efficiencies were achieved by saline solution delivered by SI and 0.5%NaOCl delivered by SI.

SEM

Neither smear layers nor microorganisms were observed on the root canal walls in the negative control; the entrance to the dentin tubules appears open (Fig.2, A1-A2). After bacterial incubation for 10 days, a heavy and dense biofilm of *E. faecalis* formed on the dentin surface, occluding the dentin tubules (Fig.2, B1-B2) was seen. The specimens treated with the Er,Cr:YSGG laser and 0.5% NaOCl showed an effective removal of both smear layer and biofilm. The root canal wall displayed open tubules and a clean surface (Fig. 2, C1-C6). In the saline + laser group (Fig. 2, D1-D6) and 0.5% NaOCl +SI group (Fig. 2 E1-E6), the *E. faecalis* biofilm and smear layer were observed on the surface of the root canal walls and inside the dentin tubules, showing that a complete biofilm removal was not achieved. None of the SEM micrographs showed signs of melting.

CLSM

In the control group (Fig. 3A and Fig. 4A) and saline+LAI group (Fig. 3B and Fig. 4B), the CLSM images showed a dense biofilm of *E. faecalis* formed on the dentin surface, formed predominantly by alive bacteria (green). The images revealed the presence of both alive and dead bacteria in the passive irrigation group with 0.5%NaOCl (Fig. 3C and Fig. 4C) with living cells predominating. Passive irrigation was not able to reach deep tooth areas. Finally, no viable cells were detected after treatment with the 0.5%NaOCl + LAI group (Fig. 3D and Fig. 4D).

Discussion

The age of the biofilms used in experimental biology is frequently a matter of discussion. Most research is conducted with 24 or 48-hour-old biofilms, while in clinics it is highly likely that we have to fight much older biofilms (up to 10 days or more). Longer bacterial incubations afford more relevant characteristics thanks to the formation of mature biofilms. The time needed for colonization by *E. faecalis* and biofilm formation varies among the different studies; while some use 24 hours of incubation [22], others use 48 hour [23], or even much longer incubation periods. We used a 10-day biofilm [21, 24] mimicking natural conditions. The bacterial colonization pattern on the dentin and inside the dentin tubules was verified by scanning electron microscopy.

There is no consensus regarding the actual time needed to completely eradicate *E. faecalis* biofilms. Radcliffe et al. [25] demonstrated that 0.5% and 1% NaOCl concentrations need at least 20-30 minutes to fully remove *E. faecalis* planktonic cells, while 5.25% NaOCl required only 2 minutes to achieve complete disinfection. In our case, 5% NaOCl released with the SI protocol was significantly more effective at removing *E. faecalis* biofilm than the other treatments with or without activation ($P < 0.001$). The main negative fact is that NaOCl at such high concentrations is extremely irritating to the periapical tissue [18]. Thus, the need to find new alternatives that can make the most of the antimicrobial activity of NaOCl but at less toxic concentrations is a matter of great interest.

It has been reported that laser-activated irrigation significantly enhances the effectiveness of root canal disinfection [14, 26]. The expansive shockwaves contribute to the overall photomechanical effect by facilitating access of the irrigant to the apical third of the canals and the deepest areas of the dentin [9]. In addition, the increased movement of NaOCl inside the root canal system increases the contact between the active chlorine molecules and the organic matter and, therefore, improves the chemical effectiveness of the irrigant [27]. However, little is known of the antibacterial effectiveness of low concentrations of NaOCl because most LAI studies have focused on working with high concentrations of NaOCl [14, 26, 28]. Working on human tooth root canals, we have demonstrated that 0.5% NaOCl combined with the Er,Cr:YSGG laser can effectively disinfect them, being the use of a less toxic concentration of NaOCl feasible. The canals irrigated with LAI 0.5% NaOCl showed a reduction of 3 logarithms in the CFU/mm2 count. The SEM revealed a large part of the canal wall and tubules as being free of microorganisms. This finding is encouraging because its

effect equaled that of 2.5% NaOCl administered by conventional irrigation. This is relevant as it demonstrates the existence of a synergic effect between the laser and low concentrations of NaOCl. Similar results were obtained by Jaramillo et al.[29], who concluded that the activation of 0.5% sodium hypochlorite with an Er:YAG laser significantly increased its antimicrobial effectiveness. By contrast, Christo et al. [20] observed that, working on a biofilm model with extracted teeth, LAI had a limited potential to increase the antibacterial effect of 0.5% NaOCl. This may be due to the fact that the work was done using an low power Er,Cr:YSGG laser (0.5W).

