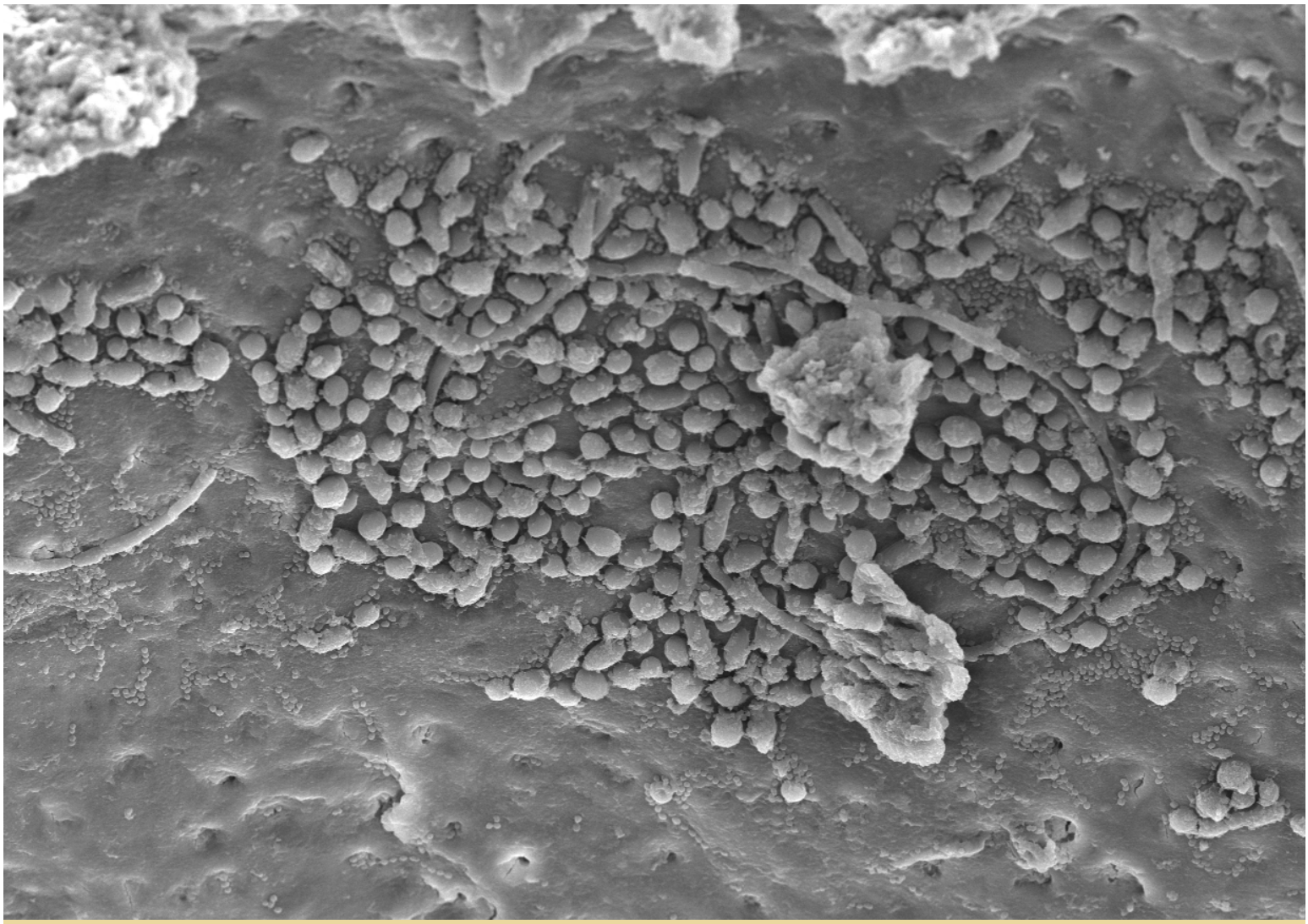

Patrícia Diogo Nunes

Photodynamic therapy applied to asepsis of root canals

Tese de Doutoramento no Programa de Doutoramento em Ciências da Saúde, ramo de Medicina Dentária, orientada pela Professora Doutora Teresa Maria Fonseca de Oliveira Gonçalves e pelo Professor Doutor João Miguel Marques dos Santos apresentada à Faculdade de Medicina da Universidade de Coimbra

Agosto de 2017

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UNIVERSIDADE DE COIMBRA

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Photodynamic therapy applied to asepsis of root canals

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Universidade de Coimbra

2017

CAPA

Imagem de biofilme misto de *Enterococcus faecallis* e *Candida albicans* (controlo) às 48h, em blocos cilíndricos de dentes monorradiculares humanos, obtida em microscópio electrónico de varrimento, Hitachi SU-70.

O trabalho experimental descrito foi realizado no Instituto de Microbiologia e na Área de Medicina Dentária da Faculdade de Medicina da Universidade de Coimbra.

À Fernanda, Adelino e André

AGRADECIMENTOS

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Esta tese resulta de um curto, mas intenso percurso académico, flanqueado pelos meus orientadores. Após a concretização do Mestrado Integrado, o interesse pela investigação prevaleceu e foi com muita dedicação e alguma sorte, como em tudo o que se faz, que iniciei o meu programa doutoral. A linha da meta, faz-me rever a linha de partida e permite recordar o meu crescimento assistido de perto pelos meus orientadores, a quem devo a excelência alcançada neste manuscrito.

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SUMÁRIO

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A antissepsia efetiva do sistema de canais radiculares é indubitavelmente uma etapa fundamental no sucesso do tratamento endodôntico, tratamento este que visa preservar as condições de assepsia ou eliminar os microrganismos do interior dos canais radiculares, promovendo a manutenção ou o restabelecimento da integridade dos tecidos periapicais. As condições de assepsia, obtidas durante a fase de preparação biomecânica, devem ser preservadas com a realização da obturação tridimensional do sistema canalar, seguida da restauração coronária.

São várias as técnicas contemporâneas da preparação canalar existentes que incluem obrigatoriamente um desbridamento químico-mecânico, dando preferência a um sistema de Endodontia rotatório mecanizado de niquel-titânio combinado com soluções de irrigação, das quais o hipoclorito de sódio se destaca como o *goldstandard* atendendo às suas propriedades antibacterianas e proteolíticas na dissolução dos tecidos orgânicos. Contudo, são vários os estudos que verificam que com as presentes técnicas e materiais disponíveis, a total erradicação de microrganismos do sistema canalar ainda não é possível.

Na pesquisa por novos métodos na desinfeção canalar, a irrigação ativada por LASER (PIPS) e a terapia fotodinâmica (PDT) destacam-se das demais. Ao passo que as PIPS promovem o desbridamento do sistema canalar com o LASER Er:YAG em níveis de potência sub-ablativos, a PDT resulta da combinação de um agente inerte (fotossensibilizador) com uma fonte de luz (LASER de baixa potência) que, na presença de oxigénio, absorve fotões de luz e com o aumento do período de irradiação, os eletrões passam a um estado energético cada vez mais elevado, mas instável. Os eletrões ao regressarem ao seu estado basal, libertam energia, que é transferida ao substrato sob a forma de radicais livre de oxigénio altamente reativos

que possibilitam a eliminação de microrganismos, sem indução de resistência microbiana e dano no hospedeiro.

A PDT tem apresentado, nos estudos experimentais um franco crescimento com resultados promissores e é economicamente mais acessível ao Endodontista do que as PIPS.

O objetivo principal do presente trabalho foi avaliar a eficácia antimicrobiana da PDT com vários fotossensibilizadores, em particular uma clorofila modificada derivada do complexo de zinco(II) de éster trimetílico da clorina e_6, Zn(II)e_6Me, extraída da clorofila a, um novo fotossensibilizador aplicado à Endodontia *versus* irrigantes clássicos. Para a concretização deste estudo, foram preparados biofilmes de *Enterococcus faecalis* e *Candida albicans* mono- e multiespécies em superfícies abióticas experimentais (lamelas de vidro); discos de dentina e blocos radiculares obtidos a partir de dentes humanos. Na primeira parte do trabalho experimental, realizou-se um estudo piloto para selecionar os agentes antimicrobianos mais eficientes. Em biofilmes de 48 h, mono- e multiespécies, foram aplicados três irrigantes clássicos; hipoclorito de sódio (NaOCl) a 3%, ácido etileno-diamino-tetra-acético (EDTA) a 17% e clorhexidina (CHX) a 2%; em três períodos de contacto diferentes (60, 90 s e 30 min). Nos grupos de PDT, testaram-se nos mesmos biofilmes quatro fotossensibilizadores, todos à mesma concentração de 0,1 mg/mL. Azul de toluidina-O (TBO), rosa bengala (RB), a porfirina catiónica sintética de tetraiodeto de 5,10,15,20-tetraquis(1-metilpiridinio-4-il)porfirina (TMPyP) e a clorofila modificada (Zn(II)e_6Me) em dois períodos de tempo (60 e 90 s).

Em resultado destes ensaios, concluiu-se que o período de tempo de 90 s é suficiente para obtermos resultados antimicrobianos de excelência, não sendo necessário alongar o mesmo até 30 min nos irrigantes clássicos, sendo este período descartado nos estudos seguintes. A clorofila modificada, Zn(II)e_6Me, em condições

in vitro quando ativada por LED em períodos de 60 a 90 s conseguiu eliminar cerca de 60% de biomassa de biofilme, sendo mais eficiente do que TBO e RB e similar à TMPyP, CHX e EDTA. NaOCl apresentou uma eficiência superior, quando comparado com a clorofila modificada, com 70-90% de remoção de biomassa de biofilme. Várias técnicas de microscopia óptica, de fluorescência confocal e eletrónica de transmissão mostraram a morfologia e ultra-estrutura dos biofilmes e permitiram aferir a eficácia antimicrobiana nos grupos de PDT com os vários fotossensibilizadores. Por microscopia eletrónica de transmissão, nos biofilmes expostos a NaOCl, foi possível perceber que ocorreu uma quase total destruição do biofilme, pois não se conseguiu biomassa suficiente para analisar.

Na segunda parte do trabalho experimental, avaliámos a citotoxicidade e comparámos o efeito antimicrobiano da clorofila modificada, Zn(II)e_6Me, na mesma concentração (0,1 mg/mL) com o Fotosan®, fotossensibilizador disponível comercialmente, e com o melhor irrigante clássico, 3% NaOCl quando aplicados a discos de dentina e blocos radiculares infectados com biofilmes mistos de 48 h de *E. faecalis* e *C. albicans*. As principais conclusões identificadas nos discos de dentina foram que a clorofila modificada quando irradiada por 90 s alcançou uma eficiência antimicrobiana de 59,13%, ultrapassada pelo NaOCl (68,12%). Contudo, quando o período de irradiação é alargado dez vezes, dos 90 para os 900 s, a eficácia de Zn(II)e_6Me aumenta para 70,88%, ao passo que o aumento do período de contacto directo nos agentes clássicos não potencia a sua eficácia antimicrobiana. Nos blocos radiculares, a clorofila modificada apresenta o melhor resultado antimicrobiano aos 90 s (79,74%) e aos 300 s (81,72%), seguido de perto por 90 s de NaOCl (75,5%). De salientar, que o padrão de desinfeção antimicrobiano é consistente nos grupos da clorofila modificada, o que não se verifica no exposto a NaOCl.

Nos testes de citotoxicidade, a clorofila modificada não induz qualquer citotoxicidade. Contrariamente, nas imagens de microscopia confocal, há um aparente reforço estrutural dos fibroblastos, quando comparado com o grupo controlo.

Como conclusão final deste trabalho pode afirmar-se que a clorofila modificada, por se ter revelado um fotossensibilizador com resultados estatisticamente semelhantes, em termos de eficácia antimicrobiana, ao que é considerado como o melhor método de assepsia do canal radicular, o hipoclorito de sódio a 3%, mas isenta de citotoxicidade, apresenta um elevado potencial para uma aplicação futura em Endodontia.

ABSTRACT

ABSTRACT

Root canal asepsis is one of the most important goals for a successful endodontic treatment, focused on microorganisms removal from the root canal system, aiming to maintain or re-establish the integrity of the periapical tissues. The endodontic treatment procedure comprises the establishment of an aseptic environment, biomechanical root canal preparation to restrain microorganisms, three-dimensional filling of the root canal system to resist potential microbial ingress, and a coronal restoration to prevent microleakage in the long-term.

Current techniques include the root canal system mechanical debridement and shaping with emphasis on various nickel-titanium (NiTi) rotary systems and endodontic irrigant solutions with sodium hypochlorite (NaOCl) considered the goldstandard because of its antibacterial and tissue dissolution properties. Nevertheless, numerous studies have verified that complete microorganisms eradication from the root canal system cannot be consistently achieved with none of the currently endodontic techniques.

In search of new methods to provide additional disinfection to the root canal system and presumably improve treatment outcome, novel techniques including PIPS (Photon Induced Photoacoustic Streaming) and photodynamic therapy (PDT), both standing out as the most promising.

PIPS is a revolutionary method for cleaning and debriding the root canal system using Er:YAG LASER energy at sub-ablative power levels, to create photoacoustic shock waves. Antimicrobial PDT (aPDT) is an approach that uses a nontoxic agent, photosensitizer (PS), that is selectively absorbed in a target tissue and when activated with a low-intensity light source, in the presence of oxygen, a series of reactions produce free radicals and singlet oxygen molecules leading to

microorganisms eradication with no microbial resistance induced and host damage.

aPDT has been a fast-growing approach at experimental studies with promising results and economically is more accessible to Endodontists when compared to PIPS.

In this study, the main goal was to assess the efficacy of aPDT with a modified chlorophyll, Zn(II)chlorin e6 methyl ester, Zn(II)e_6Me, extracted from chlorophyll a, as a new PS activated by red light versus endodontic classical irrigants.

In the first part of this experimental study, it was performed a screening to obtain the best antimicrobial agents. For this, Zn(II)e_6Me was compared with Rose Bengal (RB), Toluidine Blue-O (TBO), the synthetic tetracationic porphyrin (TMPyP) for 60 and 90 s all at the same concentration of 0.1 mg/mL, as well as classical endodontic irrigants (3% NaOCl, 17% EDTA and 2% CHX) for 60, 90 s and 30 min all applied to monospecies and mixed biofilms of *Enterococcus faecalis* and *Candida albicans*. The main conclusions obtained were that the direct contact time period of 90 s is sufficient to obtain optimal antimicrobial outcomes. It is not necessary to increase it up to 30 min in classical irrigants; for this reason this period was discarded in the following research.

Zn(II)e_6Me once activated with light for 60 or 90 s was able to remove around 60 % of the biofilm's biomass, similar to TMPyP and classical irrigants, CHX and EDTA. Zn(II)e_6Me was more efficient than TBO and RB. NaOCl revealed higher efficacy, with 70-90% of the biofilm's biomass removal. In the microscopy images (optical, confocal and transmission) it was possible to visualize the biofilms and to make a qualitative evaluation of the antimicrobial results with the respective images in the PDT groups. Nevertheless, in transmission microscopy, it was not possible to visualize the NaOCl group, because, due to the extensive destruction of cells, the biomass in the pellet was not enough to proceed.

In the second part of the experimental work, the Zn(II)e_6Me cytotoxic outcomes and antimicrobial efficacy were calculated and compared to FotoSan®, the commercially PS avaiable in the dentistry market as well the best classical irrigant solution, 3% NaOCl all applied to dentin discs and root blocks infected with 48h mixed biofilms of *E. faecallis* and *C. albicans*. The main conclusion at dentin discs identified were for the time period of 90 s, classical irrigant 3% NaOCl revealed the best antimicrobial outcome (68.12%), followed by Zn(II)e_6Me (59.13%). Nevertheless, after irradiation time of 900 s, Zn(II)e_6Me has a biofilm removal outcome of 70.88%. In root dentin blocks, Zn(II)e_6Me has the best antimicrobial approach at 90 s (79.74%) and 300 s (81.72%), followed by 3% NaOCl (75.5%) and the antisepsis pattern was consistent at inner and outer samples for aPDT group. No cytotoxic outcomes were detected for the modified chlorophyll, Zn(II)e_6Me, at 24 and 48 h and at confocal microscopy images it was observed an apparent structural reinforcement of fibroblasts cytoskeleton, when compared to the control group.

As a general conclusion, it can be claimed that the modified chlorophyll presented asepsis efficacy similar to 3% of sodium hypochlorite, but with no cytotoxicity, revealing potential future application in endodontics.

CONTENTS

CONTENTS

FIGURES AND TABLES LIST

FIGURES AND TABLES LIST

CHAPTER I

With no Figures and Tables.

CHAPTER II

CHAPTER III

CHAPTER IV

Figure 5: Scanning electron microscopy, Hitachi SU-70, micrograph of root blocks infected with 48h mixed biofilms.
Figure 6. hAPCs viability.
Figure 7. Microscopy of *in vitro* aPDT cytotoxicity in hAPCs.

Table 1. PSs effect in mixed biofilms biomass of *C. albicans* and *E. faecalis* incubated during 15 min in total absence of light.
Table 2. Luminance values for several substrates, all irradiated for 90s in total absence of natural light.

CHAPTER V

With no Figures and Tables.

CHAPTER VI

Figure 1. Different periods of exposing *E. faecalis* biofilm biomass removal (%) quantified using the safranin red (SR) assay.
Figure 2. Different periods of exposing *C. albicans* biofilm biomass removal (%) quantified using the SR assay.
Figure 3. Mixed biofilm biomass removal (%) using with SR assay after exposition to endodontic irrigant solutions at three direct contact periods.

PUBLICATIONS LIST

PUBLICATIONS LIST

The following papers were published in peer-reviewed international scientific journals during the development of this thesis:

1. Photodynamic antimicrobial chemotherapy for root canal system asepsis: a narrative literature review.

   ***International Journal of Dentistry*. (2015) Article ID 269205; doi: org/10.115/2015/269205**

2. Antimicrobial Photodynamic Therapy against endodontic *Enterococcus faecalis* and *Candida albicans* mono and mixed biofilms in the presence of Zn(II)e_6Me and other photosensitizers: a comparative study with classical endodontic irrigants.

   ***Frontiers in Microbiology*. (2017) 8: 498; doi: 10.3389/fmicb.2017.00498**

The following paper has been submitted and in under review for publication:

1. Is the chlorophyll derivate, Zn(II)e_6Me a good photosensitizer in root canal disinfection?

   International Endodontic Journal

ABREVIATIONS AND ACRONYMS LIST

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$^1O_2$ - Singlet oxygen
aPDT - Antimicrobial photodynamic therapy
ATCC - American Type Culture Collection
Au-Pd - Gold-palladium
BHI - Brain Heart Infusion
Ca^{2+} - Calcium ion
$Ca(OH)_2$ - Calcium hydroxide
CFU - Colony-forming unit
CHX - Chlorhexidine
ClO^- - Hypochlorite ion
DAPI - 4',6-diamidino-2-phenylindole
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
EDTA - Ethylenediaminetetraacetic acid
EPS - Extracellular polymeric substance
ER - Endoplasmic reticulum
FBS - Fetal bovine serum
GaAsP - Gallium arsenide phosphide
GaP - Gallium phosphide pure
GGD - Gates-Glidden Drill
GPI - Glycosylphosphatidylinisotol
hAPCs - Human apical papilla primary cells
HClO - Hypochlorous acid
LAD - Light activated disinfection
LAI - LASER activated irrigation
LASER - Light Amplification by Stimulated Emission of Radiation
LED - Light emitting diode
MB - Methylene Blue
NaOCl - Sodium hypochlorite
$NH2^-$ - Amino groups
$O_2^{-\cdot}$ - Superoxide
OmpA - Outer membrane protein A
PAI - Periapical index of Dag Ørstavik
PBS - Phosphate Buffered Saline
PDT - Photodynamic Therapy
PIPS - Photon-Induce Photoacoustic Streaming
PS - Photosensitizer
RB - Rose Bengal
RAD - Radiation
ROS - Reactive oxygen species
RPMI - Roswell Park Memorial Institute
SEM - Scanning electron microscopy
SOD - Superoxide dismutase
SR - Safranin Red
TBO - Toluidine Blue-O
TEM - Transmission electronic microscopy
TMPyP - Cationic synthetic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin
XTT - 2,3-Bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt
$Zn(II)e_6Me$ - Zn(II)chlorin e6 methyl ester

"If I have seen further than other it's

by standing upon the shoulders of Giants."

Isaac Newton

Chapter 1

BACKGROUND

BACKGROUND

In 1998, Marques *et al.* performed in Portugal a study on the prevalence of apical periodontitis, reporting an outcome of 27%. Before this study, diverse epidemiological studies on adult oral health were performed in Portugal, but none of them reported the prevalence of apical periodontitis, the root canal quality and endodontic treatment final prognosis. Marques *et al.* showed that the root filling quality of endodontic treatments majority was inadequate with 54% short and/or poorly condensed and only 22% of the endodontically treated teeth presented apical periodontitis (Marques *et al.* 1998).

In 2011, Diogo *et al.* analyzed the prevalence of apical periodontitis in an adult Portuguese population. From 856 patients, 157 were selected during the first consultations at the Dentistry Area of Faculty of Medicine of the University of Coimbra, between the 1st of January and the 30th of April with the following inclusion criteria: age over 18 years, panoramic radiography updated, more than 8 teeth in mouth and signed informed consent. The endodontic treated teeth were subject to periapical radiographs and evaluated with the Periapical index of Dag Ørstavik (PAI index) and quality of root canal filling. The prevalence of apical periodontitis in the sample was 4.4%. The prevalence of apical periodontitis in endodontically treated teeth was 29.6%, and when the root was the common denominator, the prevalence of apical periodontitis was 29.3 %, whereby the success rate was 70.7% for roots with endodontic treatment (Diogo *et al.* 2014).

Considering the global comparative analysis of these data and taking into account the doubling of endodontic treatment teeth prevalence in the sample, 3%, authors concluded that over 13 years there has been an improvement of oral health directories, revealed by increased adoption of conservative treatments not only by

patients, but also by health professionals, justifying the prevalence of apical periodontitis in 1998 sample of 27%, while the recent prevalence study is slightly higher, 29.3%. The prevalence of teeth with endodontic treatment of 31%, was lower than expected, compared with reference publications from countries with similar level of success rate, 70.4%, which may indicate the possibility of our dental care to evolve even more conservative therapies.

Some studies confirmed that endodontic treatment prognosis is affected by the progression of the disease process (Ng *et al.* 2011) and by the fact that it is performed endodontic treatment or retreatment (Wang *et al.* 2004). A follow up study of 4-6 years of initial endodontic treatment disclosed that the presence of periapical infection at the time of treatment reduced the healing rate from 92 to 74% (Friedman *et al.* 2003).

The main goal of endodontic treatment is the complete eradication of microorganisms, prevention of reinfection of treated root canal system and perpetuation of structural integrity of dentin for a successful long-term treatment outcome (Siqueira Jr *et al.* 2000). However, complete abolition of microbiota from the root canals (Nair 2006) and maintaining the structural integrity of dentin in endodontically treated teeth has been an overwhelming mission in endodontics field (George *et al.* 2005).

Approximately 60% of human infections are associated with bacterial-biofilms (Chavéz de Paz 2007). Diversified unrestricted microorganisms, especially bacteria (facultative anaerobes, obligatory anaerobes, Gram-positive and Gram-negative bacteria), existing as biofilms inside the root canal system are the main cause of endodontic disease (Ricucci and Siqueira Jr 2010). As a consequence, the incomplete microorganisms eradication has been attributed to the polymicrobial or mixed endodontic biofilms and root canal anatomy complexities (Nair 2006).

The root canal system is a sterile place, which does not comprehend a regular microbiota in healthy teeth, therefore, to each microbial species discovered in infected root canals it can be attributed an etiological role as endodontic pathogen, also playing the additional role in developing the ecology niche capable of causing endodontic disease. Endodontic infection of both primary and persistent nature demonstrated microbial colonization as typical biofilms in the apical portions of root canal system. The morphology of these microbial communities is consistent with the accepted criteria to include endodontic diseases in the biofilm-mediated diseases category (Ricucci and Siqueira Jr 2010). All infected root canals are conventionally managed using classical chemical irrigant solutions, such as sodium hypochlorite (NaOCl), chlorhexidine (CHX) and ethylenediaminetetraacetic acid (EDTA) combined with biomechanical instrumentation to achieve effective asepsis prior to filling them with an inert filling material.