Teeth treated with conventional irrigation and 0.5% NaOCl showed a minimum alteration of the *E. faecalis* biofilm since most of dentin tubules exhibited a high number of bacteria. The unability to achieve good results by NaOCl at low concentration without activation stands out the relevant interest of laser energy in the disinfection of the root canal at these low concentrations.

Despite obviously saline is not bactericidal, some bactericidal effects were observed when used as an irrigant with LAI. The related bacterial death may be due to the intense flow action created within the irrigant [20]. Although the combination of activation laser and saline produced an alteration of biofilm, *E. faecalis* remained within the dentin tubules and on the dentin surface. It should be noted that the combination of the laser and saline improved the elimination of the smear layer, supporting the observations made by Di Vito et al. [15].

It has been reported that NaOCl extrusion increases during activation by the laser. Peeters & De Moore [30] demonstrated that the likelihood of extrusion is greater the closer the apex is placed to the optical fiber. Here we performed activation with the optical fiber in the coronal portion of the tooth for the duration of the activation, thereby decreasing the likelihood of irrigant extrusion. Recently, Peeters et al. [31] studied the degree of extrusion of radiopaque contrast medium in 20 teeth with open apex using Er,Cr:YSGG LAI (1W, 35 Hz). The results showed a total absence of contrast medium in every case, demonstrating the safety of the technique. Nevertheless, extreme caution should be exercised particularly in the vicinity of apical constriction to prevent extrusion.

It has been seen that the increase in temperature caused by laser energy can produce undesirable effects in the dentin, such as cracks, small fissures or carbonization [32]. In our experiments, the laser fiber was used away from the dental apex and never made contact with the canal walls, thus protecting the structure from possible thermal damage. This was confirmed in the SEM analysis, where the undamaged dentin can be observed following treatment.

Conclusion

In a 10-day-old *E. faecalis* biofilm in extracted teeth, the Er,Cr:YSGG LAI proved to be able to improve the antibacterial properties of 0.5% NaOCl after 60 seconds of activation, reaching the same level of effectiveness as 2.5% NaOCl. This is of great clinical interest, because it demonstrates that a lower concentration of NaOCl may be useful diminishing undesired secondary effects.

Declarations.

Ethics approval and consent to participate

The study protocol was approved by the Clinical Research and Ethics Committee of the University of Barcelona (#2016-23).

Consent for publication

Not aplicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors deny any conflict of interests.

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Authors' contributions

Pablo Betancourt (PhD Student) and Josep M. Sierra (Assistant professor) performed the experimental section, did the observations, and participate in the discussions.

Octavi Camps-Font (Assistant professor) performed the statistics

Josep Arnabat-Domínguez (Assistant professor) supervised the work in relation with the dentistry clinics challenges, and participate in the discussions.

Pablo Betancourt and Miguel Viñas (Full professor) wrote the paper

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Fig 1. Bacterial counts values of *E. faecalis* cells exposed to different treatments after 60 s. Box plot of the logarithm of bactericidal index reduction by different treatments tested after 60 seconds.

Fig.3. SEM images of negative control group (A1-A2) and positive control group (B1-B2). Magnification 1. x1,000 ; 2. x10,000.

Fig.4. SEM images of the coronal, middle and apical thirds of the root canal after different treatments. C1-C6: Er,Cr:YSGG laser and 0.5% NaOCl group. D1-D6: saline +laser. E1-E6: 0.5% NaOCl +SI. Magnification **1,3,5**: x1,000 ; **2,4,6**: x10,000.

Fig.5. Representative CLMS images of *E. faecalis* biofilm on the surface of the root canal: (a) untreated biofilm,(b) Saline + Er,Cr:YSGG, (c) LAI 0.5%NaOCl + SI, (d) 5%NaOCl + SI. Green: viable bacteria; Red: dead bacteria. Scale bar: 10µm.