Occasionally, treatment-resistant strains, especially facultative anaerobe *Enterococcus faecalis* (Stuart *et al.* 2006; Iqbal *et al.* 2013) and *Candida albicans* (Waltimo *et al.* 1999; Lopes *et al.* 2014) become dominant and difficult to eradicate from the root canal system. Chemomechanical cleaning and shaping of the root canal effectively reduces microbial statistics. Nevertheless, most frequently, total disinfection cannot be accomplished, compromising the final endodontic outcome and consequent prognosis. It has been proved that covering the canal with intracanal medication between successive sessions can further reduce residual microorganisms and calcium hydroxide ($Ca(OH)_2$) (Peters *et al.* 2002; Upadya *et al.* 2011) is commonly used for this purpose. Nonetheless, specific microbes, such as *E. faecalis* (Du *et al.* 2014) and *C. albicans* (Peciuliene *et al.* 2001), mentioned above, remain resistant to $Ca(OH)_2$.

Another significant issue in endodontic treatment is that the chemical agents need to be selective in eliminating microbial biofilms while sparing the adjacent host

mammalian cells, allowing targeted antibacterial activity without cytotoxicity (Ok *et al.* 2015). Thus the two major challenges in the management of infected dental hard tissue are (i) efficient endodontic microbial biofilms removal from the root canal system (Lewis 2001) and (ii) repair of disease-mediated hard tissue changes (Gilbert *et al.* 2010).

Advanced approaches in endodontic irrigation, as LASER activated irrigation (LAI), should lead to improved endodontic treatment outcomes. Currently there is no technique in endodontics that promotes total anti-biofilm removal efficacy (Shrestha *et al.* 2014) and simultaneously do not affect negatively the ultrastructural integrity of root dentin tissue in infected teeth.

AIMS OF THE PRESENT DISSERTATION

In the last 20 years in biomedicine, researchers have been studying Photodynamic Therapy (PDT) as an antimicrobial strategy that combines the use of a nontoxic photosensitizer (PS) activated by a low energy light source in oxygen presence. It results in the production of highly reactive free radicals, such as singlet oxygen species (Lim *et al.* 2009), which are highly reactive and known to target various microbial cells spots such as cell wall, nucleic acids as well as membrane proteins. Antimicrobial PDT (aPDT) has been applied to dentistry (Gursoy *et al.* 2013), and more recently has received strong interest to ameliorate root canal disinfection (Muhammad *et al.* 2014), not only with the application of nanoparticles (Shrestha *et al.* 2014), but also searching for new endodontic PSs to improve the final endodontic asepsis outcomes without teeth discoloration detectable by human eye.

Therefore, the leading objective of this dissertation is to analyse aPDT efficacy versus classical endodontic irrigant solutions such as 3% NaOCl, 2% CHX

and 17% EDTA in an *in vitro* and *ex vivo* experimental model of mono- and mixed endodontic biofilms.

OUTLINE OF THE THESIS

The present dissertation is divided in six chapters and outcomes are shown as data compiled in submitted or under manuscripts preparation.

CHAPTER I dues to the background, investigation purpose and the main origin of the presented work.

CHAPTER II introduces the state of the art related to the introductory aspects of the thesis, reviewing the most important and relevant achievements on the field and allowing a global comprehension of the dissertation.

CHAPTER III corresponds to the characterization of Zn(II)chlorin e6 methyl ester (Zn(II)e_6Me) in aPDT efficacy against *in vitro* endodontic *Enterococcus faecalis* and *Candida albicans* mono and mixed biofilms.

CHAPTER IV deals with aPDT with 0.1 mg/mL of a modified chlorophyll, Zn(II)chlorin e6 methyl ester (Zn(II)e_6Me) applied to dentin discs and root blocks infected with endodontic mixed biofilms and its cytotoxic outcomes when applied to a human apical papilla primary cells lines.

CHAPTER V corresponds to conclusions and future perspectives and is named as final remarks.

CHAPTER VI was introduced as supplementary results since it is a pilot study that allowed establishing the minimum direct contact period with antimicrobial efficacy needed to incubate biofilms with classical irrigants.

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Chapter II

INTRODUCTION

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INTRODUCTION

Apical periodontitis is an inflammatory reaction of perirradicular tissues caused by a microbial biofilm in the root canal system (Kakehashi *et al.* 1966). A microbial biofilm (**Table 1**) is considered a community that congregates four basic benchmarks, such as autopoiesis, homeostasis, synergy and communality (Caldwell *et al.* 1997) and dental plaque is the typical biofilm example (Fontana *et al.* 2009).

Table 1. Biofilm conceptions - a chronological resume (1976-2014).

AUTHOR	BIOFILM DEFINITION
(Marshall 1976)	The involvement of very fine extracellular polymer fibrils that anchored bacteria to surfaces.
(Costerton *et al.* 1978)	Communities of attached bacteria in aquatic systems enclosed in a glycocalyx matrix.
(Bakke *et al.* 1984)	Biologically active matrix of cells and extra-cellular substances in association with a solid surface.
(Costerton *et al.* 1987)	Matrix-enclosed communities of microorganisms tightly interacting with each other and, in most cases, supported by an abiotic surface.
(Costerton *et al.* 1995)	Microorganisms adhere to surfaces and interfaces and to each other, including microbial aggregates and floccules and adherent populations within pore spaces of porous media.
(Costerton 1995)	Adhesion trigger expression of genes controlling production of bacterial components necessary for adhesion and biofilm formation. Specific genes transcribed during initial cell attachment regulate the process of biofilm formation.
(Costerton *et al.* 1999)	Cells immobilized at a substratum and frequently surrounded by an organic polymer matrix of microbial origin.
(Costerton 1999)	Bacterial cells within the matrix are characterised by their lack of Brownian motion and careful structural analysis of many micro-colonies often reveals a mushroom-like shape.
(Davey & O'toole 2000)	Accumulations of microorganisms within a complex matrix that adhere to a surface, they are closely linked with the aetiology of diverse chronic and recurrent human infections.
(Shirtliff *et al.* 2002)	Microbially derived sessile community characterised by cells that attach to an interface, embedded in a matrix of exo-polysaccharide, with an altered phenotype.
(Donlan and Costerton 2002)	Cells irreversibly attached to a surface or interface, embedded in a matrix of extracellular polymeric substances which these cells have produced, and including the noncellular or abiotic components, but also other physiological attributes of these organisms, including such characteristics as altered growth rate and the fact that biofilm organisms transcribe genes that planktonic organisms do not.
(Donlan 2002)	Microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription.
(Allison 2003)	Mixed communities of microorganisms and the products of their metabolism.
(Chávez de Paz 2007)	Multilayers of dense aggregates.
(Pierce *et al.* 2008)	Surface-associated and highly structured community of microorganisms that are enclosed within a protective extracellular matrix.
(Chávez de Paz *et al.* 2010)	Microorganisms organized in structures attached to each other or the root canal walls often involving a multitude of species.
(Hancock *et al.* 2011)	Microbial interaction at the substrate surface. The formation includes several steps, namely bacterial attachment, formation of microcolonies and microbial growth.
(Barnes & Patel 2011)	Complex communities of microbes embedded in a matrix.
(Mohammadi *et al.* 2014)	Attachment of bacteria to a surface, followed by the formation of a bacterial community characterized by cells that are firmly attached to a surface and enmeshed in a complex extracellular matrix.

Biofilm resistance mechanisms are synonymous of extracellular polymeric substance (EPS) matrix diffusion barrier, physiological state of bacteria with slower

growth relative to planktonic cells, altered microenvironment (Gilbert *et al.* 1997), changes in phenotype with differential expression of genes/proteins, (Hall-Stoodley *et al.* 2004) and horizontal genes transfer (Roberts and Mullany 2010) resumed in **Table 2**.

Table 2. Biofilm resistance mechanism and functions.

MECHANISM	FUNCTIONS
EPS matrix diffusion barrier	Improves the biofilm resistance to antimicrobials as a physical barrier and neutralizing the antimicrobial chemicals applied.
Physiological state of bacteria	Persister cells are a small population of dorman cells that contribute to antibiotic tolerance.
Altered microenvironment and phenotype	Generates a gradient of nutrients and redox potential that offers to surface bacteria the benefit of higher growth rates.
Horizontal gene transfer	Horizontal transfers of DNA, stimulated by intercellular signalling molecules, improve antibiotic resistance and biofilms development.

As microbial biofilms are considered the major cause for primary and secondary root canal infection, endodontic treatment procedures focus on microorganisms removal from the root canal system and endodontic treatment success relies on the effective biofilms eradication (Nair 2006) aiming to maintain or re-establish the periapical tissues integrity.

Endodontic treatment procedures comprises the establishment of an aseptic environment acquired with biomechanical root canal preparation, three-dimensional filling of the root canal system to resist potential microbial ingress and a coronal restoration to prevent microbial ingress in the long-term. Nevertheless, even when all therapeutic procedures are meticulously applied, in the long term, microorganisms can eventually invade the root canal via coronal access cavity, lateral canals and dentinal tubules (Santos *et al.* 2014). Once the root canal system is invaded, microorganisms can propagate through the filled canal and interact with the host's periapical tissues. This interaction results in the development of post-treatment apical periodontitis, thereby negatively affecting the outcome of endodontic treatment. It can be generally stated that microbial biofilms are essential for the development of

apical periodontitis (Kakehashi *et al.* 1966) and advanced endodontic treatment is focused on the elimination of microbial biofilms from the infected root canal system before obturation.

Biomechanical instrumentation and antimicrobial irrigation with sodium hypochlorite solution reduce near 50% of canals microorganisms-free, however the remaining canals contain small numbers of recoverable bacteria (Bystrom and Sundqvist 1985). When the biomechanical preparation is combined with an antimicrobial dressing applied to the clean canal for a suitable interval of time before root filling, microorganisms (common bacteria) can be consistently eliminated from the canal (Sjögren *et al.* 1991). Still, when endodontic treatment is performed in one visit, with no inter-appointment antimicrobial dressing, residual microorganisms may be present in the canal at the time of root filling. Only a few classical studies have evaluated the effect of infection at the time of root filling on the endodontic treatment prognosis and they have shown that the success rate of endodontic treatment is approximately 10-26% lower for teeth which yield a positive culture before obturation than for teeth which yield a negative culture (Engstrom and Frostell 1952; Sjögren *et al.* 1997).

When advanced methods for cultivating anaerobic bacteria were used to study the root canal microbiota, obligate anaerobic bacteria proved to form a high proportion of the total microbial load in infected root canals. Most of the bacteria isolated from infected root canals are oxygen sensitive and hardily can be cultivated using conventional bacteriological methods (Gambarini *et al.* 2011). In endodontic microbiological studies that have evaluated the influence of infection on the final outcome of the endodontic treatment, bacteriological techniques that were used were unfavourable for the recovery of anaerobic bacteria (Bonsor *et al.* 2006a). Therefore, microorganisms presence might have been important for the outcome of treatment

missed and cases that seemingly contained no bacteria could in fact have harboured persisting microorganisms. Another important factor in determining the outcome of endodontic therapy is the use of a large observation period after the treatment has been completed.

The periapical tissues recovery to a healthy condition is a dynamic process and it is possible that a premature evaluation of periapical healing might include teeth in which the repair process has not yet been stabilized. The majority of periapical lesions heal within 4-5 years after endodontic treatment, therefore it is considered desirable to have a follow-up period of at least 4 years to perform an adequate evaluation of the prognosis (Strindberg 1956; Bystrom and Sundqvist 1985). When using Strindberg's criteria for outcome assessment of 4 to 5 years after root canal treatment, the conventional root canal treatment procedures support healthy periapical tissues in 95% of teeth undergoing biopulpectomy and in approximately 74% of teeth with preoperative apical periodontitis. Following this results, Sjögren *et al.* reveal that the success rate of teeth filled with a negative culture before root canal obturation was 94%, which was statistically significantly higher than the success rate of 68% for teeth with a positive culture at the time of root filling (Sjögren *et al.* 1997).

Current treatment strategies are insufficient to reduce microorganisms inside root canals below detection limits before permanent root canal filling, which is required in order to achieve optimal healing conditions for the periapical tissues (Sjögren *et al.* 1997). It is mandatory to search for new methods and advanced disinfection approaches to provide optimal disinfection of the root canal system without host cytotoxic effects (Santos 2008). This achievement will lead to a clear improvement of the endodontic treatment prognosis.

MINIMALLY INVASIVE LASER ACTIVATED IRRIGATION

Minimally invasive LASER activated irrigation have been proposed in literature for efficient root canal disinfection and it is divided between Photon-Induced Photoacoustic Streaming (PIPS) (DiVito *et al.* 2012) and Photodynamic Therapy (PDT) (Seal *et al.* 2002).

PIPS were developed by Dr. Enrico DiVito along with his research group in Arizona School of Dentistry and Oral Health, USA, using an erbium LASER (Er:YAG), at 2940 nm of wavelength at sub-ablative power levels with a stationary hand piece at the teeth orifice with no intracanal fiber or a endo tip inserted to the root canal coronal third. The endodontic irrigant solutions were activated through profound photoacoustic photomechanical generated shockwaves. A faster streaming of fluids, a turbulent flow phenomenon distant from the source, in magnitudes three-fold greater than the passive ultrasonic irrigation (Pedullà *et al.* 2012). The combination of low-energy and a turbulent flow strongly contributes to the elimination of endodontic biofilms with the highest outcomes (Koch *et al.* 2016), but this methodology presents very high costs not sustainable for the majority of dentists. Otherwise, PDT was discovered by chance at the very beginning of the twentieth century, when a combination of nontoxic dye exposed to visible light resulted in microorganism cell death and John Toth renamed this therapy as PDT. Combined effect of three elements – light and photosensitizer in oxygen presence – has been termed photodynamic antimicrobial chemotherapy by Wainwright (Wainwright 1998) also recognized as photo-activated disinfection (Bonsor *et al.* 2006b) and antimicrobial photodynamic therapy (aPDT) (Garcez *et al.* 2007).

PHOTODYNAMIC THERAPY MECHANISM

PDT uses a non-toxic dye, known as photosensitizer (PS) on a target tissue, which is consequently irradiated with a suitable visible light of the appropriate, wavelength to excite PS molecule to the singlet state, in presence of oxygen, to produce reactive oxygen species (ROS) (Hamblin and Hasan 2004). When a basal state PS absorbs light that excites it to the singlet state, it can release energy by fluorescence or can undergo intersystem crossing to long-lived PS triplet state that can carry out photochemistry or lose its energy be phosphorescence. This excited state may undergo to the slightly lower energy, while longer lived, triple state can undergo for two kinds of pathways known as Type 1 and Type 2 pathways. Both photoprocesses lead to local ROS production such as superoxide ($O_2^{\cdot-}$) in Type 1 reaction (with the substrate) and singlet oxygen ($^1O_2$) in Type 2 reaction (counters with molecular oxygen). Both photoprocesses require the presence of oxygen and are cytotoxic to microbial cells with no damage to the mammalian host cells, **Figure 1**.

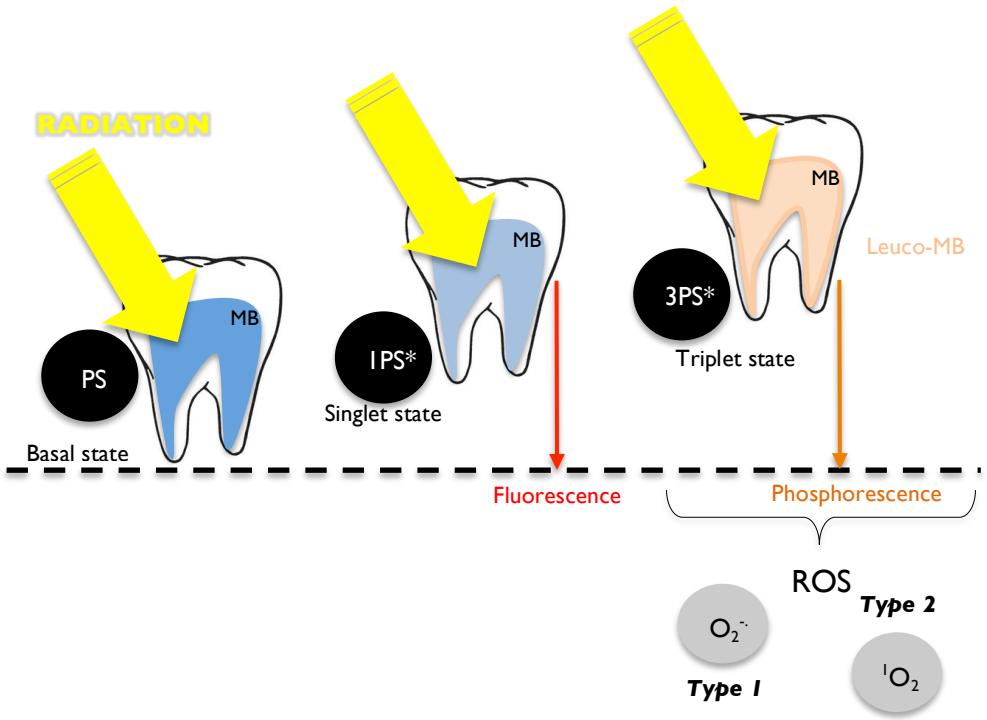

Figure 1. Photodynamic therapy operative mechanism. Schematic representation adapted from Kharkwal *et al.* 2011.

The type 1 – radical and ROS pathway – comprises an electron transfer step between the triplet PS and a substrate, with ROS generation, then the finalist is intercepted by ground state molecular oxygen yielding a variety of oxidized products. The PS at basal state (baseline) has two electrons in opposite spins in the low energy molecular orbital. Subsequent to the absorption of light, one of these electrons is boosted into a high-energy orbital but keeps its spin (first excited singlet state). This is a short-lived time species with nanoseconds of lifetime, and can escape its energy by emitting light (fluorescence) or by internal conversion into heat. Type 1 pathway frequently involves initial production of superoxide anion by electron transfer from the triplet PS to molecular oxygen (monovalent reduction) initiating radical-induced damage in biomolecules. Superoxide is not particularly reactive in biological systems and does not cause abundant oxidative damage by itself, but it can react and produce hydrogen peroxide and oxygen, a reaction known as dismutation that can be catalysed by the enzyme superoxide dismutase (SOD). The way of the electron relocation between the PS and the substrate is controlled by the relative redox potentials of the two species.

Type 2 pathways – singlet oxygen – involves an electronic energy transfer process from the triplet PS to a receptor, most frequently oxygen, which is a triplet in its ground state. The final compound is converted to a highly reactive species, the singlet oxygen ($^1O_2$). The excited singlet state PS may also undergo the process known as intersystem crossing, whereby the spin of the excited electron inverts to form the relatively long-lived, in terms of microseconds, excited triplet-state that has parallel electron spins. The long lifetime of the PS triplet state is explained by the fact that the loss of energy by emission of light (phosphorescence) is a spin forbidden process, as the PS would move directly from a triplet to a singlet-state.

Photoprocesses of type 1 and 2 depend on the initial involvement of radical intermediates that are subsequently scavenged by oxygen or the generation of the highly cytotoxic singlet oxygen by energy transfer from the photoexcited sensitizer. It is difficult to determine which of these two mechanisms is more prevalent; both types of reactions can happen simultaneously and the ratio between them depends on three singular features: oxygen available, substrate concentration and PS type (Castano *et al.* 2004).

PHOTODYNAMIC THERAPY TARGETS

Hamblin and Hasan in 2004 stated that antimicrobial PSs can be divided into three categories: (I) PSs that strongly bind and penetrate the microorganisms (ex. chlorine6), (II) those that bind weakly as Toluidine Blue-O (TBO) and Methylene Blue (MB) and (III) those that do not demonstrate binding at all such as Rose Bengal (RB) (Hamblin and Hasan 2004). It is essential to understand these principles and PDT mechanisms because in bacterial cells, outer membrane damage plays an imperative role, differently from mammalian cells, where the main targets for PDT are lysosomes, mitochondria and plasma membranes (Trindade *et al.* 2015). Typically, neutral anionic or cationic PS molecules could powerfully destroy Gram-positive bacteria, whereas only cationic PS attack the Gram-negative permeability barrier in combination with non-cationic PS (Dai *et al.* 2009). This difference in susceptibility between Gram positive and Gram-negative bacteria is explained in part by their cell wall composition. In Gram-positive bacteria, the cytoplasmic membrane is surround by peptidoglycan layer and lipotechoic acid, **Figure 2**. Different from this, the Gram-negative bacteria cell envelopes consist of lipid inner and an outer membrane, separated by a peptidoglycan layer, **Figure 3**. The outer membrane forms an

effective permeability barrier between the cell and environment. It tends to restrict the binding and penetration of several PS.

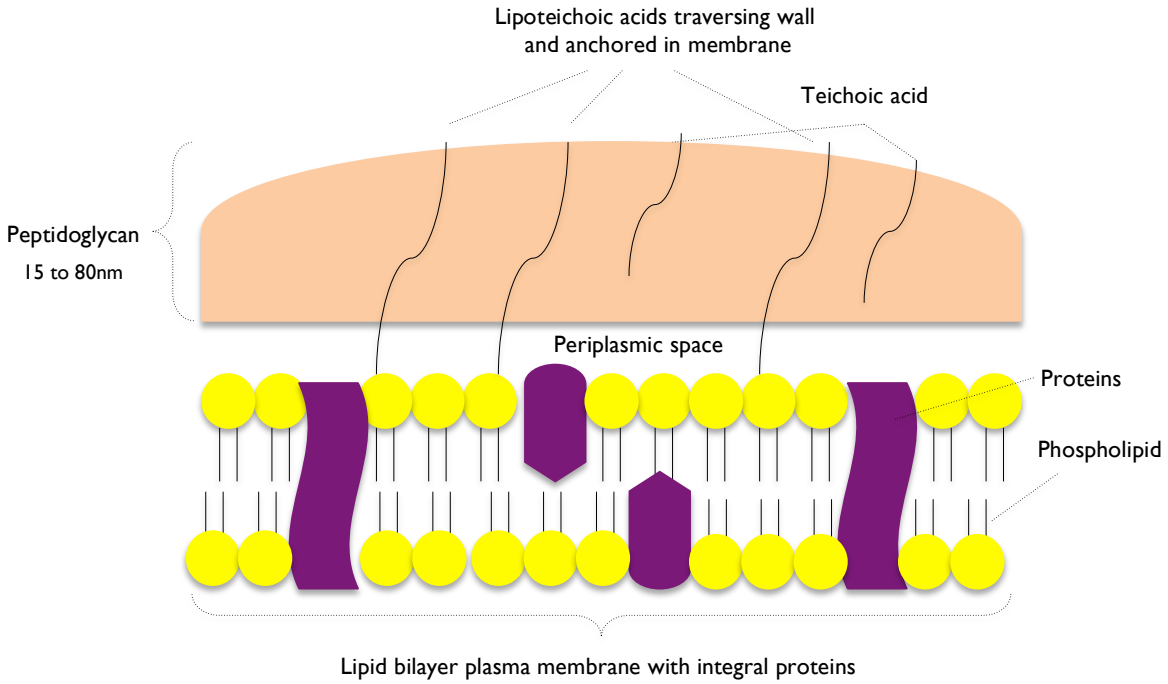

Figure 2. The cellular membrane and cell wall of Gram-positive bacteria (schematic representation).

Fungi are provided with a thick cell wall that includes glucans, mannoproteins and chitin offering among others, a permeability barrier. In terms of aPDT efficacy, fungal cell wall (**Figure 4**), was described as having an intermediate behaviour between Gram-positive and Gram-negative bacteria (Minnock *et al.* 2000).

On the basis of these considerations and from these three groups of microorganisms ultrastructural cell walls described above, it appears that Gram-negative bacteria represent the most challenging target for aPDT. These findings are relevant, because photoaction occurs firstly in direct contact with membranes and secondly by penetrating or binding at the membrane. The early attack of singlet oxygen in membranes lipids is by the specific reaction with double bonds to form allylic hydroperoxides.