Fig. 6.- CLMS representative images of the *E.faecalis* on the surface of the root canal. (a) Untreated biofilm, (b) Saline + Er,Cr:YSGG LAI , (c) 0.5%NaOCl + SI , (d) 5%NaOCl + SI . Green: viable bacteria; Red: dead bacteria. Scale bar: 40µm.

Figure

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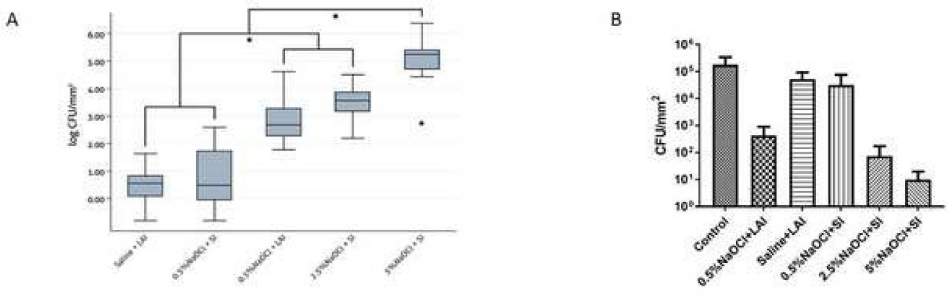

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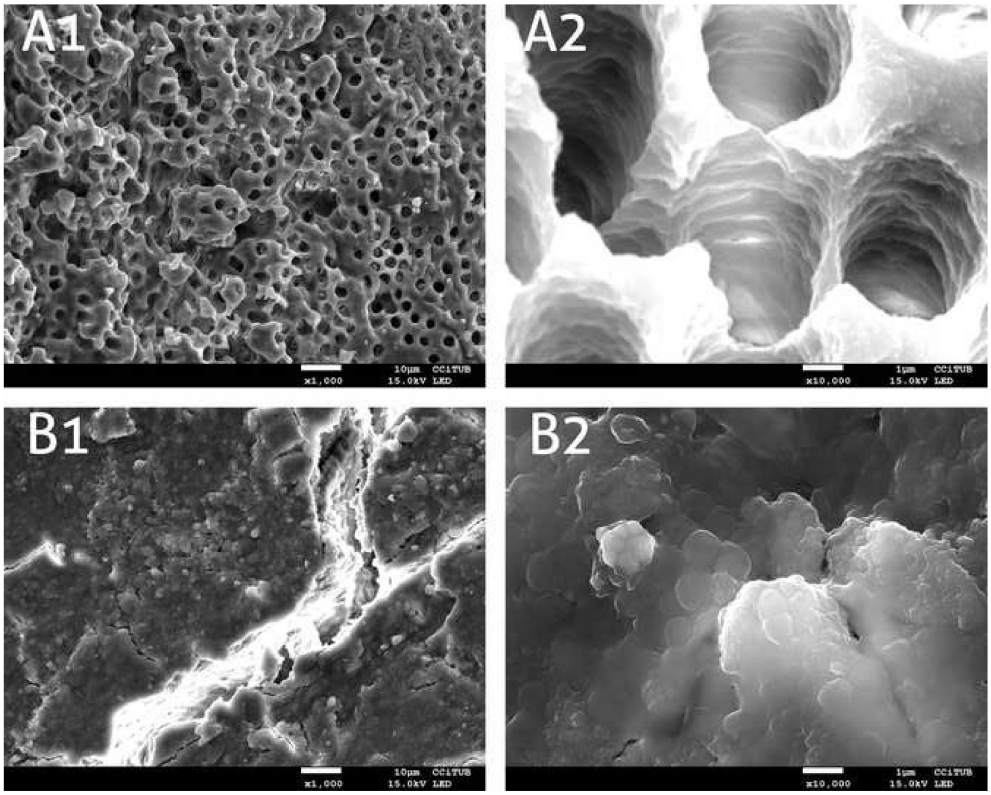