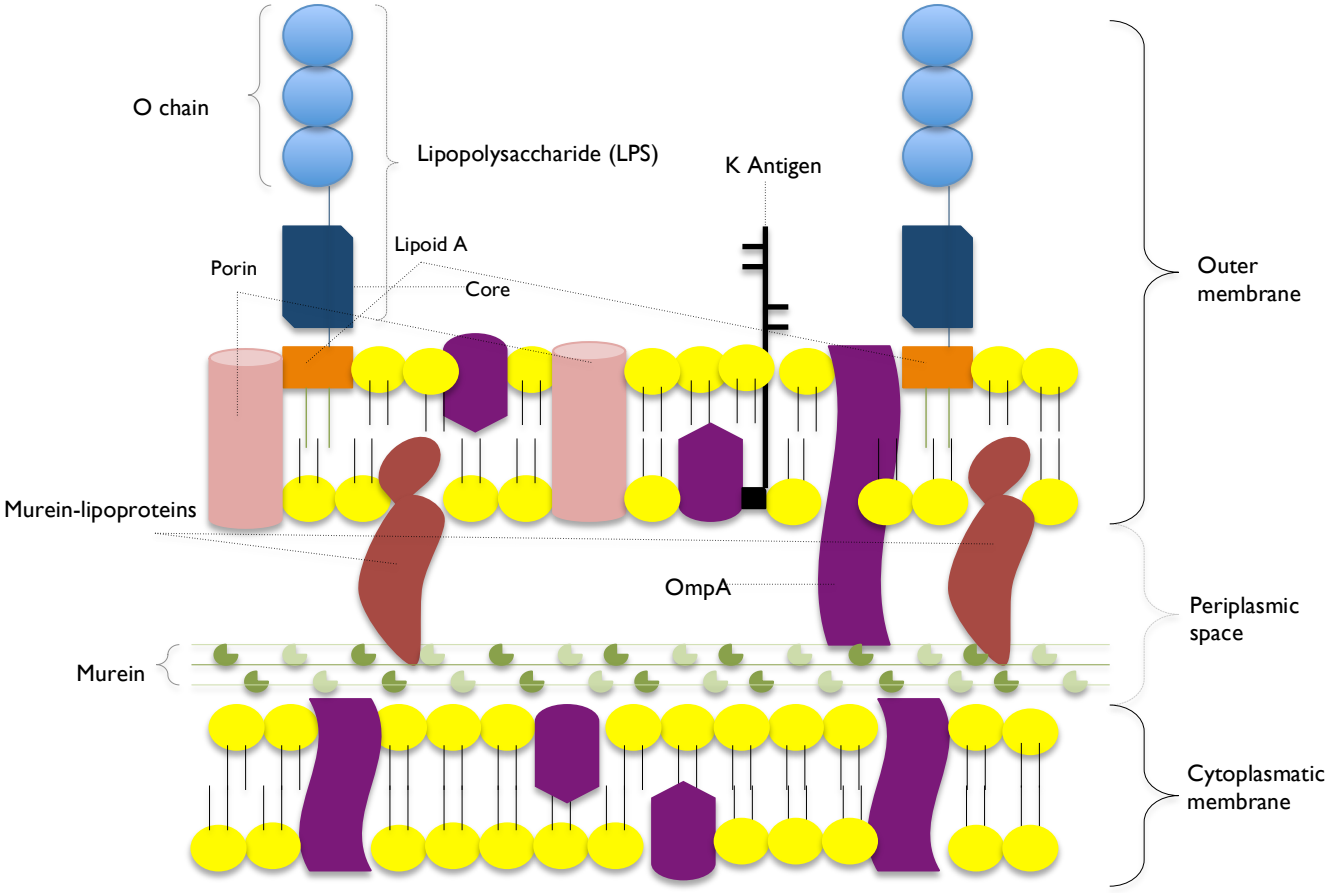

Figure 3. The cellular membrane and cell wall of Gram-negative (schematic representation).

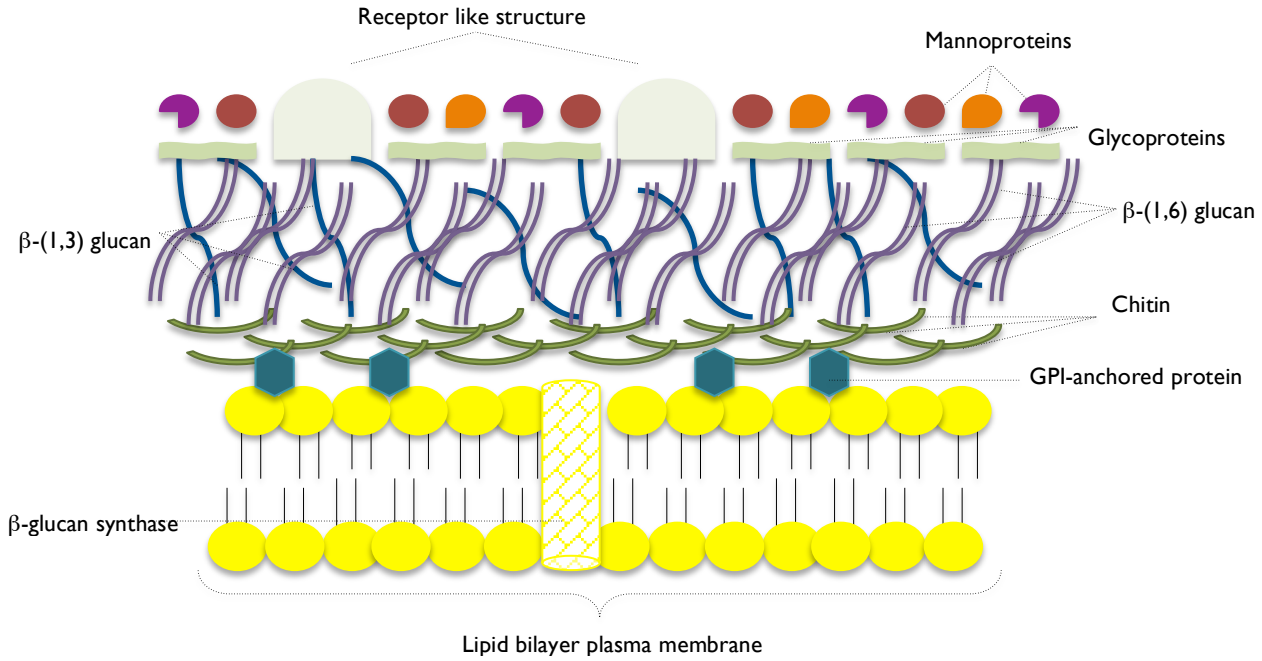

Figure 4. The cellular membrane and cell wall of fungal cell (schematic representation).

Photodynamic lipid peroxidation is an oxidative degradation of cell membrane lipids, also known as photoperoxidation and it has been related to several microbial cytotoxic effects, such as increased ion permeability, fluidity loss, inactivation of membrane proteins and cross-linking, which disrupts the intracellular homeostasis (Tardivo *et al.* 2005). Consequently, necrosis is induced as cell death process. A probable explanation is that PS bounds to the membrane, generates most of the singlet oxygen ($^1O_2$) involved in photoperoxidation (Kim *et al.* 2015), highlighting the double selectivity (light and PS cellular localization) and the fact that it works in multi-resistant strains and does not encourage microbial resistance.

PDT lethal action is based on photochemical production of ROS and not on thermal and cavitation effects as high power LASER therapy (Dougherty *et al.* 1998). One of several clinical PDT advantages is the absence of thermal side effects in periradicular tissues and this PDT property makes it highly effective in eradicating microorganisms such as bacteria (Soukos *et al.* 2006), fungi (Bliss *et al.* 2006) and viruses (Cassidy *et al.* 2009) from root canal system without causing damage of adjacent tissues due to overheating. In summary, PDT is a recent approach, but with more than twenty years of experimental level, with high potentially and proved significant antimicrobial properties in endodontic disinfection protocols (Chrepa *et al.* 2014).

While the oral applications of PDT have been extensively tested, variations in study type and design limits the ability to synthesize or pool the available quantitative data, not allowing a formal meta-analysis and a high level of evidence systematic review. Throughout the scientific literature there are several forms to present the quantification of biofilm removal by aPDT. This turns difficult the task to compare results among studies. Considering this apparent contradiction in reporting results among the studies analysed, it is difficult to provide a definitive assessment of the

research question posed. It is important to mention that aPDT efficacy is shown in colony-forming unit (CFU) or in percentage and logarithm (in form of log_{10}). Nonetheless, the authors state this is pointless without the perception of the initial biofilm concentration. As an example, if we have an initial sample from a root canal of 10^7 microorganisms and after aPDT approach we had 10^5, statistically, 99% was killed, but there are still 100.000 microorganisms left inside the root canal. Considering the variation in units at outcomes, it is difficult to analyse and compare the final outcomes.

Even though PDT has significant advantages, potential adverse events as tooth discoloration have been reported previously in root canal treatment when MB and TBO were used as PSs (Figueiredo *et al.* 2014). It is also important to mention that future clinical studies clearly reporting possible adverse events associated with PDT, clearly will be determinant to estimate the benefit-to-risk ratio. PDT outcomes in literature have been reported by the dual combination of PS and a visible light source in the presence of oxygen. However, recently Sousa *et al.* analyzed that twice-daily blue light of 420 nm, energy density of 72 Jcm^{-2} applied for 776 s without PS is a promising approach in the inhibition of five days *Streptococcus mutans* matrix-rich biofilm development (Sousa *et al.* 2015). It has remarkably inhibited the production of EPS-insoluble, which is responsible for the scaffold of the extracellular biofilm matrix. The authors suggest that this evidence is very important to improve standardization in PDT procedures in total absence of light as the evaluation of PS dark toxicity is also an important issue to considerer. The role of PDT in root canal disinfection has been tested using several combinations of PS and light sources and has shown divergent outcomes and studies revealed several limitations associated with aPDT. For a successful aPDT, a PS is required that will show enough affinity for microorganisms without catalyzing photodamage to host tissues, a light source at

a wavelength that can penetrate tissues (630-700 nm) and sufficient oxygenation to produce a level of ROS necessary to induce photodynamic lipid peroxidation and cell death by necrosis. If there is a photodamage to both tissues and microorganisms, efficacy will be sub-optimal.

Microorganisms in the root canal and their growth mode were found to influence their susceptibility to aPDT in a dose-dependent manner (Upadya and Kishen 2010) and biofilms can be difficult to eradicate not only because of their effect as barriers to PS uptake, but also their ability to diffuse or attenuate light in the root canal dentinal tubules. Even dentin, dentin matrix, pulp tissue, bacterial lipopolysaccharides and bovine serum albumin were found to significantly decrease aPDT efficacy (Shrestha *et al.* 2012) and as a consequence, an effort to enhance the aPDT by nanoparticle-based technology appears to be promising (Pagonis *et al.* 2010). Other strategies include the use of a PS solvent (deionized water, PBS buffer, BHI broth) (George and Kishen 2008), efflux pump inhibitors (Upadya and Kishen 2010) or photoactivated functionalized chitosan nanoparticles for disinfection and dentin matrix stabilization (Shrestha *et al.* 2013).

aPDT application for additional reduction of the microbial load of root canal systems seems to be promising. However, it would be beneficial to identify the ideal combination of PS and light wavelength in pre-clinical studies and conduct future randomized controlled trials to test the clinical efficacy of aPDT on root canal disinfection in *in vivo* studies.

ENDODONTIC MICROORGANISMS

When teeth are submitted to endodontic treatment under aseptic clinical conditions, the success rate reports are generally high, from 85 to 90% (Bystrom and Sundqvist 1985; Haapasalo 1989; Sundqvist *et al.* 1998; Elemam and Pretty 2011; Su *et al.* 2011; Eyuboglu *et al.* 2017). The majority of endodontic treatment failures are caused by technical problems occurred during the treatment (Sjögren *et al.* 1997). However, some clinical cases fail even when apparently well treated and a specific number of hosts factors have been identified as associated causes of endodontic treatment failure, including extraradicular infection, true cysts and extruded filling materials cause foreign-body reactions (Siqueira Jr 2001; Abbott 2002). Still, most treatment failures are caused by persistent microorganisms in the apical parts of obturated teeth root canals (Waltimo *et al.* 2005).

In 1998, two studies revealed that primary endodontic infections associated microbes are distinctive from teeth with no previous endodontic treatment. These are characterized by a smaller number of species present in each canal, Gram-positive bacteria predominance with an identical distribution between the facultative and the obligate anaerobes (Molander *et al.* 1998; Sundqvist *et al.* 1998). Microorganisms initially present in the root canal vary greatly in what regards their susceptibility to the therapeutic procedures, namely to the instrumentation, irrigation and intracanalar medication with antiseptics (Basrani *et al.* 2003).

Among the microbial genus associated with persistent infections, the most frequently isolated are *Enterococcus*, *Streptococcus*, *Actinomyces* (facultative anaerobic) and *Peptostreptococcus* (obligate anaerobe). *Enterococcus faecalis* and *Candida albicans* are two species that deserve special attention as opportunistic pathogens, with high

prevalence in persistent infections when compared to primary infections (Roças *et al.* 2004a).

Enterococcus faecallis

Enterococci are Gram-positive facultative anaerobic bacteria that can occur singly, in pairs or as short chains; catabolize several energy sources including glycerol, carbohydrates, citrate, arginine, agmatine, lactate, malate and many α-keto acids (Siqueira Jr *et al.* 1997). Enterococci survive in austere environments including extreme alkaline pH (9.6) and high salt concentrations, can grow between 10 to 45°C and survive a temperature of 60°C for 30 min, resist bile salts, detergents, heavy metals, ethanol, azide and desiccation (Stuart *et al.* 2006).

There are currently 23 Enterococci species divided into five groups based on their interaction with mannitol, sorbose and arginine. *Enterococcus faecalis* belongs to the same group as *E. faecium, E. casseliflavus, E. mundtii* and *E. gallinarum.* In general these five species form acid in mannitol broth and hydrolyse arginine, but fail to form acid in sorbose broth (Sedgley *et al.* 2004). Like other species in the *Enterococcus* genus, *E. faecalis* can cause life-threatening infections in humans, especially in the nosocomial environment, mainly due to their high levels of antibiotic resistance (Roças *et al.* 2004b).

The prevalence of *E. faecalis* is 11% at oral rinse samples from patients receiving initial endodontic treatment, midway through treatment and patients undergoing endodontic retreatment, when compared to 1% of patiens with no endodontic treatment history (Sedgley *et al.* 2005; Stuart *et al.* 2006).

E. faecalis is associated with several forms of periradicular disease including primary endodontic infections and persistent infections (Roças *et al.* 2004a). In the

primary endodontic infections category, *E. faecalis* is observed in 5% of asymptomatic chronic periradicular lesions (Siqueira Jr *et al.* 2000; 2010) significantly more often than acute periradicular periodontitis or acute periradicular abscesses. *E. faecalis* is found in 4 to 40% of primary endodontic infections and the prevalence of *E. faecalis* in persistent periradicular lesions has been shown to be much higher. In fact, failed root canal treatment cases are nine times more likely to contain *E. faecalis* than primary endodontic infections (Roças *et al.* 2004a). In detail, *E. faecalis* plays a major role in the etiology of persistent periradicular lesions after root canal treatment and in some studies its prevalence in such infections ranges from 12 to 77% (Hancock *et al.* 2001; Peciuliene *et al.* 2001; Pinheiro *et al.* 2004; Kaufman *et al.* 2005; Stuart *et al.* 2006), others repeatedly found *E. faecalis* in root canal treated teeth with prevalence values ranging from 30 to 90% of the cases. Is generally believed that the major cause of failure is the survival of microorganisms in the apical portion of the root-filled tooth as a consequence of fluid leakage via gaps at the restoration-tooth interface following coronal leakage (Chávez De Paz *et al.* 2003; Santos *et al.* 2014)

Unlike primary endodontic infections, which are polymicrobial in nature and dominated by Gram-negative anaerobic rods, the microorganisms involved in secondary infections are composed of one or a few bacterial species (Stuart *et al.* 2006). *E. faecalis* is not limited to its possession of various virulence factors (Facklam *et al.* 2002). These factors may or may not contribute to the innate characteristics of *E. faecalis* to cause disease, because *E. faecalis* relies on its ability to survive and persist within the root canal system in several ways as a result of genetic polymorphism (Haapasalo *et al.* 2003; Hubble *et al.* 2003). It has the capability to endure prolonged periods of starvation until an appropriate nutritional supply becomes available (Figdor *et al.* 2003). Once accessible, the starved cells are able to recover by utilizing serum as a nutritional source original from alveolar bone and periodontal ligament

who also helps *E. faecalis* binding to type I collagen (Love 2004). *E. faecalis* inside dentinal tubules has been shown to resist intracanal dressings of calcium hydroxide for over ten days (Orstavik and Haapasalo 1990) especially when a high pH is not maintained (Tronstad *et al.* 1981; Lin *et al.* 2003; McHugh *et al.* 2004). *E. faecalis* is able to form a biofilm that helps it resist destruction by enabling the bacteria to become 1,000 times more resistant to phagocytosis, antibodies and antimicrobials than non-biofilm producing organisms (Stuart *et al.* 2006).

E. faecalis ability to attach to dentin, invade dentinal tubules and form biofilm communities contributes to its resistance to endodontic classical irrigant solutions and intracanal medicaments (Du *et al.* 2014). *E. faecalis* is known to colonize dentinal tubules up to depth of 600-1000 μm, whereas conventional irrigants penetrate no more than 100 μm (Bumb 2014; Gergova *et al.* 2014).

Clearly, *E. faecalis* is the most studied pathogen, because it is commonly associated with recurrent endodontic infections and endodontic failure (Dai *et al.* 2009) and is easily reproduced in laboratory (Pinheiro *et al.* 2004). Few microbes, such as *E. faecalis* and *Candida albicans* survive extreme pH (pH 11) and have been shown to be resistant to antimicrobials (Gergova *et al.* 2014) causing endodontic failure. For all the reasons described above, *E. faecalis* is often chose to induce *in vitro* mono and multispecies biofilms in dentinal tubules for disinfection studies (Iqbal *et al.* 2013; Siddiqui *et al.* 2013; Almeida *et al.* 2016; Cieplik *et al.* 2016).

Candida albicans

An important consideration in endodontic treatment is the microorganisms removal, including fungi, from the complex three-dimensional root canal system. Yeasts can be detected in 7-18% of infected root canals (Waltimo *et al.* 2004) and

they are normally associated with persistent cases of apical periodontitis (Nair *et al.* 1990), but can also be isolated from primary apical periodontitis (Waltimo *et al.* 1997). Peciuliene *et al.* reported that yeasts constitute less than 1% of the total oral cultivable microbiota in most root specimens (Peciuliene *et al.* 2001) and caries has been considered as the portal of entry for fungi to the root canal system, through coronal microleakage. However, the most common body niches showing asymptomatic colonization by *Candida* are gut, vagina (Jenkinson and Douglas 2002) and oral cavity, where one third of the healthy individuals carry fungi in their normal microbiota (Waltimo *et al.* 2004). These can be either commensal or opportunistic pathogens with the ability to cause a variety of infections, ranging from superficial to life threatening and accounts for up to 75% of all candidal infections (Jenkinson and Douglas 2002).

The essential oral yeasts present in root canal system belong to the genus *Candida* that comprises more than 200 fungal species (Baumgartner *et al.* 2000), and *Candida* cells were found in 55% of root canals (Sen *et al.* 1995). *Candida albicans* is the predominant species, followed by *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida guilliermondii*, *Candida kefyr* and *Candida parapsilosis* (Baumgartner *et al.* 2000; Waltimo *et al.* 2004).

Candida albicans is an opportunistic dimorphic fungus, the most encountered commensal and pathological yeast in the oral cavity. The tongue dorsum is considered to be the primary oral reservoir of *C. albicans*, and other sites such as mucosa and plaque covered tooth surfaces may be colonized secondarily (Hisajima *et al.* 2008). Particularly, *C. albicans* is associated with persistent apical periodontitis (Sen *et al.* 1995), from infected root canals, typically found together with Gram-positive bacteria such as *Streptococci*, but can also be isolated in pure culture, which is an indication of its pathogenicity (Moyes *et al.* 2016).

It is an adaptive microorganism that demonstrates different growth forms such as the unicellular yeast form, pseudohyphae and hyphae. Besides the ability of *C. albicans* to switch its morphology between yeast and hyphal form is crucial for its ability to adhere to surfaces and colonize tissue (Saville *et al.* 2003). It is proficient with several virulence factors, such as adhesins, yeast-to-hypha switch, thigmotropism (contact sensing), enzyme secretion and phenotypic switching (Naglik *et al.* 2003).

In the oral cavity, *C. albicans* grows and survives by competing and cooperating with an estimated 300 or more bacteria species (Jenkinson and Douglas 2002). It is important to recognize that *C. albicans* has the ability to live in harmony with the host, inside the resident oral complex microbiota present on mucosal surfaces, but also inside dentinal tubules during endodontic infections as an opportunistic pathogen. Adherence to dentinal tubules and the interaction with a wide variety of host extracellular matrix molecules is a prerequisite for colonization and therefore infection establishment (Baker *et al.* 2017).

The resistance of *C. albicans* strains against some locally disinfecting agents and classical antifungals such as fluconazole has increased, which drives the search for new therapeutic alternatives mandatory (Viana *et al.* 2015) and there is a solid evidence that their eradication from the root canal is essential.

Mixed biofilms of C. albicans *and* E. faecalis

In most natural environments, microorganisms exist predominantly as biofilms rather than as planktonic or free-floating cells (Jenkinson and Douglas 2002). Mixed biofilms are expressed as structured microbial communities that are attached to biotic or abiotic surfaces encased in a matrix of exopolymeric material,

consisting of a single microbial species or a mixture of bacterial or fungal species. (Costerton *et al.* 1999). This has strong clinical repercussions as it is now estimated that a significant proportion of all human microbial infections involve biofilm formation (Davey and O'Toole 2000). Crucially, biofilm-embedded organisms tend to exhibit amplified resistance to antimicrobial therapy and to withstand host immune defenses (Lewis 2005).

Persistent microorganisms, such as *E. faecalis* or *C. albicans*, are often present in root canal infections resistant to conventional therapy. Therefore, attention should be paid to adequate strategies including selection of irrigant solutions potentially efficient against microorganisms that can persist in root canal system and help each other in survival rate.

Waltimo *et al.* studied *C. albicans* and *E. faecalis* penetration into human dentine concluding that *C. albicans* growth into dentinal tubules was relatively poor in comparison with *E. faecalis* due specially to *C. albicans* size. However, both organisms were able to penetrate through a 2 mm thick human dentine disc (Waltimo *et al.* 1997) and both can coexist and cooperate in symbiosis in biofilms community.

The necrotic pulp tissue, root canals space and adjacent dentine tubules, although a harsh ecological environment provides essentials conditions for mixed biofilms at endodontic persistent infection.

In this context, it should be notice that classical endodontic studies recommend a copious irrigation (Ciucchi *et al.* 1989), temperature rising (Seal *et al.* 2002; Bonsor *et al.* 2006), high concentrations (Shabahang and Torabinejad 2003) and long exposure time (Bergmans *et al.* 2006) to expand the biofilm removal efficacy of all endodontic classical irrigant solutions.

CLASSICAL IRRIGANT SOLUTIONS

The primary goal of root canal treatment is to remove pulp tissue, necrotic debris and microorganisms from the root canal system. For this, irrigant solutions have to be used in conjunction with mechanical preparation for the five following purposes: i) debris removal created during instrumentation; ii) lubrication, iii) microorganisms removal; iv) soft tissue dissolution and finally v) smear layer removal (Basrani and Haapasalo 2012). There are several irrigant solutions available and different techniques for their delivery, but none of those can satisfy all the demands. Therefore, an optimal irrigation regime advocates the use of different irrigants in sequence.

Complete dentine disinfection is a main goal arduous to achieve with the presently endodontic irrigating solutions and root canal medicaments. One of the reasons for the poorer clinical *in vivo* effectiveness, when compared with *in vitro,* results from partial or total inactivation of the antimicrobial effect of the various medicaments by dentine and other substances potentially present in the necrotic root canal environment. Nowadays, the most used classical irrigant solutions are sodium hypochlorite (NaOCl), ethylenediamine tetraacetic acid (EDTA), and chlorhexidine gluconate (CHX), and as intracanal medicament, the most expended is calcium hydroxide, $Ca(OH)_2$.

Therefore, it is reasonable to search for better disinfecting protocols by combining existing and new disinfectant products/approaches that are not affected or are potentiated by dentine instead of inhibition and do not affect the host cells. Recent reports in this field had indicate the potential of photodynamic therapy for root canal dentine disinfection as extensively discussed in the first part of this introduction and reviewed in Diogo *et al.* 2015.

Sodium hypochlorite – 3% NaOCl

The worldwide endodontic irrigant solution is sodium hypochlorite (NaOCl) due mainly to its effective antimicrobial activity (Bystrom and Sundqvist 1981) and an excellent organic solvent for vital, necrotic and fixed tissues (Siqueira Jr *et al.* 1997). The recommended concentrations already studied range from 0.5% (Dakin's solute), 1% (Milton's solute) to 5.25% (Jeansonne and White 2006). However, only 1% concentrated solution presents acceptable biological compatibility with small antimicrobial activity (Basrani and Haapasalo 2012).