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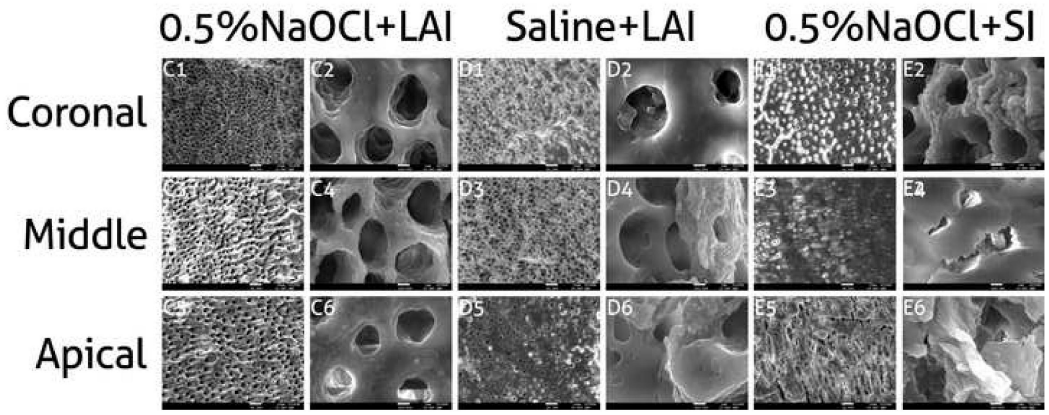

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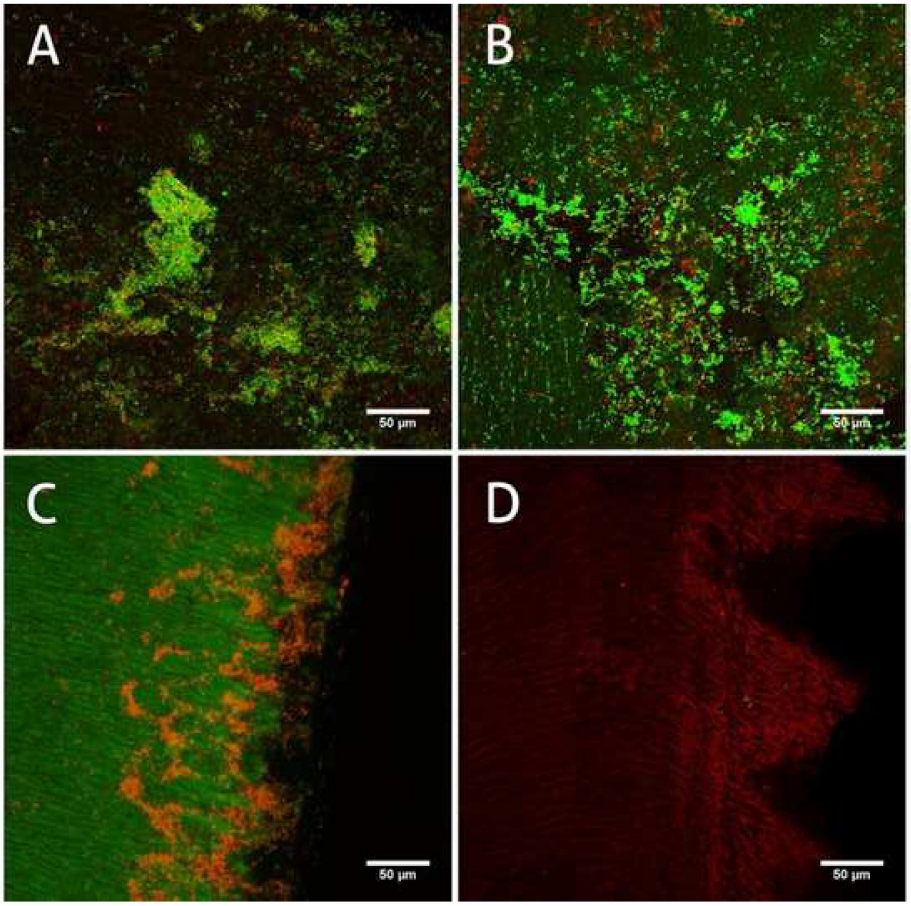