In terms of chemical reactions, it can be observed that NaOCl acts as an organic and fat solvent, degrading fatty acids, transforming them into fatty acid salts (soap) and glycerol (alcohol), thus reducing the surface tension of the remaining solution, known as a saponification reaction. NaOCl neutralizes amino acids creating water and salt, recognized as neutralization reaction. With the hydroxyl ions exit, it is detected a pH reduction. Hypochlorous acid (HClO), present in NaOCl solution, when in contact with organic tissue acts as a solvent, liberates chlorine that, combined with the protein amino group, forms chloramine, this leads to an amino acid chloramination reaction, that interferes in cell metabolism. Chlorine (a strong oxidant) reveals antimicrobial action by inhibiting bacterial enzymes leading to the reaction of chlorine with amino groups ($NH2^-$) and an irreversible oxidation of sulphydryl groups of essential bacterial enzymes (cysteine), interfering with their metabolic or membrane activities (Estrela *et al.* 2002). HClO and hypochlorite ions (ClO^-) lead to amino acid degradation and hydrolysis.

Considering NaOCl physicochemical properties, as a strong base (pH>11) and hydroxyl ions action, its antimicrobial effectiveness is based in its high pH and its mechanism is similar to calcium hydroxide (Basrani *et al.* 2003; Turk *et al.* 2009). The NaOCl, driving high pH, affects the cytoplasmic membrane integrity with an

irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation observed in lipid peroxidation. Increasing NaOCl concentration enhances its ability to dissolve organic components, especially pulp tissue, however this increase leads to periapical tissue irritation and host tissue toxicity (Mohammadi 2008).

NaOCl is an effective deproteinating agent that results in the heterogeneous removal of organic substrate from dentin, debris removal and organic matter cleaning from root canals, although, it leaves the prepared canal walls covered with smear layer (McComb and Smith 1975).

Ethylene diamine tetra-acetic acid - 17% EDTA

The smear layer in the root canal consists mainly of moistened calcified debris, which has been compacted against the dentine wall and into the tubule openings as a result of the rasping and trowelling action of the instruments (Mader *et al.* 1984). Ethylenediaminetetra-acetic acid (EDTA), a chelating agent, is the most widely irrigant solution used for smear layer inorganic component removal (Hülsmann 2013). Its reaction with calcium ions in dentine results in calcium chelation, promoting dentine decalcification at approximate depths of 20-30 μm within 5 min (von Der Fehr and Ostby 1962). The chelating ion reacts with the calcium present in the hydroxyapatite crystals, removing them from the root canal. EDTA effectiveness depends on various factors, for example, root canal length, depth of penetration of the solution, dentin hardness, time of contact, pH and concentration of the substance. It has been demonstrated that longer periods of EDTA and NaOCl irrigation can instigate the progressive peritubular and intertubular dentine dissolution and showed that EDTA reduces the mineral portion

of the dentin by increasing surface roughness and, as a consequence, dentin surface modifications can cause poor adaptation of the filling material and root canal walls, creating an opportunity for microbial infiltration (Poudyal *et al.* 2014).

EDTA has been used in several concentrations and combinations within the root canal, however weak acids, such as citric acid (Scelza *et al.* 2001) and maleic acid (Attur *et al.* 2016) have been evaluated at different concentrations for smear layer removal but all, including EDTA, had shown a harmful effect on periapical tissues (Silva *et al.* 2013).

Chlorhexidine gluconate - 2% CHX

Chlorhexidine is a wide-spectrum antimicrobial agent, effective against Gram-positive, Gram-negative bacteria and yeasts. Proper to its cationic nature, CHX is capable of binding to the negatively charged surfaces of microorganisms, damaging the outer layers of the cell wall and rendering it permeable. It also can disturb bacterial metabolism in several other ways such as abolishing the activity of the phosphotransferase system sugar transport system and inhibiting acid production in some bacteria (Basrani and Haapasalo 2012). CHX is a strongly basic molecule and is stable as a salt. CHX gluconate salt is easily soluble in water and depending on its concentration, CHX can be either a bacteriostatic or bactericidal agent. CHX has the ability to be adsorbed onto hydroxyapatite and dentin, also to bind to proteins such as albumin and this reaction is reversible. This reversible reaction of uptake and release of CHX leads to substantive antimicrobial activity and it is referred in literature as CHX substantivity (Santos 2008).

Chlorhexidine gluconate has been recommended as a root canal irrigant and medicament. It is a potent antimicrobial agent and its best property is due to CHX substantivity and low grade of toxicity (Guerreiro-Tanomaru *et al.* 2013). CHX is

unable to dissolve pulp tissue and its clinical application remains over CHX antimicrobial activity for up to 12 weeks (Mohammadi and Abbott 2009) at liquid form, as used in this thesis, because liquid form has better antimicrobial activity than gel presentation (Sena *et al.* 2006).

ENDODONTIC IRRIGANT TECHNIQUES AND DEVICES

Irrigation is an essential part of root canal debridement because it allows cleaning beyond what might be achieved by root canal instrumentation alone. Still, there is no technique that can meet all requirements, even with the use of methods as lowering pH (Christensen *et al.* 2008), temperature rising (Macedo *et al.* 2014b), as well as surfactants to increase the wetting irrigant efficacy (Lui *et al.* 2009).

Several endodontic irrigant studies were done (Hülsmann 2013; Plotino *et al.* 2016; Tawakoli *et al.* 2016) but, the majority of them describe *in vitro* outcomes and literature revisions solely reflect these studies, because no well-controlled clinical study is available (Gu *et al.* 2009).

Effective irrigant delivery and agitation techniques are pre-requisites for successful endodontic treatment and current technological advances through the last two decades have brought to the development of new devices and techniques that rely on numerous irrigation mechanisms divided in two broad categories as manual and machine-assisted agitation devices (Gu *et al.* 2009) summarized in **Figure 5**. From all manual irrigation techniques, the simplest is the manual irrigant agitation with dynamic movements applied to activate endodontic files or gutta-percha cones until reaching the working length within the root canal (Schäfer and Zapke 2000).

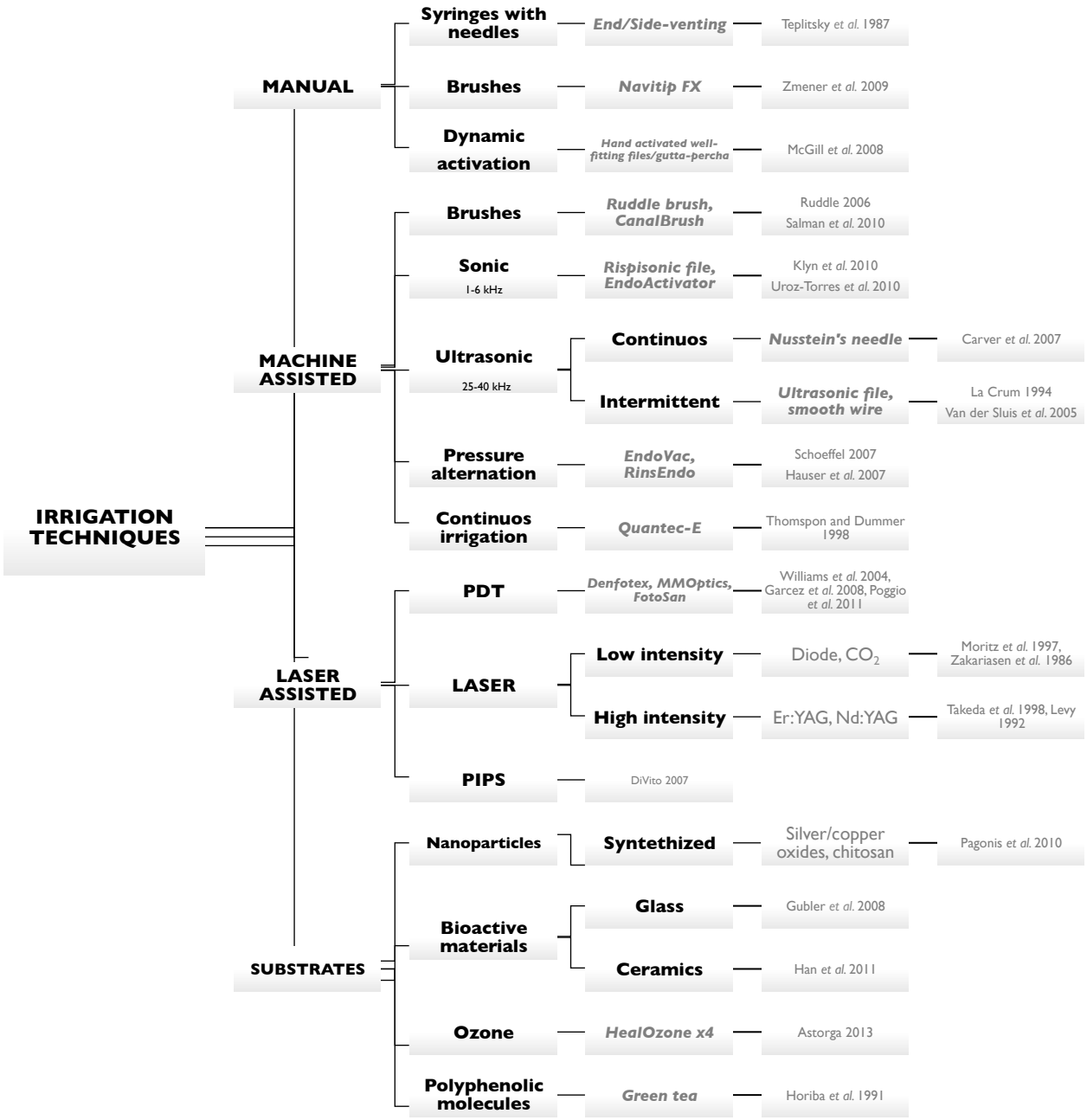

Figure 5. Endodontic irrigant techniques based on Gu *et al.* 2009, except when mention differently, in the scheme.

Syringes with needles and brushes were suggested in order to move the irrigant more effectively within the canals (Wu *et al.* 2003) and after conventional syringe needle irrigation, inaccessible canal extensions and irregularities are likely to harbour debris and microorganisms, making full canal debridement difficult (Nair *et al.* 2005). Sonic activation has shown to be an effective method to disinfect the root canals (Macedo *et al.* 2014a), and most actual systems have smooth plastic tips of different sizes activated at sonic frequency by a hand piece and it is generally found to be less effective in removing debris than the ultrasonic systems. The effectiveness of ultrasound in the irrigation is determined by its ability to produce cavitation, minimized and limited to the instrument tip, and acoustic streaming is more significant than cavitation (Macedo *et al.* 2014a). In order to deliver the irrigant into the root canal for the entire length and to obtain a good flow of fluid, apical negative-pressure systems have been introduced to simultaneously release and remove the irrigant. Most of the studies on this technique have shown that it is very effective to ensure a greater volume of irrigant in the apical third, excellent debris removal in this area and in inaccessible areas, with outcome similar to those obtained with the ultrasonic activation techniques (de Gregorio *et al.* 2009; Bago *et al.* 2013). Plotino *et al.* in 2006 anticipated that sodium hypochlorite and ultrasonic activation will be the goldstandard as both remain until the present as the most commonly used (Plotino *et al.* 2016). The interaction between LASER and the irrigant in the root canal outlines a new area of interest in the field of endodontic disinfection. This concept is the base of the LASER Activated Irrigation (LAI) (De Moor *et al.* 2010) and the so-called PIPS technology (Photon-Initiated Photoacoustic Streaming) (Pedullà *et al.* 2012). Some studies have concluded that the LASER irradiation is not an alternative, but rather a possible integration to existing protocols to disinfect root canal system (Diogo *et. al* 2015), however there is still no strong evidence to support

the application of high-power LASER for direct disinfection of root canals (Leonardo *et al.* 2005).

Nowadays, substrates are added to improve the irrigant technique, such as the classical example, the addition of chitosan to rose bengal to improve antimicrobial PDT efficacy (Shrestha *et al.* 2010; 2012; 2013); bioactive glass and ceramics to improve endodontic root canal disinfection (Farmakis *et al.* 2013). Recently, green tea started to be used as irrigant solution based on the principle that polyphenolic molecules had lower antimicrobial properties (Jagani *et al.* 2009).

ENDODONTIC IRRIGANT SOLUTIONS TIME CONTACT

Classical endodontic studies recommend 30 minutes cycles of a copious and prolonged irrigation for antimicrobial purpose (Sobhani *et al.* 2010; Haapasalo *et al.* 2010; Basrani and Haapasalo 2012). Contrary to this idea, Senia *et al.* in 1975, reported that 5.25% NaOCl one minute contact is sufficient to obtain total absence of several microorganisms (Senia *et al.* 1975).

It is known that *in vitro* antimicrobial activity of an environment depends upon the pH of the substrates in plates or tubes, drug's sensitivity, microbial source (wild or collection strains species) of bacteria inoculated number, incubation period, and the metabolic activity of the microorganisms (Gomes *et al.* 2001). Nonetheless, to understand the antimicrobial asepsis pattern in the present investigation, it was analysed the efficacy of several classical irrigants such as 3% NaOCl, 17% EDTA and 2% liquid CHX for 60 s, 90 s and 30 minutes over 48h mono and mixed biofilms of *E. faecalis* and *C. albicans*.

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Chapter III

ANTIMICROBIAL PHOTODYNAMIC THERAPY APPLIED TO ENDODONTIC *IN VITRO* BIOFILMS

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ABSTRACT

Endodontic biofilms eradication from the infected root canal system remains as the primary focus in endodontic field. In this study, it was assessed the efficacy of antimicrobial Photodynamic Therapy (aPDT) with the Zn(II)chlorin e6 methyl ester (Zn(II)e_6Me) activated by red light against monospecies and mixed biofilms of *Enterococcus faecalis* and *Candida albicans*. The results were compared with the ones obtained with Rose Bengal (RB), Toluidine Blue-O (TBO), the synthetic tetracationic porphyrin (TMPyP) as well as classical endodontic irrigants (3% NaOCl, 17% EDTA and 2% CHX). The efficacy of aPDT toward monospecies and mixed biofilms was quantified resorting to safranin red method. The changes of biofilm organization and of cellular ultrastructure were evaluated through several microscopy techniques (light, confocal and transmission electron microscopy). Zn(II)e_6Me once activated with light for 60 or 90 s was able to remove around 60% of the biofilm's biomass. It was more efficient than TBO and RB and showed similar efficacy to TMPyP and classical irrigants, CHX and EDTA. As desirable in a PS, Zn(II)e_6Me e in the dark showed smaller activity than TMPyP. Only NaOCl revealed higher efficacy, with 70–90% of the biofilm's biomass removal. The organization of biofilms and the normal microbial cell ultrastructure were extensively damaged by the presence of Zn(II)e_6Me. aPDT with Zn(II)e_6Me showed to be an efficient strategy deserving further studies leading to a future clinical usage in endodontic disinfection.

INTRODUCTION

Apical periodontitis is an inflammatory reaction of periradicular tissues caused by a microbial infection in the root canal system (Siqueira Jr *et al.* 2000; Nair 2006). Microbial biofilms are considered the major cause for primary and secondary root canal infection and the success of endodontic treatment relies on the effective eradication of such biofilms (Nair 2006). Conventionally, this is accomplished by chemo-mechanical disruption with instruments and antimicrobial chemicals used topically inside root canals. However, current treatment strategies are insufficient to reduce microorganisms inside root canals below detention limits before permanent root filling. This is mandatory to achieve optimal healing conditions for the periapical tissues (Sjögren *et al.* 1997). Therefore, advanced disinfection approaches are required to effectively eradicate biofilms and increase the endodontic treatment success rate.

It is widely accepted that the main reason for endodontic treatment failure is the insufficient root canal microorganisms eradication (Siqueira Jr *et al.* 2000). As residual species are not reachable to the host's immune system, propagation and re-colonization is highly possible, endorsing microbial spread inside root canal system, which leads to endodontic infections. In 1965, apical periodontitis was recognized as a microbial mediated infection, which was later reinforced by ultrastructural microscopic techniques, revealing microorganisms organized as biofilms within the infected root canals (Nair 1987). Moreover, histopathological studies have also contributed to the concept that apical periodontitis is indeed a microbial biofilm-mediated disease (Carr *et al.* 2009; Ricucci *et al.* 2009; Ricucci and Siqueira Jr 2010).

As described by Donlan and Costerton, a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, imbedded in a matrix of extracellular polymeric

substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transfer (Donlan and Costerton 2002). In addition, it is accepted that microbial cells comprising the biofilm are more resistant than the planktonic counterparts and mixed biofilms are more resistant to drugs than monomicrobial biofilms (Costerton *et al.* 1999). As such, polymicrobial biofilms diseases are associated with worse clinical outcomes than monomicrobial infections for decades (McKenzie 2008). Although the endodontic biofilm is constituted by multiple microorganisms (Tan *et al.* 2015), most of *in vitro* studies have been made in monospecies biofilms of bacteria or combining that with *C. albicans* (Sabino *et al.* 2014).

The problem of endodontic biofilms eradication from the infected root canal system remains as the primary focus in endodontic field. In recent years, photodynamic therapy (PDT) has been applied with success in several types of tumours (Ochsner 1997; Gomes *et al.* 2015), age-related macular degeneration (Kawczyk-Krupka *et al.* 2015) and also in the photoinactivation of several microorganisms (Almeida *et al.* 2014). In the endodontic field, aPDT has emerged as an optional extra to classical irrigation solutions in root canal asepsis, (Bonsor *et al.* 2006a; Bonsor *et al.* 2006b) such as sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX) and ethylenediamine tetraacetic acid (EDTA). The NaOCl solution is the most widely used in endodontic treatment (Siqueira Jr *et al.* 2007; Mohammadi 2008; Vaziri *et al.* 2012; Wang *et al.* 2015) albeit with some degree of toxicity. To avoid this toxicity, other root canal asepsis approaches with lower or insignificant toxicity should be implemented. Therefore, aPDT has emerged with promising experimental results, anticipating a possible new era in endodontic disinfection (Siddiqui *et al.* 2013; Chrepa *et al.* 2014). aPDT involves the combination of a non-toxic photosensitizer (PS) with a harmless visible light source in oxygen presence.

After being excited by light, the PS releases its energy or electrons to molecular oxygen producing highly reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), which induce microorganism's injury and death with no host cell damage. Also, it has been indicated as bearing a strong potential in the fight against antimicrobial resistance (Hamblin and Hasan 2004; Tavares *et al.* 2010; Costa *et al.* 2011). aPDT has also been studied as an auspicious approach to eradicate oral pathogenic microorganisms that cause, not only endodontic diseases, but also periodontitis, peri-implantitis, caries lesions and mucositis (Diogo *et al.* 2015).

In this study, we analysed the aPDT efficacy against monospecies and mixed biofilms of *E. faecalis* and *C. albicans* using the following PSs: Toluidine Blue-O (TBO), Rose Bengal (RB), a synthetic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) and Zn(II)chlorin e6 methyl ester (Zn(II)e_6Me) obtained from chlorophyll a, **Figure 1**. The outcomes obtained by aPDT approach were compared with the ones achieved with three endodontic classical irrigants 3% NaOCl, 2% CHX and 17% EDTA toward *in vitro* biofilms.

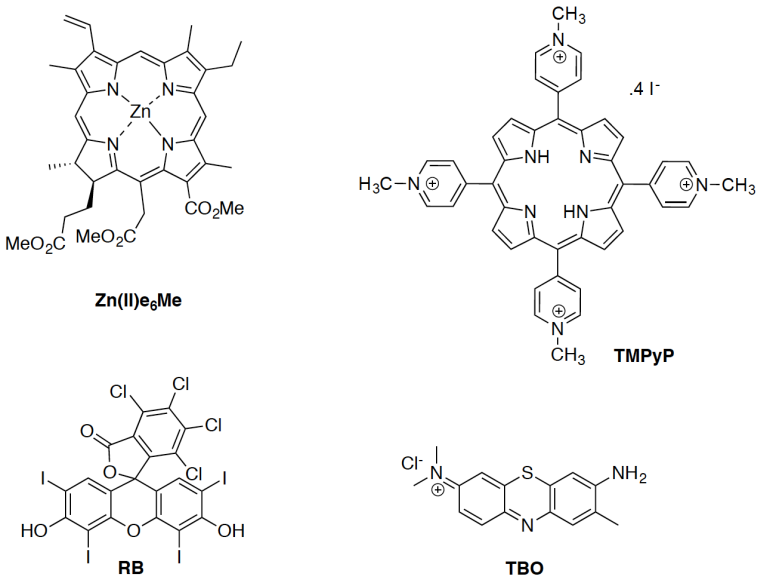

Figure 1. Chemical structures of the photosensitizers tested.

MATERIALS AND METHODS

Strains and media

The strain of *C. albicans* (YP0037) used in this study was obtained from the Pathogenic Yeast Collection of FMUC, University of Coimbra. *E. faecalis* (ATCC29212) was purchased from the American Type Culture Collection (ATCC). Microorganisms were stored at -80ºC in 25% glycerol. When needed, pre-cultures were prepared by defrozen microbial cells in appropriate media, brain-heart infusion (BHI) for *E. faecalis* and YPD (0.5% yeast extract, 1% bacto-peptone and 2% glucose) for *C. albicans*. For *C. albicans* growth it was used YPD broth. *E. faecalis* growth and biofilm formation was obtained in BHI liquid medium (Difco, Detroit, MI, USA). *C. albicans* biofilms and mixed biofilms consisting of *C. albicans* with *E. faecalis* were obtained in RPMI (Roswell Park Memorial Institute) 1640 medium (R8755, Sigma–Aldrich®).

In vitro *biofilm formation*

For *E. faecalis in vitro* biofilm formation, bacterial cells were previously grown in 4 mL of BHI overnight at 37°C. These cells were harvested by centrifugation (Biofuge Fresco, Heraeus, UK), at 16,000 rpm during 5 min at 4°C, and washed twice in sterile BHI. A bacterial cell suspension with a density of $1.5x10^8$ cells/mL (0.5 McF of McFarland scale) was obtained; 200 μL of this suspension was pipetted to sterile 96-well polystyrene microtiter plates (Nunc F, Nalgene, Denmark). These plates were covered and sealed with parafilm and incubated during 48 h at 37°C without agitation.

For the preparation of *C. albicans* biofilm, a loopful of cells from the solid

stock cultures was used to inoculate 20 mL of YPD and incubated overnight in an orbital shaker (120 rpm) at 30°C. The cells were harvested by centrifugation (16,000 rpm for 5 min at 4°C), and washed twice with phosphate buffered saline (PBS). The final pellet was resuspended in prewarmed RPMI-1640 at 37°C. The resulting cell suspension was diluted in RPMI to obtain a final suspension with a cell density of $1.0x10^6$ cells/mL. This was used to prepare *C. albicans* biofilms in 96-well polystyrene microtiter plate (Nunc F, Nalgene, Denmark). For that 200 µL of *C. albicans* suspension was pipetted to the plate wells. After sealing with parafilm, they were left to incubate during 48 h at 37°C, without agitation.

For the mixed biofilm of *E. faecalis* and *C. albicans,* the two microbial species were pre-grown overnight and prepared as described for monospecies biofilms preparation. The two microbial suspensions were mixed in 1:1 ratio and resuspended in 37°C prewarmed RPMI-1640 (R8755, Sigma-Aldrich®). The mixed biofilm was formed during 48 h at 37°C without agitation.

Biofilms eradication with classical irrigants

The classical irrigants tested were 3% NaOCl, 17% EDTA and 2% CHX (CanalPro™, Coltene®, Whaledent, UK). The biofilms were exposed to the irrigants for 60 and 90 s. A longer period of irrigation (30 min) was also tested because some authors defend that a continuous irrigation and time are important factors for the efficacy of classical irrigating solutions (Bystrom and Sundqvist 1985, Haapasalo *et al.* 2010). After each period, the supernatants were removed and the chemical reactions were stopped using adequate inhibitors: 200 µL of sodium thiosulfate (S7026, Sigma-Aldrich®) was added to the NaOCl treated group; 3% Tween 80 (T2575, Sigma-Aldrich®) was used to neutralize CHX. Finally, 200 µL sterile distilled water was

applied to dilute the 17% EDTA. Controls were made without the irrigants, in which the stop solutions were added, proving that these stop solutions, especially 3% Tween 80, did not interfered with the biofilm biomass quantification.