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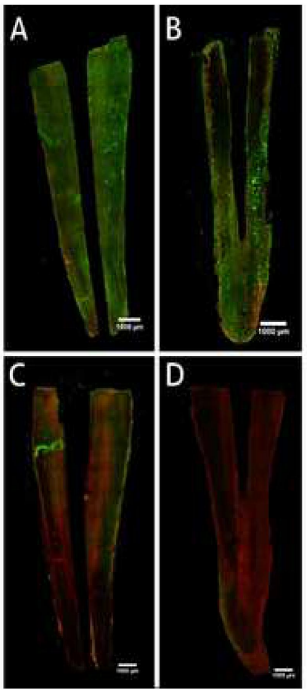

4. DISCUSSION

4. DISCUSSION

The science devoted to light generation, detection, manipulation and uses is called Photonics. Photonics is acquiring relevancy in many fields and also in medicine. The use of laser in many medical specialties is progressing fast although at the beginning laser was defined with a certain caustic humor as "a solution looking for a problem". The laser was only recognized as a useful tool in medicine several decades after its discovery. It is worthy to note that, at least in part, the growth of laser applications is attributable to military research. Nevertheless, applications of laser exceed nowadays the military field and have acquired a wide variety of applications including dentistry. Bacterial persistence within the root canal system is the major etiologic cause of endodontic infection and subsequent endodontic failure. [144] *E. faecalis*, an anaerobic-aerotolerant Gram-positive coccus, is the most prevalent specie among those isolated from endodontic failures samples, due to its ability to survive in adverse environments as root canals characterized by lack of nutrients, alkalinity and dryness. The antiseptic irrigating solutions that are delivered conventionally with end-vented or side-vented needles lack a discrete turbulent flow, limiting the ability to reach complex areas, such as istmus or lateral canals. It has been demonstrated that endodontic instruments leave 35% or more of the dentinal surface untreated (without contact with the disinfectant).[110] This leads to a significant probability of bacterial survival and persistence and subsequently of unsuccessful endodontic treatment.

Recently, LAI has been proposed as an adjuvant to conventional chemo-mechanical therapy to improve both cleaning and disinfection. Erbium lasers (Er, Cr: YSGG 2780nm - Er: YAG 2940nm) are the most commonly used based on their high affinity (of such a wavelength) for

water. The absorption of the energy supplied by laser, generates an instantaneous superheat, causing cavitation vapor bubbles in the fluid, which expand and implode, generating shock waves and high speed streaming of fluid. [145] The generated pressure waves move first at a supersonic speed (shockwaves) and then at a sonic speed (acoustic waves), being able to remove bacterial biofilms and smear layer from complex anatomical areas. Morphological injuries in the membrane of bacterial cells have been demonstrated through atomic force microscopy after LAI (Figure 3, paper 1). One of the main advantages of LAI is that the laser fiber is placed at the entrance of the root canal during the entire activation, reducing the possibility of extrusion of the irrigant and minimizing the thermal side effects.

A challenge in endodontics is to find alternatives to reduce the toxicity of NaOCl without losing the antibacterial activity. Thus, the study of eventual synergistic effects between laser and low concentrations of NaOCl becomes a field of great interest. Jaramillo *et al.*[146] reported that the activation of buffered 0.5% NaOCl improved its antibacterial capacity against 4 weeks-old biofilm of *E. faecalis* in extracted teeth. Nevertheless, not all contributions are in agreement. Christo *et al.*[147] failed in demonstrating improvement of 0.5% NaOCl in identical biofilms. We have performed series of experiments to validate and enlarge this previous knowledge.

The use of erbium lasers to activate irrigating solutions inside the root canal, have opened a new field in endodontics. Activation systems seem to be a good alternative to improve the irrigant delivery through the root canal system, above all, to the areas where the instruments cannot reach.