Photodynamic inactivation of biofilms

In the aPDT experiments all the PSs tested (TBO, RB, TMPyP and Zn(II)e_6Me) were used at the same concentration (0.1 mg/mL). This concentration was chosen based on market formulation FotoSan agent®, in which the active substance is TBO at 0.1 mg/mL. This formulation is available in the dentistry market with a light source device (FotoSan®: 630, CMS Dental A/S, Glyngore, Roslev, Denmark) (Rios *et al.* 2011). The cationic porphyrin TMPyP and the modified chlorophyll, Zn(II)e_6Me, were synthetized and isolated according with literature (Carvalho *et al.* 2010; Menezes *et al.* 2014). Their ^{1}H NMR and UV–vis spectra were consistent with literature data and their purity was confirmed by thin layer chromatography and ^{1}H NMR (data not shown). TBO and RB used were purchased from Sigma-Aldrich® (T3260 and 330000-1G, respectively). Stock solutions (10 mg/mL) of each porphyrinic derivative were prepared in dimethyl sulfoxide (DMSO). For biological assays, the stock solutions of photosensitizers were diluted to the final concentrations in PBS.

The irradiations of the PSs in the aPDT experiments were performed in the presence of adequate light emitting diode (LED) source setup to comply with the 96-well plates used in this study, **Figure 2**. The LED sources were built at request by the Telecommunications Institute, Informatics, Electronics and Telecommunications Engineering Department of the University of Aveiro, Portugal.

RB was irradiated with a green LED with a wavelength peak centred at 557

nm, made with gallium phosphide pure (GaP), an output of 62.5 mW, continuous waves, density power of 42 $mW.cm^{-2}$, energy fluence of 3780 $J.cm^{-2}$ and voltage of 2.5 V.

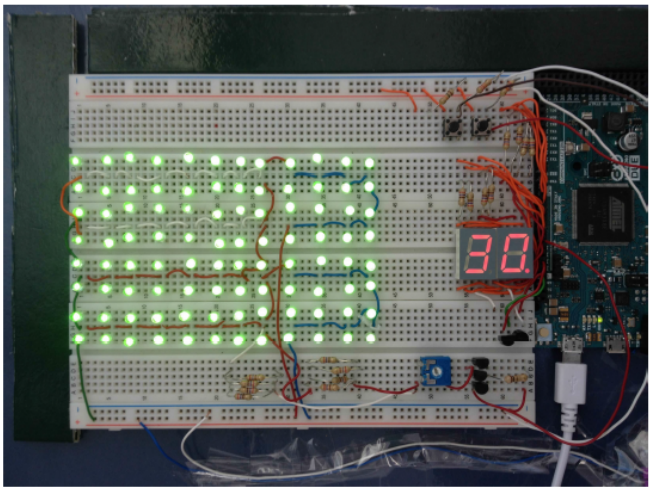

Figure 2. Model of the experimental LED sources. Green light diode (GaP) with 557 nm of wavelength.

TBO, TMPyP and $Zn(II)e_6Me$ were irradiated with a red LED device with a wavelength peak centred at 627 nm, a gallium arsenide phosphide on gallium phosphide (GaAsP/GaP), with an output power of 75 mW, continuous waves, density power of 35 $mW.cm^{-2}$, energy fluence of 3150 $J.cm^{-2}$ and a voltage of 2.5 V.

The experimental methodology included a pre-incubation period of the biofilms with the PSs, in total absence of light, for 15 min, to allow the entrance of PSs into the cells (Diogo *et. al* 2017). After that, light activation was performed for 60 or 90 s. Also, in each assay it were included controls in which the PSs were not added, to control the impact of irradiation in the biofilms.

Biofilm biomass quantification

The biofilm biomass was quantified using the safranin red (SR) assay (Kueng *et al.* 1989). After each experiment, 200 µL of methanol was added to each well of the

96-well plate. After 15 min, the content of each well was aspirated and let to dry. After drying, 0.1% SR solution was added and incubated for 20 min. The resulting solution was removed with a Pasteur pipette and two washes were made with distilled water. Two hundred microliter of acetic acid 33% (v/v) was added and 20 min after, the absorbance was measured at 590 nm on a microplate reader (SpectraMAX Gemini XM, Molecular Devices, USA). The results were expressed as a percentage of biofilm removal when compared with the control biofilm biomass quantified before irradiation or before exposure to the irrigants.

Microscopic study of the biofilms

E. faecalis and *C. albicans* were grown and prepared as described above. One mL of the final suspensions was added to sterile 12-well polystyrene microtiter plates with glass coverslips (CBAD00120RA1#1.5, ThermoScientific-Menzel®) coated with poly-D-lysine (P1149, Sigma-Aldrich®). After seeding, the entire microtiter plate was covered and sealed with Parafilm® and incubated for 48 h at 37°C without agitation. For confocal fluorescence microscopy, fresh cultures of biofilms were used. *E. faecalis* was stained with Syto 13 Green Fluorescent Nucleic Acid Stain (ThermoFisher Scientific®). *C. albicans* in monospecies biofilm was probed with polyclonal primary antibody Acris Antibodies Gmbh® RGTX40096 with anti-rabbit secondary antibody Alexa Fluor® 594 (RA21207, Invitrogen®). Images were obtained with a Carl Zeiss Cell Observer Spinning Disk with Alpha Plan-Apochromat objective, at a magnification of 100x. For light microscopy, it was used an Olympus BX-40 microscope at 400x total magnification. Images were recorded on an Olympus C-200 digital camera.

For transmission electronic microscopy (TEM), samples of 48 h-biofilms

were fixed with 2.5% glutaraldehyde in 0.5 M sodium cacodylate buffer (pH 7.2) for 2 h. Post-fixation was performed using 1% osmium tetroxide for 1 h. The samples were rinsed with the same buffer and dehydrated in a graded ethanol series (30 to 100%). Impregnated and embedded in Epoxy resin (Fluka Analytical). Ultrathin sections (~70 nm) were mounted on copper grids (300 mesh) and stained with uranyl acetate 2% (15 min) and 0.2% lead citrate (10 min). Observations were carried out on a FEI-Tecnai G2 Spirit Bio Twin transmission electron microscope at 100kV.

Statistical analysis

Data were analyzed using Prism (version 5) software (GraphPad Software, Inc., La Jolla, CA). Statistical differences between groups were assessed with the independent samples t-student test or Mann-Whitney test and a significance level of 0.05 was assumed.

RESULTS

Biofilm removal

Before initiating our comparative study of the efficacy of aPDT and classical irrigants in the clearance of biofilms, it was important to verify if the PSs selected, TBO, RB, TMPyP and Zn(II)e_6Me, had the ability to disturb the biofilms in the dark (*i. e.*, in the absence of light activation) at the same concentration used in the aPDT studies. It was clearly desirable that the PSs had zero or very low activity in total absence of light indicating that aPDT efficacy results strictly from the ROS generated by PS light activation. The results obtained from the biofilm biomass analysis, using the SR assay, showed that upon 15 min of biofilms exposure to the PSs, in the dark,

there was a decrease of the biofilms biomass in values ranging from 5.7% to 16.6% (**Table 1**).

Table 1. PSs effect in biofilm biomass[(1)] in total absence of light during an incubation period of 15 min.

Biofilm	TBO (%)	TMPyP (%)	Zn(II)e_6Me (%)	RB (%)
E. faecalis	15.2	13.1	12.7	14.7
C. albicans	16.6	15.5	8.6	10.0
Mixed	12.4	10.0	5.7	7.5

[(1)] The quantification of biofilm biomass was obtained with the SR assay and results are expressed as a percentage of biofilm removal when compared to the control biofilm biomass (n=3).

Following a pre-incubation period in the dark with the PSs, each preparation was irradiated with the appropriate LED. Thus, TBO, TMPyP and Zn(II)e_6Me were irradiated with a wavelength of 627 nm while 557 nm was used for RB. Three periods of irradiation were tested, 60, 90 s and 30 min. Since there were no differences between the 90 s and the 30 min periods, this longer period was abandoned (data not shown; see Supplementary Results at the Chapter VI of this Thesis). Also, the controls of the impact of light irradiation in the biofilms during the 60 or 90 s of irradiation showed no damage of the biofilm, as assessed by the SR assay and by microscopic observation of the biofilms morphology (results not shown).

The results summarized in **Figure 3** (upper left panel) shown that Zn(II)e_6Me, is more effective in the removal of *E. faecalis* biofilm than the other PSs used, in both irradiation periods (60 and 90 s) (P=0.0079). Similar reduction values of *E. faecalis* biofilm were obtained using TMPyP and RB (**Figure 3**; upper left panel). For *C. albicans* biofilm, Zn(II)e_6Me and TMPyP had similar efficacies in decreasing biofilm biomass upon 90 s of irradiation. However, a shorter period of irradiation, 60 s, revealed a significant difference between the efficacies of both dyes in the capacity to remove biofilm biomass (**Figure 3**; middle left panel). Otherwise, Zn(II)e_6Me was

much more effective than the other PSs used, TBO (P=0.0317), and RB (P=0.0079) after 90 s of irradiation and at the same concentration.

The mixed biofilm seems to be less susceptible to aPDT than the monospecies biofilm, especially when using TBO as PS, (P=0.0013), (**Figure 3**; lower panel). In this mixed community of *E. faecalis* and *C. albicans,* Zn(II)e_6Me demonstrated to be the most efficient PS removing 58.98% of biofilm biomass (P<0.001).

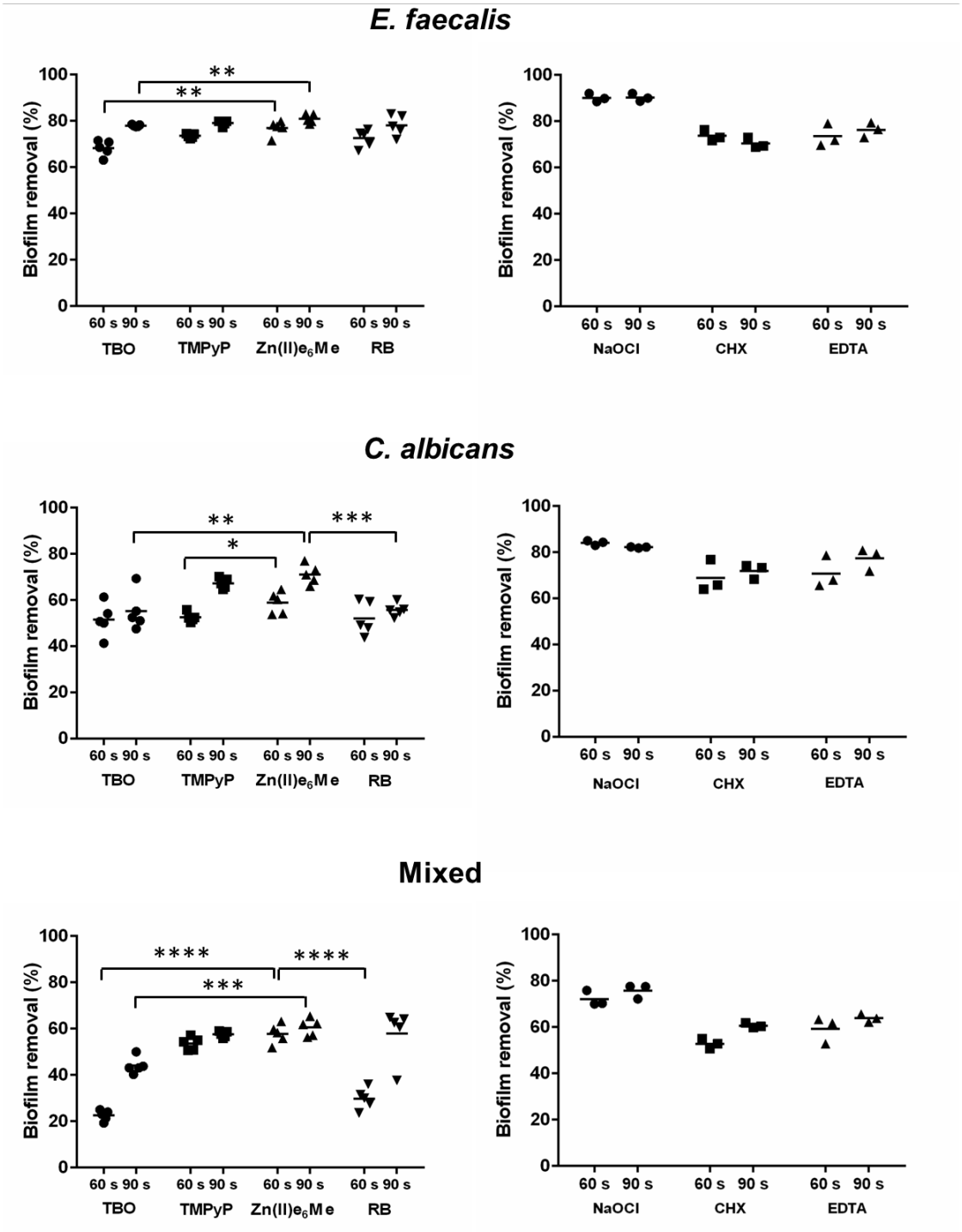

Figure 3. Biofilm biomass removal upon aPDT and treatment with classical irrigants. Monospecies biofilms and mixed biofilms of *E. faecalis* and of *C. albicans* were treated by aPDT using several photosensitizers (left panels) and classical irrigants (right panels). The quantification of biofilm biomass was performed with the safranin red (SR) assay. The percentage of biofilm biomass loss was calculated in relation to untreated biofilm. Differences were analyzed by Student's t test using Prism software and considered significant at *P* values of < 0.05. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

When compared to classical irrigants, Zn(II)e_6Me was not as effective as NaOCl, the treatment that causes the higher damage, regardless of the type of biofilm (**Figure 3**; right panels). In fact, Zn(II)e_6Me (with an irradiation period of 90 s) was more effective in removing *E. faecalis* biofilm than EDTA or CHX (**Table 2** and **Figure 3**; upper panel). Zn(II)e_6Me reveal the same effect of CHX or EDTA treatment toward *C. albicans* biofilms (90 s) and toward mixed biofilms, either with 60 s or with 90 s of irradiation (**Table 2** and **Figure 3**; middle and lower panels).

Table 2. Statistical analysis[(1)] of the efficiency of Zn(II)e_6Me against microbial biofilms in comparison with classical irrigants in the clearance of *E. faecalis*, *C. albicans* and mixed biofilms.

	E. faecalis		*C. albicans*		Mixed	
	60s	90s	60s	90s	60s	90s
Zn(II)e_6Me *vs* NaOCl	***	***	***	**	**	**
Zn(II)e_6Me *vs* CHX	ns	***	ns	ns	ns	ns
Zn(II)e_6Me *vs* EDTA	ns	*	*	ns	ns	ns

[(1)] Differences were analyzed by Student's t test using Prism software and considered significant at P values of < 0.05. *, P < 0.05; **, P < 0.01; ***, P < 0.001, ns: no significant difference.

Biofilms disturbance by aPDT in the presence of Zn(II)e_6Me and NaOCl

The study of biofilms morphology was performed after 48 h of biofilm maturation, because 48 h- and 72 h-old biofilms had similar morphologies. The changes observed in the biofilm organization developed for 48 h when treated with Zn(II)e_6Me and NaOCl (the classical irrigant with the best outcome) were compared with the untreated biofilms (control).

Zn(II)e_6Me eliminated most of the *E. faecalis* (**Figures 4A,B**) but *C. albicans* preparations retained some hyphae and yeast cells (**Figures 4D,E**). Otherwise, NaOCl eliminated all the cells adhered to the glass slide, either in *E. faecalis* or *C. albicans* biofilms (**Figures 4C,F**). Using light microscopy, it was observed that while NaOCl lead to an almost complete loss of living cells (**Figure 5C**), aPDT with

Zn(II)e_6Me resulted in a mixed biofilm with less *E. faecalis* cells and less *C. albicans* hypha, with a predominance of pear shaped cells (**Figure 5B**), when compared with the morphology of the untreated mixed biofilm (**Figure 5A**).

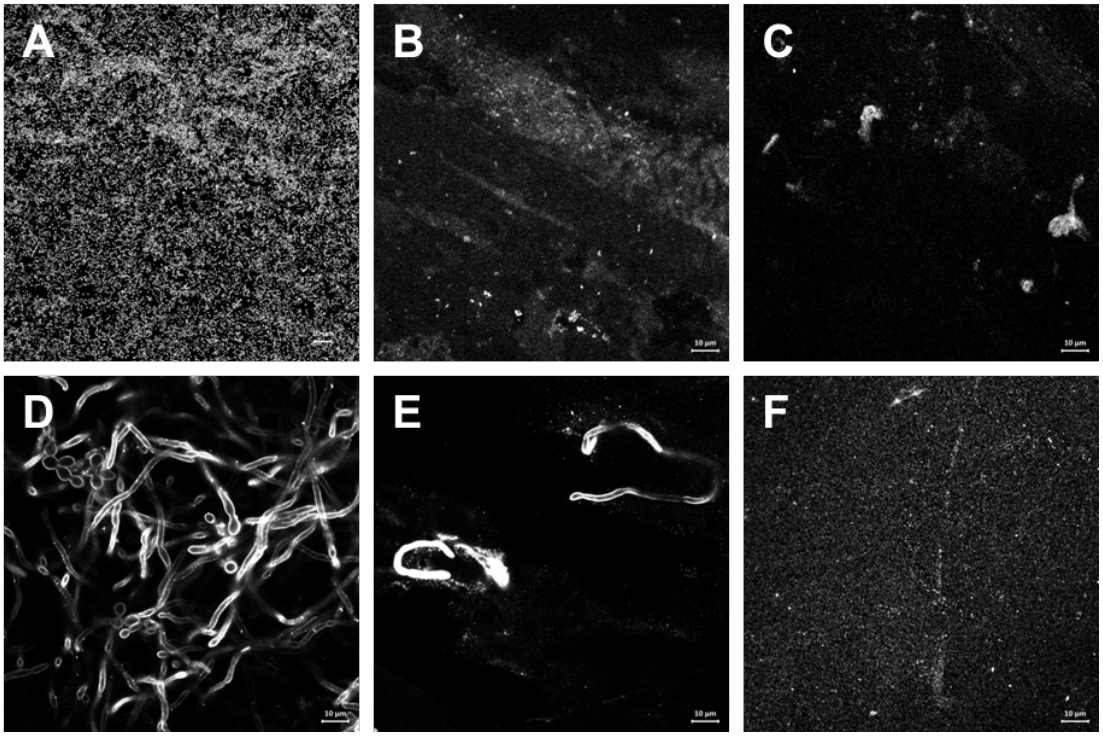

Figure 4. Effect of aPDT with Zn(II)e_6Me as PS compared with the classical irrigant NaOCl in the morphology of monospecies biofilms. Endodontic *in vitro* 48 h-biofilms of *E. faecalis* and *C. albicans* were obtained and treated as described under Materials and Methods. (**A-C**) *E. faecalis* was stained with Syto 13 Green Fluorescent Nucleic Acid Stain. (**D-F**) *C. albicans* was probed with polyclonal primary antibody and with anti-rabbit secondary antibody Alexafluor® 594. Representative images of biofilms untreated (**A,D**), treated with Zn(II)e_6Me as PS activated for 90 s (**B,E**), and treated with NaOCl (**C,F**). Images were obtained with a Carl Zeiss Cell Observer Spinning Disk with Alpha Plan-Apochromat objective (100x). Bars: 100 μm.

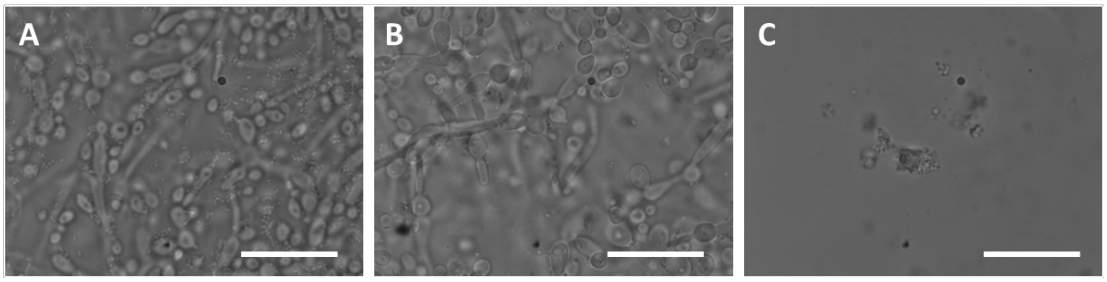

Figure 5. Effect of aPDT with Zn(II)e_6Me as PS compared with NaOCl in the morphology of mixed biofilm. The images (**A**) untreated controls, (**B**) Zn(II)e_6Me activated during 90 s, and (**C**) with NaOCl were obtained with using an Olympus BX-40 microscope at 400x total magnification. Images were recorded at different time periods on an Olympus C-200 digital camera. Bars: 20μm.

Ultrastructure modification of microbial cells in biofilms

The different morphological aspects observed in the fluorescence confocal microscopy lead us to study the ultrastructural changes using TEM. After several attempts, it was realized that the remainings of the biofilms (either monospecies or mixed biofilm) treated with NaOCl were drastically damaged and no signs of cells were observed in the epoxy resin blocks sections (data not shown). The ultrastructural modification of bacterial and fungal cells were studied in biofilms exposed to Zn(II)e_6Me with an activation period of 90 s. In *E. faecalis* monospecies biofilm it was observed the existence of cell wall "ghosts", *i.e.*, bacterial cell wall forming a structure with typical morphology of *E. faecalis*, without its intracellular content (**Figure 6A,D-F**).

The complexity of the cellular ultrastructure of *C. albicans*, a eukaryote, allowed the observation of induced modifications. Most of the yeast cells showed an atypical irregular cell wall thickness and the cytoplasmic membrane integrity was lost, with cell membrane invaginations (**Figure 6G**), caused by 90 s of Zn(II)e_6Me-aPDT treatment. The cell membrane was damaged and the cell wall surface was rougher (**Figure 6H**) than in control cells (**Figure 6B**). Abnormal intracellular membrane arrangements probably corresponding to endoplasmic reticulum (ER) whorls (**Figure 6I**) were also observed. Some *C. albicans* cells exhibited big vacuoles with electrodense materials (**Figure 6J**). A general view of the mixed biofilms showed *E. faecalis* cell wall structures devoid of the intracellular content and irregular *C. albicans* cell walls (**Figure 6K**). In fact, in mixed biofilms microbial cells ultrastructural modifications were similar to those observed in monospecies biofilms, including invaginations of the cell membrane found in *C. albicans* cells (**Figure 6L**).

Additionally, in mixed biofilms, *C. albicans* cells showed persistent extracellular vesicles, at the surface of the cell wall, with different sizes and shapes

(**Figure 6M**). In **Figure 6N** it is also possible to observe several ultrastructural features of mixed biofilms treated with Zn(II)e_6Me by PDT: in the extracellular matrix, besides the spread of electrodense materials typical of a biofilm matrix, fragments of membranes or of fibrous materials were also observed, which were not observed in *E. faecalis*-*C. albicans* mixed biofilms untreated (**Figure 6C**); also, the cytoplasm of *E. faecalis* showed electrodense agglomerates and some fungal cells showed a twisted irregular shape.

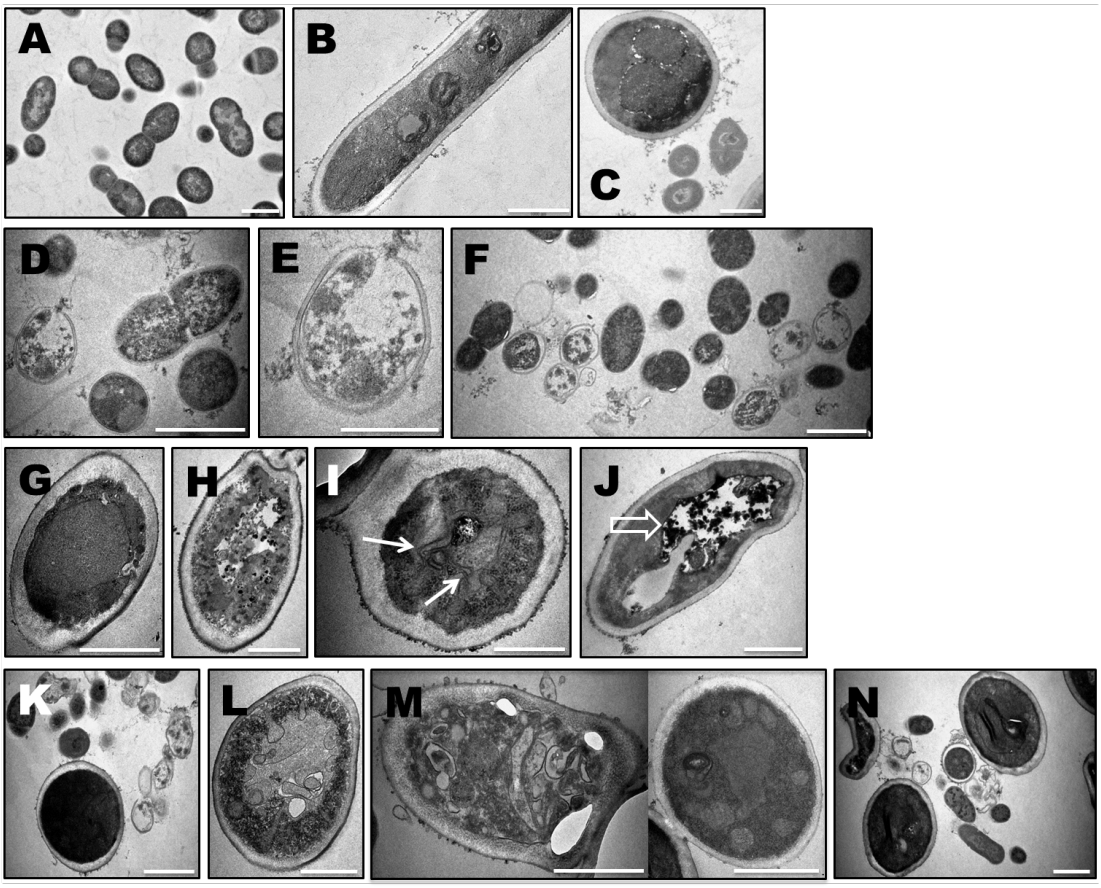

Figure 6. Ultrastructural modification of microbial cells upon aPDT with Zn(II)e_6Me. 48 h-biofilms of *E. faecalis*, *C. albicans* and mixed biofilms with both microorganisms untreated (**A-C**) and treated with aPDT with Zn(II)e_6Me as photosensitizer (*E. faecalis* biofilm: **D-F**), (*C. albicans* biofilm: **G-J**), (mixed biofilms: **K-N**). Bars: 1000 nm, except for E and I, 500 nm.

DISCUSSION

The aim of this work was to compare the efficacy of Zn(II)e_6Me to disturb *in vitro* models of endodontic biofilms comparatively with three other PSs (TBO, RB and TMPyP) and with endodontic classical irrigants. For this, monospecies biofilms of *E. faecalis* and *C. albicans* were used, together with a mixed biofilm model with both microorganisms. The main conclusion is that Zn(II)e_6Me had a better antimicrobial efficacy than the clinically used PSs (TBO and RB). Although the efficacy of Zn(II)e_6Me and TMPyP is similar, one of the main advantage of using Zn(II)e_6Me is its availability from natural sources, associated to a lower toxicity in the total absence of light. It also presents the same antimicrobial potential than the clinically used irrigants, CHX and EDTA. It is worth mentioning that TBO (Seal *et al.* 2002; Bergmans *et al.* 2008; Rios *et al.* 2011) is available in the market under the name of Fotosan® agent (Gambarini *et al.* 2011; Rios *et al.* 2011) and RB has been widely studied (Shrestha *et al.* 2012; 2014; Persadmehr *et al.* 2014). As expected, 3% NaOCl had the best final outcomes. In fact, NaOCl at different concentrations, is considered an excellent irrigant solution in endodontics (Jeansonne and White 1994; Siqueira Jr *et al.* 2007; Mohammadi 2008), nevertheless it also displays high toxicity levels towards the host tissues (Estrela *et al.* 2002; Önçağ *et al.* 2003; Trevino *et al.* 2011; Wang *et al.* 2015).

The antimicrobial effect of aPDT is dependent both on the cellular localization of the PS, which may be determined by its physiochemical properties (Castano *et al.* 2004) and on the diffusion of singlet oxygen that should be sufficient to inactivate microorganisms structure and biomolecules. There have been several reports on the use of aPDT to kill both yeast and bacteria, however, fungi are much more complex targets than bacteria. Nevertheless, similarities with mammalian cells

should be considered and this may indicate the use of cationic PSs, rather than their anionic counterparts, since the later exhibit facile uptake by mammalian cells (Bonnett 1995). The biochemical and functional effects of photosensitization include peroxidation of lipids, resulting in cell membranes disruption, lysosomes and mitochondria lysis and consequently autophagy (Schuck *et al.* 2014). The phenothiaziniums, such as TBO and MB, are known to target plasma membrane of yeast and bacteria (de Melo *et al.* 2013; Baltazar *et al.* 2015), TBO was described as increaasing cell wall permeability (Wainwright *et al.* 1997), whereas MB produces bacterial DNA damage (Menezes *et al.* 1990).

The use of cholorophylls in endodontic root canal treatment was previously described (Mohammadi *et al.* 2013) and there are evidencies showing that clorophyll present in green tea can be used in endodontic root canal treatment due to its antibacterial effects (Horiba *et al.* 1991).

In this work, we describe Zn(II)e_6Me, obtained from the natural chlorophyll a, as an encouraging PS candidate displaying consistent antimicrobial outcomes. The ultrastructural study of microbial cells upon aPDT demonstrated that using Zn(II)e_6Me as PS, results in the irreversible damage of *E. faecalis* cells (mono and mixed biofilms), displaying "cell ghosts", empty of its cellular content but with almost intact cell walls. The presence of these inactive "ghost" cells was corroborated by the biomass loss assessed by SR assay. Before, it was described that *E. faecalis* elimination with aPDT resulted in bleb formations suggestive of damage of membrane components (López-Jiménez *et al.* 2015), shrunken, bacterial cell diameter reduction, rough and fractured appearance of the bacterial cells (Cheng *et al.* 2012). It was also described the presence of bacterial cell membrane shrivelling and alterations including loss of cocci or bacilli shape, grooves on the cell surface and draining of the intracellular components (Garcez *et al.* 2013).

According to our observations, *E. faecalis* cell wall destruction was sporadic and not a massive one, which appear to indicate that the induced damage was directed to proteins and/or lipids of the cytoplasmic membrane, resulting in the leakage of cellular contents, as described by others (Girotti 2001). It is also known that the extension of biochemical changes induced by aPDT is dependent on PS nature and on the irradiation period (Dai *et al.* 2009). In this study the aPDT proceeded during a short period (60 or 90 s) can justify the punctual cell wall destruction in *E. faecalis*.

In *C. albicans* monospecies and in *C. albicans* cells in mixed biofilms, it was noticed several changes in the cellular organization, with a cytoplasmic membrane disruption, vacuoles morphology and organelles damage including signals of autophagy (e.g. ER whorls, and organelles inclusion in vacuoles) as described by others (Prates *et al.* 2011; Schuck *et al.* 2014). The intracellular damage induced by aPDT with Zn(II)e_6Me is probably dependent on the entry of this PS, since this feature is crucial for aPDT efficacy and outcomes (Hamblin and Hasan 2004; Baltazar *et al.* 2015). This lead us to speculate that the pre-incubation period of biofilms with PSs during 15 min in total absence of light, before the short irradiation period (60 and 90 s) most certainly contributed for the interaction between the PS and the cell. This would lead to intracellular PS distribution (due to its hydrophobic nature), impacting in the genesis of the intracellular damage observed. The questions raised by these observations highlight the importance of future further studies to unravel the intracellular distribution of Zn(II)e_6Me.

The Zn(II)e_6Me antimicrobial potential, that we showed by quantification of biofilm biomass loss and by a microscopic study of the biofilm morphology and of the cellular ultrastructure, leads to the importance of defining the mechanism by which this modified chlorophyll affects the endodontic biofilms. Based on this,

further research will be mandatory to improve the antimicrobial efficacy of aPDT in the root canal system, such as the ones recently published (Tennert *et al.* 2015; Cieplik *et al.* 2016) using human tooth models, ultimately leading to an optimization of light delivery and new PS formulations.

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Chapter IV

ANTIMICROBIAL PHOTODYNAMIC THERAPY APPLIED TO INFECTED HUMAN TEETH

This chapter is under submission for publication in *International Endodontic Journal* as:

Is the chlorophyll derivate Zn(II)e_6Me a good photosensitizer in root canal disinfection?

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ABSTRACT

Aim: To assess antimicrobial efficacy and cytotoxic outcomes of a chlorophyll based photosensitizer Zn(II)chlorin e_6 methyl ester (Zn(II)e_6Me), versus other photosensitizers (PSs) and 3% NaOCl when applied to human dentin discs and root blocks infected with 48 h mixed biofilms.

Methodology: Dentin was infected with mixed biofilms of *Enterococcus faecalis* and *Candida albicans*, then exposed to 0.1 mg/mL of Zn(II)e_6Me, Toluidine Blue-O (TBO) or Fotosan® for 15 min before irradiation with red light (627 nm, 75 mW, 3150 J/cm^2) for 90 seconds (s). Biofilm removal was calculated with safranin red assay and biofilm cells viability with XTT® assay. Microscopy samples were analysed with scanning electron microscopy (SEM). Antimicrobial Photodynamic Therapy (aPDT) cytotoxicity outcomes were evaluated at 24 and 48 h over human apical papilla primary cell line with AlamarBlue® assay and microscopy with widefield fluorescence.

Results: At dentin discs, after a treatment period of 90 s, classical irrigant 3% NaOCl revealed the best antimicrobial outcome (68.1%), followed by Zn(II)e_6Me-PDT (59.1%). Nevertheless, a biofilm removal of 70.9% was obtained with Zn(II)e_6Me after an irradiation period of 900 s. In root blocks, Zn(II)e_6Me had the best antimicrobial approach after an irradiation period of 90 s (79.7%) and 300 s (81.7%), followed by 3% NaOCl (75.5%); this antisepsis pattern was consistent at inner and outer samples. No dark or photoinduced cytotoxic outcomes were detected for Zn(II)e_6Me over human cells at 24 and 48 h.

Conclusions: The chlorophyll derivative, Zn(II)e_6Me, showed the best antimicrobial efficacy, performing better in mixed biofilm removal than TBO/Fotosan® and no cytotoxic effects over human apical papilla cells were identified.

INTRODUCTION

Microorganisms that invade the root canal system have a fundamental role in initiating and sustaining periapical disease. Microbial studies in root canal infections have identified a certain number of bacteria that persist after root canal treatment, which are mainly Gram-positive facultative anaerobic bacteria, including enterococci, streptococci, lactobacilli and actinomyces (Molander *et al.* 1998; Sundqvist *et al.* 1998; Roças *et al.* 2004). The predominance of these Gram-positive bacteria is based on their ability and resilience to persist under adverse conditions (Chávez De Paz 2012).

In 1997, Waltimo *et. al* found that in apical periodontitis yeasts are present in 7% of the culture-positive samples from persistent root canal infections. When yeasts are isolated, they are most often organized with facultative Gram-positive bacteria, while Gram-negative isolates are rare (Waltimo *et al.* 1997). Enterococci are present in nearly 50% of the root canal systems with refractory infection (Molander *et al.* 1998). *Enterococcus faecalis* is usually isolated in pure culture or as a major component of the microbiota in endodontic treated teeth with chronic apical periodontitis (Stuart *et al.* 2006; Guerreiro-Tanomaru *et al.* 2013). These microrganisms are difficult to eradicate from root canal and one mechanism behind it seems to reside in its proven resistance to endodontic antiseptics (Dahlén *et al.* 2000, Peciuliene *et al.* 2001) including calcium hydroxide (Basrani *et al.* 2002).

The most frequent yeast species isolated from the root canal system is *Candida albicans*, one of the microorganisms responsible for development of pulpal and periapical infections (Turk *et al.* 2009). Eradication of endodontic infection and preventing of reinfection is a major aim in root canal treatment. Nevertheless, with the current techniques total asepsis is mostly impossible to achieve (Sjögren *et al.* 1997).

To improve the prognosis, innovative therapeutic methods that can effectively control antisepsis need to be explored (Santos *et al.* 2014). Towards this goal, antimicrobial Photodynamic Therapy (aPDT) (Siddiqui *et al.* 2013; Chrepa *et al.* 2014) has recently emerged and provided excellent experimental results, anticipating a new era in endodontic disinfection. aPDT is an original approach involving the combination of a non-toxic photosensitizer (PS) and a harmless visible light source. Excited PS interacts with molecular oxygen to produce highly reactive oxygen species (ROS), leading to the inactivation of microorganisms without the risk of promoting microbial resistance. aPDT has been studied as a promising approach to eradicate not only endodontic infections (Jurič *et al.* 2014, Diogo *et al.* 2015) but also periodontitis and peri-implantitis (Eick *et al.* 2013), mucositis and caries lesions (Konopka and Goslinski 2007). Recently, we described that Zn(II)chlorin e_6 methyl ester (Zn(II)e_6Me), a modified chlorophyll is effective against 48 h *in vitro* mono and mixed biofilms (Diogo *et al.* 2017).

The main goal of this study is to investigate the efficacy of 0.1 mg/mL of Zn(II)e_6Me versus Toluidine Blue-O (TBO) and Fotosan® (a commercial TBO formulation) as PSs and, 3% NaOCl as classical irrigant solution to remove mixed biofilms of *E. faecalis* with *C. albicans* from infected human dentin discs and root blocks. Additionally, the *in vitro* viability of human apical papilla cells was assessed at 24 and 48 h after exposure to Zn(II)e_6Me versus contemporary PSs: FotoSan®, Rose Bengal (RB) and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP).

MATERIALS AND METHODS

Ex vivo *mixed biofilm of* E. faecalis *with* C. albicans

The strains, media, stock and growth conditions used in the preparation of this mixed biofilm of *E. faecalis* and *C.* albicans, were performed according to protocols described elsewhere (Diogo *et al.* 2017). Briefly, the two microbes suspensions at a concentration of 1.0 x 10^6 cells/mL were mixed in pre-warmed RPMI-1640 1:1 ratio and incubated at 37 °C during 48 h to allow biofilm formation over sterile dentin discs and inside root blocks.

Dentin disc samples preparation

Seventy dentin discs, **Figure 1A**, obtained from freshly extracted intact human third molars with fully developed roots were immersed in 0.5% NaOCl for 48 h to remove organic debris and surface disinfection. After roots removal at the cement-enamel junction, dentin slices of 1 mm thickness were prepared with the aid of a diamond-cutting disk (Low-speed saw, Accutom-5, Struers, Denmark) under abundant water irrigation. Organic and inorganic debris, including the smear layer, were removed by immersing the dentin discs for 5 min in a ultrasonic bath containing 17% EDTA followed by another 5 min in 5.25% NaOCl. Next, dentin discs were incubated for 96 h with RPMI medium at 37 ºC to confirm the sterility process by the absence of turbidity.

In order to prevent bias, dentins discs were evenly distributed by seven groups (n=10), regarding the area. Negative control was RMPI-medium and the five test groups and positive control were inoculated with *E. faecalis* and *C. albicans* and incubated for 48 h at 37 °C without agitation, to allow mixed biofilm formation,

Figure 1.

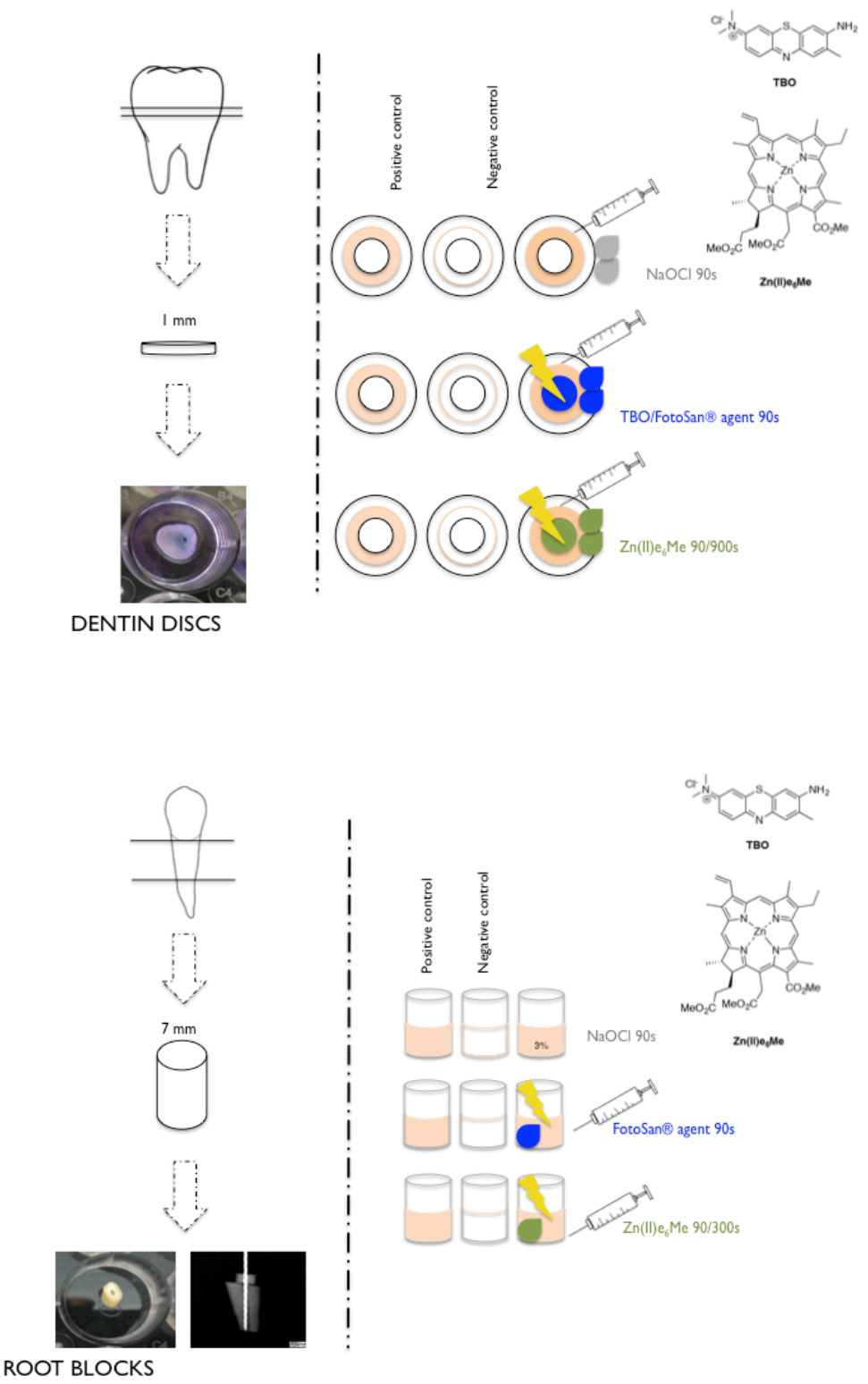

Figure 1: Schematic representation of the methodology. **A.** Experimental groups at dentin discs. **B.** Experimental groups with root blocks.

Root block samples

Root blocks (n=42) were obtained from freshly extracted single-rooted human teeth without carious lesions and fully developed roots, **Figure 1B**. They were immersed in 0.5% NaOCl for 48 h to remove organic debris and perform surface disinfection. After crown removal at the cement-enamel junction, standardized root blocks with 7 mm long were prepared. The root canal of each specimen was enlarged with a Gates-Glidden Drill no.3 (GGD; Dentsply/Maillefer, Ballaigues, Switzerland) to normalize internal diameter to 0.90 mm and kept in distilled water during all procedures to prevent dehydration. Disinfection, smear layer removal and sterilization protocol was the same applied to dentin discs.

From 42 root blocks samples, six experimental groups (n=7) were implemented; two control (negative and positive) and four test groups (3% NaOCl, 90 s FotoSan®, 90 s and 900 s of $Zn(II)e_6Me$). Each root was sealed to the bottom of a 12-well plate with a self-etch scotchbond adhesive (Scotchbond™ Universal Adhesive – 3M®) and a flowable light-curing Ormocer® (A2 Admira Flow - VOCO®). All roots were coated at the external wall with nail varnish. Finally, the four test groups and the positive control were inoculated to form a mixed biofilm during 48 h; the negative control group was incubated with RPMI medium. From each root block, three samples were obtained, adapted from a previous protocol (Basrani e*t al.* 2002). The first sample, from the canal itself was reached with sterile GGD#3 while the two other samples, the "inner" and the "outer" with GGD#4 and GGD#5, respectively. Each sample was obtained with a single in-and-out drill movement inside root canal and collected with the GGD into a sterile 96-well plate containing 100 μL of RPMI medium.

Biofilm biomass and microbial cells viability quantification

The biofilm biomass was quantified using the safranin red (SR) assay described before (Diogo *et al.* 2017) and the results were expressed as a percentage of biofilm removal when compared with the initial biomass of the control group. All antimicrobial approaches were applied inside a 12-well plate and the supernatants were transferred to microtiter plates, with transparent bottoms for further spectrophotometer readings. Microbial cells viability was assessed immediately using XTT® assay 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)-carbonyl]-2H-tetrazolium hydroxide (Sigma-Aldrich®), according to the manufacturer's instructions. Three independent measurements were done for each condition.

Classical irrigant – 3% NaOCl

CanalPro™ 3% NaOCl (Coltene®, Whaledent, UK) was used. After the experiments, the inhibitor sodium thiosulfate, $Na_2S_2O_3$, (S7026, Sigma-Aldrich®) was added to stop the chemical reactions: 200 μL (for dentin discs) and 50 μL (for root blocks).

Photosensitizer (PS) agents and light source setup

Low viscosity FotoSan® agent (FotoSan®, CMS Dental A/S, Glyngore, Denmark], Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein disodium salt), 5,10,15,20-tetrakis(1-methylpyridinium-4-yl) porphyrin (TMPyP) and Zn(II)chlorin e_6 methyl ester (Zn(II)e_6Me) were used as PSs, all at the same concentration, 0.1 mg/mL. This concentration was chosen based on FotoSan® agent. This formulation is available in the market accompanied by a light source device (FotoSan®: 630, CMS Dental A/S (Glyngore, Roslev) – Denmark) (Rios *et al.* 2011).

The cationic porphyrin TMPyP and the chlorophyll derivative, Zn(II)e_6Me, were synthetized and isolated according with literature (Carvalho *et al.* 2010, Menezes *et al.* 2014). Stock solutions (10 mg/mL) of each porphyrinic derivative (TMPyP and Zn(II)e_6Me) were prepared in dimethyl sulfoxide (DMSO). For biological assays, the stock solutions were diluted to the final concentrations in phosphate buffered saline (PBS). TBO and RB were purchased from Sigma-Aldrich® (T3260 and 330000-1G).

For the aPDT treatments of dentin discs, two light emitting diode (LED) source setup were built at request to comply with the 12 well plates (for antimicrobial purpose) and 96-well plates used for *in vitro* cytotoxicity assessment. RB was irradiated with a green LED with a wavelength peak adjusted at 557 nm, made with gallium-phosphide pure (GaP), with an output of 62.5 mW, continuous waves, density power of 42 mW.cm^{-2}, energy fluence of 3780 J.cm^{-2}; voltage of 2.5 V (Diogo *et al.* 2017).

FotoSan®, TMPyP and Zn(II)e_6Me were irradiated with a red LED, wavelength peak centred at 627 nm, prepared from gallium-arsenide-phosphide on gallium-phosphide (GaAsP/GaP), with an output power of 75 mW, continuous waves, density power of 35 mW.cm^{-2}, energy fluence of 3150 J.cm^{-2} and a voltage of 2.5 V. The experimental methodology included a PS pre-incubation period in total absence of light for 15 min, which was followed by light activation of 90 s or 900 s.

Experimental light quantification parameter

To endorse the power and specific wavelength of LED setup, spectral range was performed with a spectrometer (USB 2000+, Ocean Optics®). To confirm if the light delivered by the LED setup irradiate through the 12 wells polystyrene, dentin discs and root blocks, luminance was obtained with a colour screen handheld LED

power and energy meter (Vega P/N (7Z01560), Ophir®). All experiments were performed three times in total absence of light.

Scanning electron microscopy (SEM)

Specimens were obtained and fixed in 2.5% glutaraldehyde (diluted from Glutaraldehyde 25%, Agar Scientific Ltd, Unit 7, Essex, United Kingdom) and dehydrated in an increased alcohol sequence (50, 75, 95, 100%). Specimens were submersed in conductive carbon cement for 24 h and a gold-palladium (Au-Pd) and a conductive material was deposited on samples surface. The specimens' microstructures were evaluated using SEM analysis in a field emission scanning electron microscope, Hitachi SU-70.