As indicated previously, the most frequent bacterial specie involved in root canal infections is *E. faecalis*.[148] This is the reason why this specie has been used along all experimental section of this work. Most of the research exploring the antiseptics to be used in endodontics, have been performed with planktonic bacteria.[149] Nevertheless, oral infections are caused mostly by sessile bacteria and endodontic infections are not an exception. A major issue of the research done in biofilms is the age of biofilms, since bacteria react in different manner not only when living as planktonic or sessile but also differences may be noted between cells living in young and old biofilms.[147, 150, 151] We have used 24-h-old and 10 days old biofilms.[152, 153] The age of the biofilms used in experimental biology is frequently a matter of discussion. Most research is conducted with 24 or 48-hour-old biofilms, while in clinic practice it is highly likely that we have to fight much older biofilms (up to 10 days or more). Longer bacterial incubations afford more relevant characteristics, due to the formation of mature biofilms. The time estimated for colonization by *E. faecalis* and the subsequent biofilm formation varies among the different studies; while some use 24 hours of incubation, [154] others use 48 hours.[155] Other authors have used much longer incubation periods. As indicated in the papers we used up to 10-days old biofilm to mimic as much as we can the natural conditions. [153, 156] (Figure 3, paper 3; Figure 2, paper 4).

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 [146, 147, 157], infected artificial root canals [158], dentine slices from infected bovine teeth [159], and slices of human root dentin. [160] In all cases, it seems that irrigants cannot reach the distal extremity of canals. We have designed 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 although walls are formed by glass instead of tooth material (Figure 1, paper 1). The device limits the forward expansion of the vapor bubble generated by the laser and prevents the expulsion of irrigant out of the canal.[161] It was observed that direct laser irradiation in agar plates or microtubes effectively kills *E. faecalis.* [150, 162] Some regions of the root canal systems remain out of contact with the irrigant. In fact, these observations were confirmed since a slighty bactericidal effect of Er,Cr:YSGG laser (without irrigant) 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. [146, 153, 161]

In order to reduce undesired thermal effects and the subsequent damage to the apical area, the distance between the tip and the apex should be enlarged. Thus, the laser tip was placed at 5 cm to the closed end of the pipette and kept there during the cycle. Furthermore, the expanding shockwaves contribute to the photomechanical effect, since they favor the access of the irrigant to the apical third of the canals. [136] The limits and size of the microenvironment of the root canal are on the basis of the idea that the induced laser pumps are going to remove smear layer and cellular debris while disrupting the microbial biofilms.[163] In principle the combination of these three effects would generate morphological, structural and functional alterations in bacterial membranes. In fact, we succeed in demonstrating such alterations by using atomic force microscopy (Figure 3, paper 1).

Our experimental work, similarly to other studies, [163, 164] made us aware of the fact 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 macroscopically seen immediately after each pulse.

The use of LAI allows overcoming the surface tension. In fact, surface tension is the physical reason why penetration is prevented. On the contrary PUI did not, since we have observed that the irrigant remained unable to reach the extremity. These differences in the behavior of both methods may be attributed to the higher ability of LAI to create cavitation that is much more effective than PUI.[165] These considerations have great interest since it is well known that the effectiveness of NaOCl strongly depends upon the time of contact biofilm/irrigant, thus an efficient distribution on the entire surface to be treated during the experimental time will result in a much more efficient killing effect. In summary, LAI contributes through three different actions: a physical effect derived from their intrinsic properties, a direct bactericidal effect and the optimization of the contact between the chemical disinfectant and biofilm.

As expected, results demonstrate that saline lacks antibacterial effect. Nevertheless, when it is used as irrigant in LAI, that is to say allowing physical but not chemical attack, some antibacterial effect was seen; this is probably due to bacterial death originated by the intense streaming and flushing action created within the irrigant by the effect of activation, although it failed significantly in bacterial elimination (physical effect derived from their intrinsic properties). This is in agreement with results of other groups. [147]

It has been demonstrated that some strains of *E. faecalis* may exhibit a certain tolerance to NaOCl.[166] For this reason a period of 30 min of contact with 0.5% NaOCl has been recommended in order to achieve a complete disinfection. Higher concentrations do not need such long period. This is the case of 5.25% NaOCl; when using such a concentration a 2 min period of contact is enough to achieve good disinfection. The use of chemicals is frequently conditioned by the toxicity. It has been demonstrated that cytotoxicity of NaOCl is dose-dependent. Most studies have tested LAI with high concentrations of NaOCl, [151, 161, 167] but little is known about the effectiveness of Er,Cr:YSGG LAI in eliminating bacteria when using 0.5% NaOCl concentration.

Here, we have 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 figure 3 (paper 1), cell envelopes were broken and cytoplasmic content was leaked out of the bacteria. This may be regarded as the reason why changes in roughness were observed (Figure 4, paper 1).