Culture of Human Apical Papilla primary cells (hAPCs)

Cell cultures of hAPCs were obtained according to protocols described elsewhere (Sequeira *et al.* 2016). Briefly, hAPCs were collected from human third molars with incomplete rhizogenesis. Apical papilla was detached, minced and digested with dispase (Gibco, ThermoFisher Scientific®) and type I collagenase enzyme (Sigma-Aldrich®) mixture for 1 h at 37ºC. Next, cells were seeded in culture dishes with KnockOut DMEM (Gibco, ThermoFisher Scientific®) supplemented with 10% FBS, Glutamax and penicillin-streptomycin (Gibco, ThermoFisher Scientific®) and incubated in a humidified atmosphere at 37ºC, 5% CO_2. After reaching confluence, cells were used for experimental procedures.

Quantification of aPDT cytoxicity

According to ISO 10993-5:2009 (E) a compound is cytotoxic when it reduces

the cell viability by more than 30%. For this reason, cell viabilities less than 70% were used as cut-off values and outcomes were analyzed at 24 h and 48 h. The proliferation rate of hAPCs growing in the presence of several PSs was evaluated by AlamarBlue® Cell Viability Reagent (DAL 1025, ThermoFisher Scientific). All PSs were assessed after an incubation period of 15 min in total absence of light and after exposure to an irradiation period of 90 s.

The AlamarBlue® assay was carried out according to manufacturer's instructions. Briefly, medium was removed and the cells were washed twice with phosphate buffered saline (PBS). The cells were incubated in DMEM D5030 (Sigma-Aldrich®) without phenol red supplemented with non-inactivated FBS 3.7 g/L sodium bicarbonate, 4 mM L-glutamine, 4.5 g/L glucose, 1% penicillin–streptomycin and 10% AlamarBlue® solutions for 4 h. As negative control, AlamarBlue® was added to the medium without cells and the absorbance was read on a microplate reader (SpectraMAX Gemini XM, Molecular Devices, USA). In this assay, PSs were assessed and compared with the chlorophyll derivative Zn(II)e_6Me. Data result from five independent measurements.

Microscopic study of the in vitro *induced-PS cytotoxicity in hAPCs*

hAPCs were cultured as previously described and plated on glass coverslips in the well of 12-well plates, overnight at 37 °C in a humidified atmosphere with 5% CO_2. In the following day, cells were treated with aPDT. Then, the culture media was removed from the wells, the adhered cells washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C. Then, cells were washed with PBS twice, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich®) for 10 min at room temperature. Cell actin was stained using Phalloidin Fluorescein Isothiocyanate

(Sigma-Aldrich®) at a 1:100 dilution at room temperature for 40 min. Cellular nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific®), at room temperature for 5 min, and the cells washed twice with PBS. The coverslips were removed from the wells and mounted with specific medium (DAKO Denmark A/S) in microscope slides. Representative images were obtained with a Carl Zeiss Axio Observer Z1 with Alpha Plan-Apochromat objective, at a magnification of 63X.

Statistical analysis

Data were analyzed using Prism (version 5) software (GraphPad Software, Inc., La Jolla, CA). The validity of results was evaluated with inferential analysis assessing the average differences between groups with the independent samples t-student test or Mann-Whitney test. The significance level assumed was 0.05.

RESULTS

Antimicrobial PDT outcomes in dentin discs

A period of irradiation of 90 s with Zn(II)e_6Me performed better than all other PSs. However, Zn(II)e_6Me showed an inferior outcome than 3% NaOCl, 59.1% and 68.1%, respectively ($P \leq 0.0185$). The aPDT outcome of chlorophyll derivative Zn(II)e_6Me was also assessed after 900 s of irradiation and resulted in 70.9% of biofilm removal, **Figure 2**.

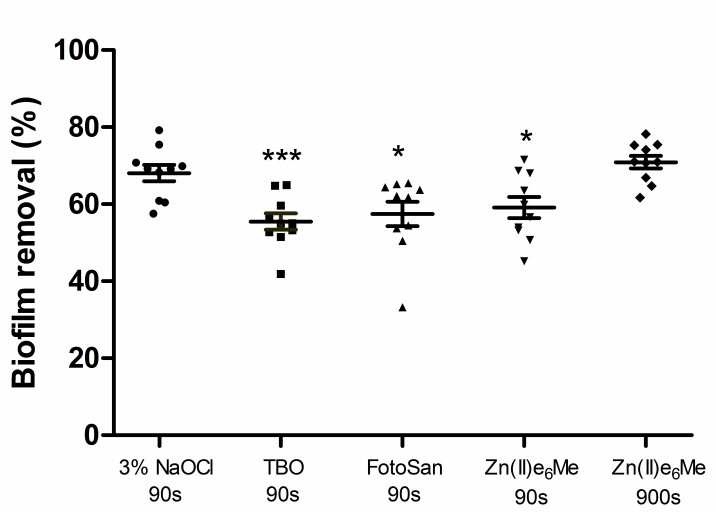

Figure 2. aPDT outcomes in dentin discs. aPDT outcomes of biofilm removal (%) obtained in dentin discs with SR assay compared with control group. $*P \leq 0.05$,$***P \leq 0.001$.

PSs effects were evaluated over mixed biofilms in total absence of light and final results are shown at **Table 1.**

Table 1. PSs effect in mixed biofilms biomass of *C. albicans* and *E. faecalis* incubated during 15 min in total absence of light.

SUBSTRACTS	PHOTOSENSITIZERS		
	TBO (%), OD_{450nm}	FotoSan® (%), OD_{450nm}	$Zn(II)e_6Me$ (%), OD_{550nm}
Dentin Discs	13.78	12.87	6.23
Root Blocks	11.91	10.36	3.57

Antimicrobial PDT outcomes in root blocks

aPDT outcomes in root blocks are shown as percentage (%) of biofilm removal, calculated either using SR or XTT® assay (canal samples). Three samples were collected (canal, inner and outer wall) from each root block. The best antimicrobial outcomes were obtained from the aPDT group with the chlorophyll derivative ($Zn(II)e_6Me$) irradiated for 90 and 300 s, **Figure 3A**.

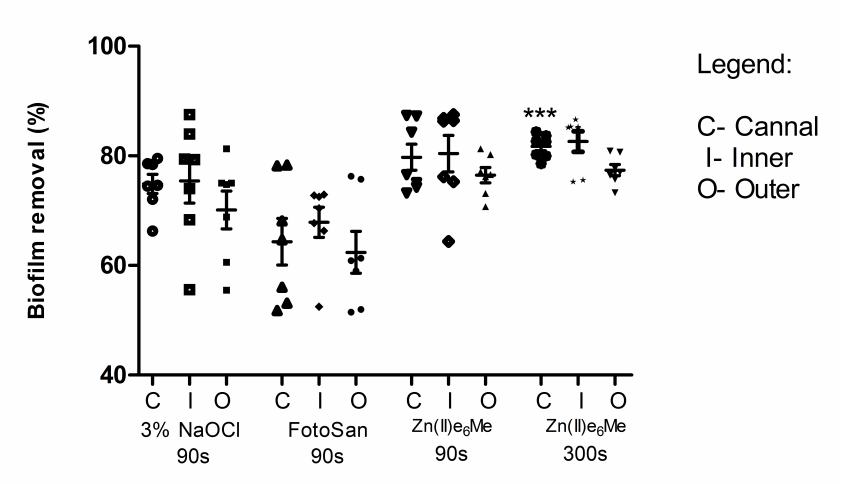

Figure 3A. aPDT outcomes in root blocks. aPDT outcomes of biofilm removal (%) obtained in root blocks at three different samples (canal, inner and outer wall) with SR assay compared with control group. ***$P \leq 0.001$.

It is worth to mention that there is no statistically significant difference between Zn(II)e_6Me irradiated for 90 s and 300 s ($P \leq 0.4$). In conclusion, when comparing with the other substances also applied in root blocks for 90 s (NaOCl and FotoSan), aPDT with Zn(II)e_6Me showed the best performance for biofilm removal and the most consistent results among the three studied depths samples.

Microbial cell viability quantification of canal samples with XTT® assay

Canal samples of mixed biofilms after 3% NaOCl and aPDT procedures with 0.1 mg/mL of FotoSan® and Zn(II)e_6Me were evaluated with XTT® assay in root blocks after 90 and 300 s of light irradiation. Samples were obtained with GGD#3, GGD#4 and GGD#5. However, data from GGD#4 and GGD#5 were not used to quantify cell viability because dentinal debris affected the final reading lectures at spectrophotometer, which were not consistent. Based on this fact, only data from GGD#3, equivalent to the root main canal size preparation were expressed as cell viability in **Figure 3B**.

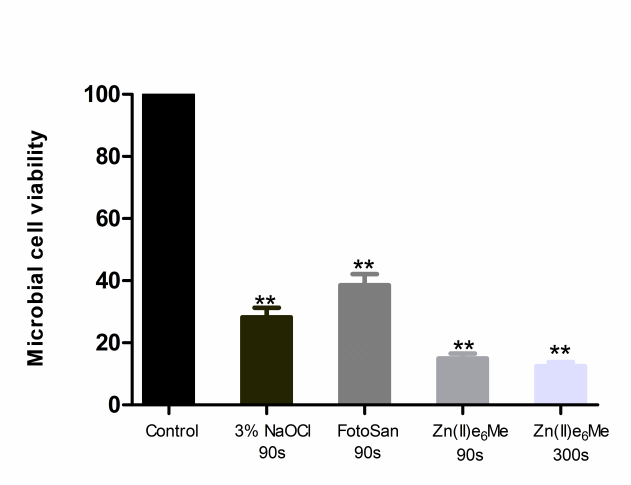

Figure 3B. Mixed biofilm cells viability in root blocks. Mixed biofilm cells viability (% of control) obtained with XTT assay immediately after NaOCl and aPDT procedures. ** $P \leq 0.01$.

Zn(II)e_6Me had the best antimicrobial approach with 15.0% and 12.4% ($P \leq 0.0021$) of mixed biofilm cells viability for 90 and 300 s, respectively, followed by 3% NaOCl with 28.2% after 90 s of exposure. FotoSan® had the worst outcome with 38.5% of mixed biofilm cells viability.

Experimental light quantification parameter

A LED setup was built to emit at a wavelength close to the FotoSan® 630 LAD pen. This LED emitted a non-coherent light with a wavelength peak centered at 627 nm. In order to assess the ability of the light to pass through several materials, the luminance was obtained for each substrate after being irradiated for 90 s with either LED setup (wells and dentin discs) and FotoSan® 630 LAD pen (root blocks). **Table 2** presents the outcome average values compared from three independent measurements (n = 3). It is possible to confirm that irradiation occurs successfully through all substrates. However, light spreads better inside root blocks with FotoSan® 630 LAD pen than through dentin discs with 627 nm LED setup ($P<0.05$).

Table 2. Luminance values for several substrates, all irradiated for 90s in total absence of natural light.

SUBSTRATES	LUMINANCE (Lux)
Experimental 627 nm LED	17.7
Total absence of light	0.01
Empty 12 well	17.3
12 Well infected with 48h mixed biofilm	16.0
Dentin disc	7.4
Dentin disc infected with 48h mixed biofilm	10.6
FotoSan® 630 LAD pen	23.7
Root dentin block[1]	20,7
Root dentin block infected with 48h mixed biofilm[1]	21,7

NOTE: 1Lux = 1 lumen per square meter (lm/m^2).

[(1)] Results expressed were obtained with FotoSan® 630 LAD pen.

Scanning electron microscopy (SEM) of the biofilms in the dentin surface

Images of dentin discs (**Figure 4**) and root blocks (**Figure 5**) were acquired in the control and also in Zn(II)e_6Me-PDT and 3% NaOCl groups, which present the best antimicrobial outcomes achieved. Images were performed to understand the infection/antisepsis pattern. SEM control images showed mixed biofilm adhered at the dentin surface (**Figure 4**, A-A_2), and inside dentinal tubules system of root blocks (**Figure 5**, D-D_2); *E. faecalis* was located on dentinal tubules surface where they link together and form chains (**Figure 4**, A_1 and B_2) that anchorage around *Candida albicans* (**Figure 5,** D_1-D_2, E-E_2). Mixed biofilm submitted to Zn(II)e_6Me-PDT was mostly disrupted and dentin surface with open dentinal tubules could be appreciated (**Figure** 4, B-B_2).

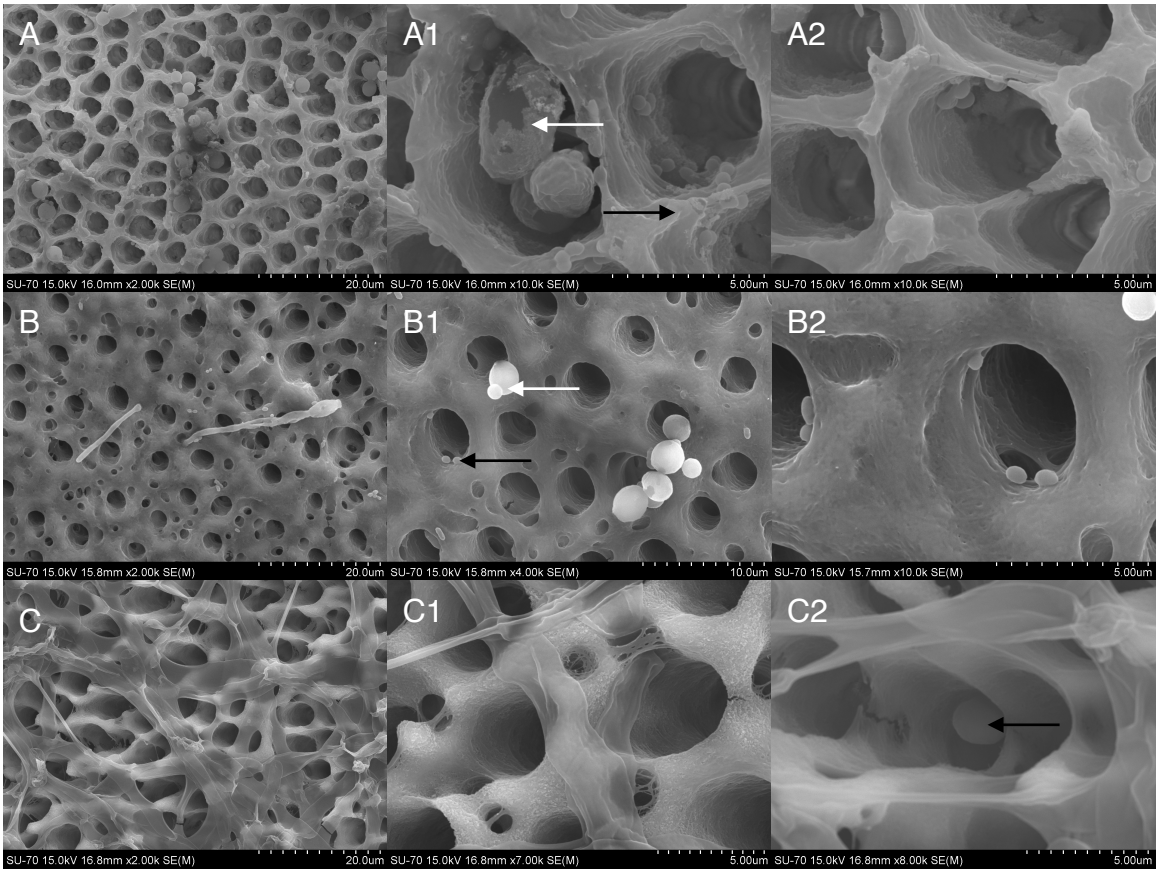

Figure 4. Scanning electron microscopy, Hitachi SU-70, micrograph of dentin discs infected with 48h mixed biofilms. Control group (A-A_2), antimicrobial Zn(II)e_6Me-PDT group (B-B_2) and 3% NaOCl group (C-C_2). From A to A_2, B to B_2 as C to C_2 magnification increases to reveal coronal dentin details and it is possible to notice exposed dentin tubules infected with mixed biofilms of *Candida albicans* (white arrow) with *Enterococcus faecalis* (black arrow). From C to C_2, advance microscopy images supports the best asepsis outcomes obtained with NaOCl with detailed coronal dentin properties description: intertubular dentin, peritubular dentin and dentinal tubules with absence of smear layer. Among A, B and C it has independent coronal dentin pattern due to anatomy variations, but all correspond to a transversal dentin disc cut from a human intact third molar.

At root blocks, microorganisms were sparse, corroborating the SR assay outcomes (**Figure 5**, E-E_2). Viable microorganisms distribution was significantly reduced and biofilm architecture was disrupted following Zn(II)e_6Me-PDT. Biofilms were damaged under NaOCl treatment with the presence of dead microorganisms (**Figure 5**, F-F_1).

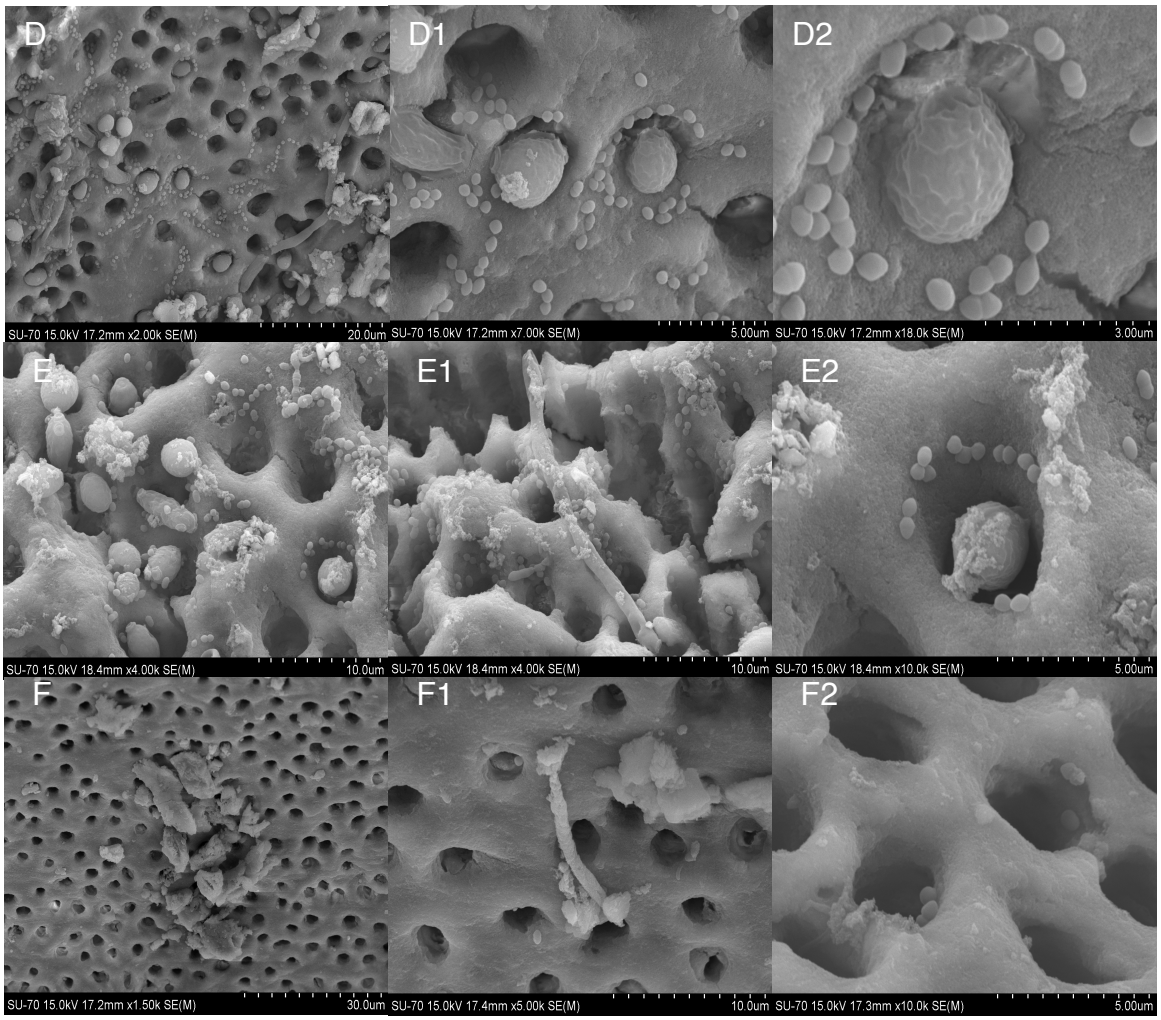

Figure 5. Scanning electron microscopy, Hitachi SU-70, micrograph of root blocks dentin infected with 48h mixed biofilms. Control group (D-D_2), antimicrobial Zn(II)e_6Me-PDT group (E-E_2) and 3% NaOCl group (F-F_2). From D to D_2, E to E_2 as F to F_2 magnification increases to reveal root dentin wall and biofilms details. From F_2 it is possible to see root dentin properties description: intertubular dentin, peritubular dentin and dentinal tubules with smear layer absence. Among D, E and F it has independent root dentin pattern due to individual anatomy variations, but all specimens correspond to a longitudinal cut proper of root middle third.

In vitro *PSs dark and aPDT cytotoxicity in hAPCs*

Zn(II)e_6Me, FotoSan® agent, RB and TMPyP were applied as PSs in total absence of light and as aPDT to cytotoxicity evaluation of hAPCs primary cells lines. RB was irradiated with a green LED of 557 nm; Zn(II)e_6Me, FotoSan® agent and TMPyP were irradiated with 627 nm LED setup. No relevant dark cytotoxicity effects were registered for any PSs, although Zn(II)e_6Me was the most biocompatible PS with constant outcomes (97.4%) at 24 h and (97.4%) at 48 h, followed by TMPyP

(94.4%) and FotoSan® agent (93.1%), **Figure 6a**. At 24 h RB had the worst outcome (82.7%). At 48 h, Zn(II)e_6Me remains as the most biocompatible, followed by FotoSan® agent (94.8%), TMPyP (85.2%) and RB (82.7%). In summary, when considering the PSs assessed without activation by light, RB is the most aggressive to hAPCs, although with no general cytotoxicity as all of them showed cell viability higher than 70%.

After irradiation, in aPDT groups, cell viability in the first 24 h revealed that the chlorophyll derivative (95.0%) and RB-PDT (94.8%) were the groups with lower cytotoxicity, with cellular viabilities similar to the control group (untreated), followed by TMPyP-PDT (75.6%), **Figure 6b**. FotoSan®-PDT was the most cytotoxic with 67.5% of viable cells. After 48 h, cell viability pattern remains similar, however overall viability decreases. At that period, the best aPDT outcome was obtained with the modified chlorophyll (87.9%) and FotoSan®-PDT reduced the number of viable cells to 66.1%. Considering 70% as the cut-off value, FotoSan®-PDT group was the only cytotoxic to the hAPCs.

As a control of the experimental conditions it was evaluated the effect of irradiating with the wavelengths used in the activation of PSs, in the total absence of natural light. The cell cultures were irradiated during 90 s and then evaluated at 24 and 48 h, **Figure 6c**. Primary cell lines of hAPCs irradiated at 557 nm have a cell viability reduction of 23.5% at 24 h and 21.1% 48 h after exposure, when compared to control group (no radiation, NO rad). The 627 nm light causes a lower damage. However, in the 557 nm group, 48 h after exposure cells had a recovery showing final outcomes similar to the 627 nm light.