Similar results were obtained by Jaramillo *et al.*[146] who concluded, as mentioned before, that the activation of buffered 0.5% NaOCl by Er:YAG laser significantly increases its antimicrobial effectiveness. On the contrary, Christo *et al.* [147] reported that in a biofilm model using extracted teeth, LAI had a limited potential of increasing the antibacterial effect of 0.5% NaOCl. In any case, a consensus regarding the actual time needed to completely eradicate *E. faecalis* biofilms does not exist. Indeed, some authors such as Radcliffe *et al.* [166] reported that 0.5% and 1% NaOCl

concentrations need at least 20-30 minutes to achieve a fully removal of *E. faecalis* biofilms, while 5.25% NaOCl do it in so much shorter periods. In our experience at this work, the release of 5% NaOCl with the SI protocol was significantly more effective at removing *E. faecalis* biofilm than the other treatments, irrespective of activation ($p < 0.001$). However, it should be taken into account that, NaOCl at such concentrations results to be extremely irritating for periapical tissues.[121] Thus, the search for less aggressive and new alternatives reaching a similar antimicrobial activity of NaOCl but being less toxic is a matter of great interest. Since as it has been pointed out the toxicity of hypochlorite is dose-dependent, a reduction in the concentration of the disinfectant is one of the strategies to be explored. In addition to the enhancement of antimicrobial effect due to LAI, the expansive shockwaves contribute to the overall photomechanical effect by facilitating access of the irrigant to the apical third of the canals and the deepest areas of the dentin. [145,168,169] In addition, the promoted movement of NaOCl in the root canal system has as a direct consequence the increase of episodes of contact between active chlorine molecules and the organic matter. Therefore, the cooperation improves the chemical effectiveness of the irrigant.[92] Despite all of this mechanisms, the antibacterial effectiveness of low concentrations of NaOCl has been poorly studied and the knowledge on this is very limited. This is, at least in part, due to most studies related to LAI have been done at high concentrations of NaOCl. [168, 169, 170]

Working on human root canals, we succeed in demonstrating that at concentration of 0.5% NaOCl, can effectively disinfect them when appropriately combined with (activated by) the Er,Cr:YSGG laser. Obviously this would allow to drastically proceed by the use of a less toxic concentration of NaOCl. SEM revealed a wide regions of the canal wall

and tubules as being free of microorganisms but may not reveal if the small proportion of remaining microorganisms are dead or alive. In any case, this finding was seen by us as a reinforcing argument for the use of laser in endodontics. It was apparent that its effects equaled those ones with 2.5% NaOCl when applied by conventional irrigation. This is relevant as it demonstrates that when activated by laser, low concentrations of NaOCl are as effective as much higher concentrations.

It should be noted that the *E. faecalis* biofilm in teeth treated with conventional irrigation at 0.5% NaOCl showed a minimum alteration. Moreover, bacteria inside the dentin tubules may be observed since were not completely eliminated. In addition, one can observe that in most of dentin tubules, bacteria and debris fill the canals. The limited results obtained with NaOCl at low concentrations without activation, stands out the relevant interest of laser energy in the disinfection of the root canal at these low concentrations. We have pointed out that saline alone has no bactericidal effect; although the combination of activation laser and saline produced visible alterations on the biofilm and improves the elimination of the smear layer. This supports the observations made by Di Vito *et al.*[163]

It has been reported that NaOCl extrusion increases during activation by the laser. Peeters & De Moore demonstrated that the likelyhood of extrusion is greater the closer the apex is placed to the optical fiber. [171] Here, we have performed the activation by using the optical fiber in the coronal portion of the tooth during activation, thereby decreasing the likelihood of irrigant extrusion. Recently, Peeters *et al.*[172] have studied the level of extrusion of radiopaque contrast medium in 20 teeth with open apex using Er,Cr:YSGG LAI (1W, 35 Hz) showing a total absence of contrast medium in all cases, demonstrating the safety of the technique.

Nevertheless, to prevent undesired effects, extreme caution should be exercised when used in mouth, particularly in the proximity of apical constriction since in this portion the extrusion may be more probable. Temperature also may be significant here since it has been pointed out that increases of temperature caused by laser energy may generate undesirable effects on the dentin, such as cracks, small fissures or even carbonization. [173] In our experiments, we have used the laser fiber far away from the dental apex and in any case contact with the tip and the canal walls was permitted. By this way a protection of the structure from possible thermal damage was achieved. The effectiveness of this "homemade artisan" procedure was confirmed by SEM which revealed intact dentin after treatment.

Finally, it was a central point of interest to compare the different alternative methods of root canal disinfection. Experiments were performed to explore if 0.5% NaOCl used activated by LAI and PUI gave different antimicrobial efficacies. The teeth treated with 0.5% NaOCl + LAI showed a reduction of 3 logarithms count (CFU/mm^2) while reduction by ultrasounds was much more mild (1 logarithm (CFU/mm^2)). These differences may be attributable to the fact that LAI has the capacity to create cavitation much more effectively than PUI. These results strongly disagree with those of Juric *et al.*[153] when they failed in detecting any significant differences in the bacterial elimination inside the root canal between LAI and PUI. This is most probably due to the fact that they used a higher concentration of NaOCl (2.5%) and in such conditions the chemical killing effect masks the differences between the method used for activation. That is to say: at high concentrations of NaOCl no activation is needed. It should be also stated that strongly curved root canals increase the risk of contact between the tip and the root canal walls. This may

result in decreasing amplitude and the reduction of the irrigant's streaming speed, thus, restricting its mechanism of action. In view of those factors, the use of the LAI seems to be more efficient by positioning the laser tip only at the pulp chamber, without needing to approach the root apex, so it could work well in both straight and curved root canals.

Some studies have suggested that the increase in the taper of the apical preparation improves the distribution of the irrigating solution to the root apex. [117, 118] In this thesis the irrigant was activated in straight canals, prepared with an ISO 55# /0.02 file, which ensured a free oscillation by the ultrasonic tip, which may explain the bactericidal effect obtained. It has recently been reported that the use of an Er:YAG laser + NaOCl laser achieve an effective bacterial reduction in root canals with minimally invasive endodontic preparations. [169] This is relevant and novel, because it shows that the action of LAI, from the most coronal part, would help maintain a large part of the tooth structure intact, which improves the prosthetic prognosis of the tooth.

5. CONCLUSIONS

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In vitro artificial root canal model

1. The colonization and biofilm formation on the walls of Pasteur pipettes used as a model to simulate the conditions inside a root canal, were feasible as demonstrated by AFM. Thus, the device may be used as a laboratory model.

2. Irrigation with Er, Cr: YSGG laser-activated 0.5% NaOCl significantly improved the antimicrobial efficacy of 0.5% NaOCl non-activated. Quantitative determination of the antimicrobial action demonstrates that the effect was similar to the one obtained by 5% NaOCl. In contrast, passive ultrasonic irrigation did not increase the antibacterial effect of 0.5% NaOCl.

3. Biofilm of *E. faecalis* was not significantly affected by the action of irrigation with saline solution either activated by laser or by passive ultrasonic. That is to said chemical attack is needed.

4. The action of Er, Cr: YSGG laser- activated with 0.5% NaOCl was capable to alter cell morphology, turgency and the integrity of the bacterial wall. Cell surface increased the nano-roughness as a result of treatment.

In extracted Teeth

1. The antibacterial action of 0.5% NaOCl was greater when activated by Er, Cr: YSGG than when passive ultrasonic was used to activate the solution. Its effect was identical of 2.5% NaOCl, and close to the one reached at 5% NaOCl.

2. When saline was used, an slighty antibacterial effect was observed, suggesting certain streaming and flushing action created within the irrigant by the effect of activation which may destabilize the biofilm through a strictly physical action. This effect was not observed in the model, probably due to the differences in diameter.

3. After 60 seconds of activation by Er,Cr:YSGG laser, 0.5% NaOCl, demonstrated an effective removal of both smear layer and biofilm from dentine surface and dentinal tubules.

4. No viable cells were detected after treatment with the 0.5% NaOCl and Er,Cr:YSGG laser by confocal laser scanning microscopy. The penetration of 0.5% NaOCl into dentinal tubules is greatly improved by Er,Cr:YSGG laser activation.

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