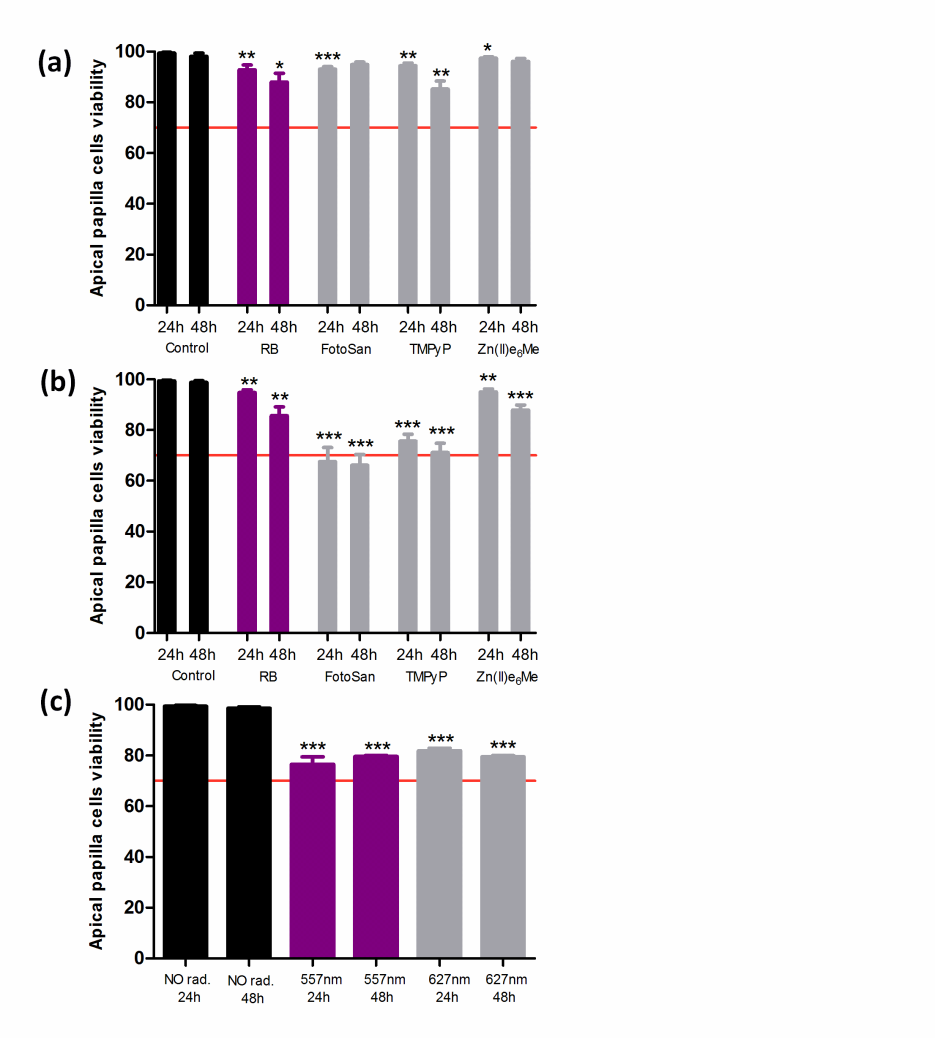

Figure 6. Apical papilla cells viability.
a) PSs dark toxicity in total absence of light applied to hAPCs evaluated at 24 and 48 h. Data are represented as mean ± SD. *P≤0.05, **P≤0.01, ***P≤0.001, compared with control cells.
b) aPDT cytotoxicity applied to hAPCs assessed at 24 and 48 h. Data are represented as mean ± SD. **P≤0.01, ***P≤0.001, compared with control group.
c) LEDs (557 and 627 nm) applied for 90s to hAPCs calculated at 24 and 48 h in total absence of light. Data are represented as mean ± SD. ***P≤0.001, compared with control cells.

In vitro *hAPCs microscopy*

The cytotoxicity effects of 90 s aPDT in the morphology hAPCs were evaluated after 24 h using fluorescence microscopy. The study showed that RB-PDT and Fotosan®-PDT were the most aggressive groups to hAPCs, followed by TMPyP-PDT, **Figure 7**.

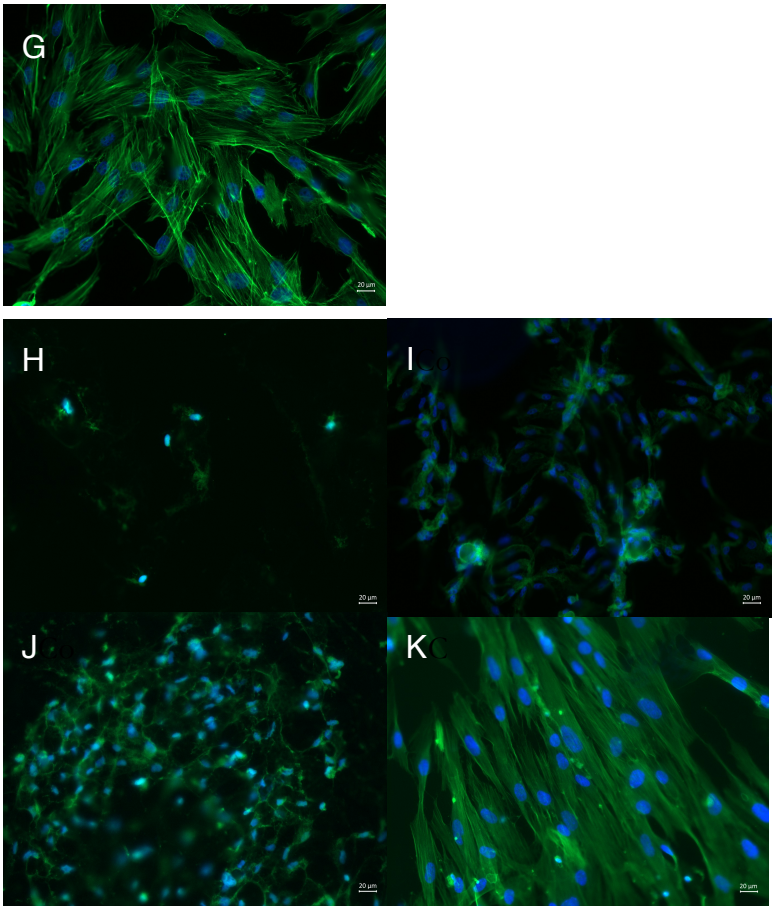

Figure 7. Microscopy of *in vitro* aPDT cytotoxicity in hAPCs.
Human apical papilla primary cells incubated with 0.1 mg/mL of PSs (900s in total absence of light) and irradiated for 90s with 557 nm LED (RB) and 627 nm LED (FotoSan®, TMPyP and Zn(II)e_6Me). Cell nucleus was stained at blue with DAPI (ThermoFisher Scientific®) and F-actin filaments were stained with Phalloidin Fluorescein Isothiocyanate (Sigma-Aldrich®). Representative images are 20 intensity projections of the top view obtained at Carl Zeiss Axio Observer Z1 with alpha plan-apochromat objective. **G** - Control; **H -** RB; **I** - FotoSan® agent; **J** - TMPyP; **K** - Zn(II)e_6Me. Bars: 20 µm.

Conversely, the chlorophyll derivative Zn(II)e_6Me-PDT application did not affect the cells as a toxic dye and, surprisingly, displayed a protective effect over hAPCs. After exposure to this PS, cells become elongated, actin cytoskeleton was more pronounced and the nuclei more marked, **Figure 7K**.

DISCUSSION

The long-term success of endodontic treatment depends on effective elimination of intracanal microorganisms and this goal is difficult to achieve with current treatment methods (Sjögren *et al.* 1997). *E. faecalis* and *C. albicans* are two of the most resistant microorganisms found in infected root canals and have a higher prevalence in secondary infections, when compared with primary infections (Molander *et al.* 1998; Peciuliene *et al.* 2001; Rios *et al.* 2011).

Antimicrobial assays on planktonic cultures are considered less relevant, because in the root canal system, they prevail in a biofilm configuration (Nair *et al.* 2005). Also, biofilms can be up to 1000X less susceptible to disinfection (Nickel *et al.* 1985) and more resistant to starvation than their planktonic counterparts (Ramage *et al.* 2001, Gao *et al.* 2016). Therefore, it was performed a mixed biofilm of both microorganisms to have a better insight into their behavior towards the tested PSs (Diogo *et al.* 2017). Earlier studies have shown that substrate is relevant for the results and human dentin is recommended for studies of disinfection of the root canal system (Basrani *et al.* 2002, Bonstein *et al.* 2010, Guerreiro-Tanomaru *et al.* 2013; Chrepa *et al.* 2014), so we decided to pursue the PSs evaluation in these *ex vivo* models.

In the current study, the best antimicrobial outcomes were obtained with aPDT performed in the presence of Zn(II)e_6Me. However, when the dentin discs were used as substrate, the chlorophyll derivative after an irradiation period of 90 s could not reach the results of 3% NaOCl treatment for the same exposure time. Even when the aPDT treatment period with Zn(II)e_6Me was enlarged ten times, for dentin discs, the antimicrobial outcomes did not rise proportionally, as it only increased 1.2 times, revealing a presumed experimental PS photobleaching.

To understand the antimicrobial disinfection pattern inside dentinal tubules, in root blocks, it was performed SEM visualization in three samples from successive depths: the infection pattern increase from the canal to the inner wall and then decrease to the outer canal in the control group, in this mixed biofilm. At the canal samples, the chlorophyll Zn(II)e_6Me had the best antimicrobial outcomes, followed by 3% NaOCl. Antimicrobial final disinfection pattern differs from aPDT to 3% NaOCl and it is possible to confirm a better Zn(II)e_6Me diffusion from the canal to the outer wall, when compared to NaOCl.

The results from the current study suggest that antimicrobial Zn(II)e_6Me-PDT has better root canal disinfection pattern inside root dentinal tubules than NaOCl. Possible explanations for this could be related to the inactivation of NaOCl by organic matter (Moorer & Wesselink 1982) and also to its compromised diffusion in biofilms (van der Waal *et al.* 2017).

Dentin discs had the worst antimicrobial outcomes when compared to root blocks and this warrant particular attention. This differential final outcome could relay into three major reasons. First, the diversity between the substrate, dentin crown/root pattern and total substrate microbial area. Second, the two LED setups have different density powers (35 $mW.cm^{-2}$ from 627 nm LED setup versus 2000-4000 $mW.cm^{-2}$ from Fotosan® 630 LAD pen). Third, FotoSan® 630 LAD pen uses an endo tip while the experimental 627 nm LED was not provided with a similar device to direct the light beam. As a consequence, dentin discs were irradiated at distance from the light source, only on one side. In what regards the root blocks, all the canal wall could be 360° irradiated with dynamic movements to optimize the distribution of light through the infected canal length, which enhances the aPDT efficacy as demonstrated in other studies (Soukos *et al.* 2006; Fimple *et al.* 2008; Beltes *et al.* 2017). The manufacturer's protocol indicates that irradiation have to be carried out

for 30 s with FotoSan® agent, however, a previous study already demonstrate better antimicrobial efficacy when irradiation time was augmented to 90 s (Poggio *et al.* 2011). In this investigation, even with this extended irradiation time, FotoSan® was less effective than 3% NaOCl in microbial reduction which corroborates other studies (Gergova *et al.* 2014).

LED luminance through human dentin discs and root blocks infected with mixed biofilms was assessed and light transmission was detected in all conditions. A previous study evaluated direct irradiation through 1 mm dentin discs infected with 48 h *Streptococcus intermedius* suspension and calculated LED power density through dentin discs. Final outcomes revealed Photofrin®-PDT exposure to QTH dental curing (500 $mW.cm^{-2}$, 400-780 nm for 5, 15 and 60 s) was effective in reducing the viability of bacteria, concluding that irradiation through dentin may be done successfully within a clinically relevant time frame (Bonstein *et al.* 2010). The aPDT efficacy was also studied when irradiation was applied from outside the tooth and with simulated surrounding tissues. The inactivation of a planktonic suspension of *Enterococcus faecalis* using two PSs: methylene blue (MB) and TMPyP were successful, demonstrating that light activation of an intracanal PS from outside of the tooth may be possible at wavelengths ≥ 430 nm (Cieplik *et al.* 2016), which is in agreement with our findings.

The potential cytotoxicity of the tested PSs was assessed against human apical papilla cells in antimicrobial effective concentrations. From the combination of cytotoxic outcomes with fluorescence microscopy images, it is possible to endorse $Zn(II)e_6Me$ as the safest PS evaluated in this assay, followed by RB which has been proven an efficient and safe PS in previous studies (Upadya and Kishen 2010; Shrestha *et al.* 2014). There are several aPDT systems available for root canal asepsis (Chrepa *et al.* 2014, Diogo *et al.* 2015). However, the antimicrobial performance and

excellent biocompatibility evidenced by Zn(II)e_6Me-PDT in this study brings new expectations to the field, deserving further evaluation to assess its clinical relevance and potential use in endodontic therapy.

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Chapter V

FINAL REMARKS

Conclusions and Future perspectives

CONCLUSIONS

Apical periodontitis is a biofilm-related disease and the root canal system is a complex structure with small anatomical intricacies that cannot completely be reached by chemomechanical root canal debridement.

In the context of chemical root canal debridement, one key element has not been yet studied in detail: how the classical endodontic irrigants affects the biofilm matrix. Biofilm matrix consists of extracellular polymeric substances, such as extracellular polysaccharides, proteins, nucleic acids, lipids and other biopolymers and the matrix core components are exopolysaccharides, consisting of monosaccharides linked by glycosidic bonds.

The anti-biofilm properties of root canal irrigants studied in the course of this work were only analyzed in experimental studies, however, the current findings were limited to *in vitro* and *ex vivo* conditions and no direct clinical conclusions should be drawn. Nonetheless, the results obtained strongly indicate that further clinical studies will highlight its importance. Before, studies have focused on the proportion of live versus dead microorganisms in the biofilm; other endodontic studies used live/dead staining with CLSM to analyze bacterial viability within biofilms, but most live/dead stains differentiate bacteria into viable or dead by means of their membrane integrity. Calculations on the total biomass and biovolume of live and dead bacteria run the risk of an overestimation due to an overlapping of the double-stained bacteria. However, conclusions based on viable bacteria alone do not sufficiently represent treatment efficacies at all. Research has suggested that endodontic irrigant solutions are uniquely effective against oral biofilms and physically remove the biofilm structure, which contrasts with other common disinfectants.

Since there is no perfect endodontic irrigant solution, further approaches have been narrated and, among all, aPDT presents auspicious outcomes. The work presented in this dissertation leads to the main conclusion that a modified chlorophyll, Zn(II)e_6Me, is a promising PS to be applied in Endodontic field and arguments to draw this conclusion are several, as follows:

I. The *in vitro* and *ex vivo* models of 48h endodontic biofilms and experimental LEDs used in this thesis are reproducible with success and could be applied to other fields.

II. Ultrastructural modification of microbial cells upon antimicrobial photodynamic therapy (aPDT) is possible to endorse, as it was performed with success using Zn(II)e_6Me as photosensitizer.

III. In general Zn(II)e_6Me-PDT had an antimicrobial efficacy similar to 3% NaOCl, the goldstandard endodontic irrigant solution, used in clinical practice.

IV. The cytotoxic outcomes were quantified and the modified chlorophyll, Zn(II)e_6Me, had the lowest dark toxicity from all PSs at 24 and 48h.

V. The final conclusion to be drawn out of this work is that Zn(II)e_6Me was the best PS option, combining better antimicrobial outcomes with lower host cells toxicity, revealing its potential to be used in Endodontic field.

FUTURE PERSPECTIVES

During the development of the present work several questions arose, much remain to be elucidated, although some of these doubts are presently being addressed.

As mentioned along the thesis, microorganisms and host cells might behave differently in clinical conditions and interactions among them must be firstly clarified. For this reason, it is considered an important issue in further studies, to investigate how Zn(II)e_6Me-PDT affects the microorganisms' cells walls in bacteria and fungi in specific biochemical pathways and the biofilm matrix.

With the surprising antimicrobial outcomes obtained with the modified chlorophyll, Zn(II)e_6Me, it is concluded that it emerges as a promising PS for antimicrobial purpose in endodontic field. However, this PS should be analyzed in detailed to understand if it affects the human root structure resistance or if it promotes tooth pigmentation, as discoloration was not visually detectable by the human eye. This must be explored in a future perspective to improve knowledge in this field before starting clinical studies with patiens. Also, before clinical trials, a new and optimal LED is essential to be constructed, to optimize the modified chlorophyll outcomes in experimental phases and also at clinical ones.

Chapter VI

SUPPLEMENTARY RESULTS

INTRODUCTION

Instrumentation with several biocompatible irrigant solutions complemented with intracanalar medication aims to have the best antimicrobial outcomes (Chong and Ford 1992), minimizing its harmful effect on periapical tissues and the majority of classical endodontic studies recommend 30 minutes (min) cycles of a copious and prolonged irrigation for antimicrobial and proteolytic purpose (Haapasalo *et al.* 2010; Sobhani *et al.* 2010; Basrani and Haapasalo 2012). In opposition, Senia *et al.* earlier in 1975 reported that 60 seconds (s) of 5.25% NaOCl is sufficient to obtain effective antimicrobial outcomes against several endodontic microorganisms at planktonic state (Senia *et al.* 1975). It is known that *in vitro* antimicrobial activity depends upon the substrate, pH, drug's sensitivity, microbial source, number of inoculated microorganisms as well as its metabolic activity and incubation period (Gomes *et al.* 2001). In fact, *in vitro* experiments are the first step in a study to evaluate the endodontic irrigant solutions efficacy, followed by *ex vivo* experiments and at last clinical trials. Also, several components of the host, namely dentin (i. e. hydroxylapatite), as inflammatory exudate entering the apical root canal in purulent infections (rich in albumins) inhibits the classical endodontic irrigants potential effects applied in root canal treatment (Haapasalo *et al.* 2000; Portenier *et al.* 2001). The root canal environment is a complex mixture of organic and inorganic components, difficult to reproduce in a laboratory setting. This dissimilarity explains, at least in part, the differences between experimental and clinical outcomes of studies concerning the efficacy of root canal antimicrobials.

Further, in clinical conditions, with a robust host environment, it is commonly recommended to employ endodontic classical irrigants in copious amounts (Ciucchi *et al.* 1989; Basrani and Haapasalo 2012), for larger period times to

eradicate the aggressive polymicrobial biofilms in endodontic infections (Chávez de Paz 2007; Lundstrom *et al.* 2010) from the root canal system.

RESULTS AND DISCUSSION

This experiment was designed to optimize the overall methodology to be followed, namely by studying the *in vitro* efficacy of clinical methodology currently used with the classical endodontic irrigants. In this study was analyzed the antimicrobial efficacy of 3% NaOCl, 17% EDTA and 2% CHX (Coltene®, Whaledent, UK) all at liquid form against 48 h mono and mixed *in vitro* biofilms, to reproduce conditions similar to the clinical practice. Furthermore, it was tested three direct contact periods of exposing the biofilms to the classical irrigants (60 s, 90 s and 30 min).

All results were obtained with safranin red (SR) assay, repeated three times for periods of seconds and five times to 30 minutes. To evaluate the validity of results, an inferential analysis was employed, namely the assessment of average differences between groups with the independent samples t-student and Mann-Whitney test. Significance level assumed was 0.05.

When applied to *E. faecalis* biofilms, all classical irrigants solutions showed the highest scores in terms of biofilm biomass removal, when compared to *C. albicans* and mixed biofilms, **Figure 1.** As classical irrigants, 3% NaOCl (90%) is the best antimicrobial irrigant solution, followed by 17% EDTA (73.7%) and 2% CHX (73.7%) with no statistically significant difference between them at the three times contact period (P=0.725).

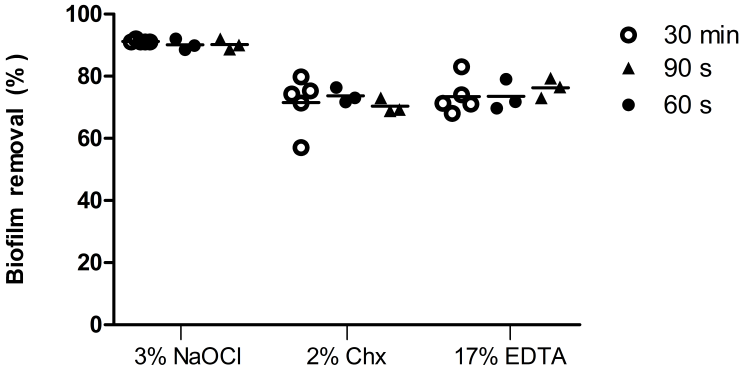

Figure 1. Different periods of exposing *E. faecalis* biofilm biomass removal (%) quantified using the safranin red (SR) assay.

For *C. albicans* biofilms, the antimicrobial pattern is comparable with *E. faecalis* biofilms, **Figure 2**. As classical irrigant solutions, 3% NaOCl (84%) is the best antimicrobial irrigant solution, followed by 17% EDTA (71%) and 2% CHX (69%), with no statistically significant difference between them at the three times contact period (P=0.608).

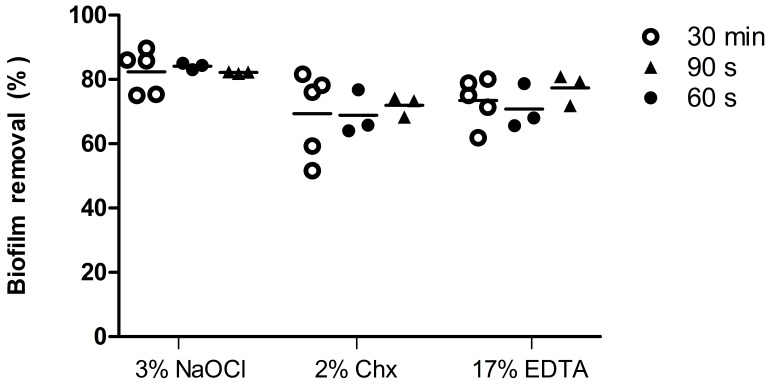

Figure 2. Different periods of exposing *C. albicans* biofilm biomass removal (%) quantified using the SR assay.

For mixed biofilms, **Figure 3**, it was obtained the lowest antimicrobial outcomes with percentage of biofilm biomass removal, <80%.

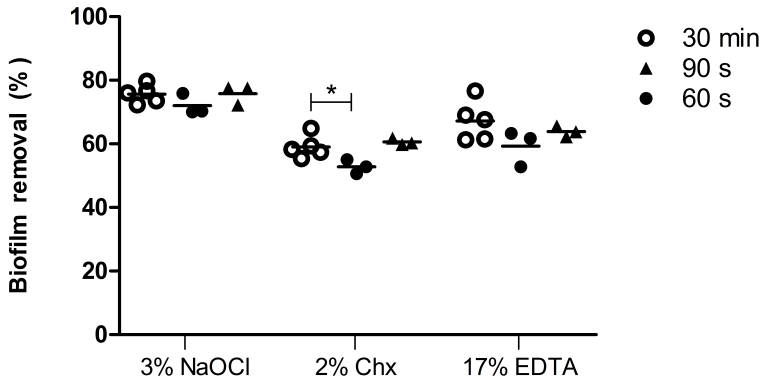

Figure 3. Mixed biofilm biomass removal (%) using with SR assay after exposition to endodontic irrigant solutions at three different direct contact periods. (* $p = 0.0357$)

The antimicrobial pattern of 3% NaOCl remains as the best irrigant solution (72%), distinctive from 17% EDTA (59.3%) and 2% CHX (53%). Although for mixed biofilms there was statistically significant difference between 2% CHX applied for 30 min and 60 s ($P \leq 0.05$) and no statistically significant difference among 90 s and 30 min ($P=0.25$). EDTA and CHX were able to remove few more than half of mixed biofilm biomass.

In terms of antibiofilms properties, 3% NaOCl was the best classical irrigant solution, followed by 17% EDTA and 2% CHX. NaOCl is very reactive with biofilms, although rapidly inactivated by organic matter (van der Wall *et al.* 2017). NaOCl high reduction-oxidation potential and high pH contributes to their saccharolytic effect (Jungbluth *et al.* 2011), by broking glycosidic bonds and dissolving glycoconjugates in the biofilm matrix. (Tawakoli *et al.* 2017). On a similar note, alkaline capacity of NaOCl solutions can contribute to their proteolytic effect (Jungbluth *et al.* 2011) and it also lysed bacterial cells.

EDTA has a recognized disruptive effect on biofilm matrix (Cavaliere *et al.* 2014) by binding of divalent cations, sequestering calcium, magnesium, zinc and iron; and dispersing bacterial cells from the matrix (Finnegan and Percival 2015). CHX

showed similar outcomes to EDTA efficacy in some studies (Hope and Wilson 2004; Bonez *et al.* 2013), while in others appear to be ineffective (Del Carpio-Perochena *et al.* 2011).

In terms of direct contact period, for the three *in vitro* biofilms types and for endodontic irrigants used, there was no statistical difference between 90 s and 30 min (P=0.29). Therefore, 90 s is the minimum direct contact period necessary to accomplish antimicrobial outcomes alongside 48h *in vitro* biofilms with no need to enlarge the period through 30 min.

For *in vitro* further investigations during the course of this project, the 30 min period was discarded in favour of 90 s. As such, in Chapter III of this Thesis only the results obtained at 60 s and 90 s were mentioned and compared with those obtained by aPDT.

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Review Article

Photodynamic Antimicrobial Chemotherapy for Root Canal System Asepsis: A Narrative Literature Review

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Aim. The aim of this comprehensive literature review was to address the question: Does photodynamic therapy (PDT) improve root canal disinfection through significant bacterial reduction in the root canal system? *Methodology.* A comprehensive narrative literature review was performed to compare PDT effect with sodium hypochlorite as the comparative classical irrigant. Two reviewers independently conducted literature searches using a combination of medical subject heading terms and key words to identify relevant studies comparing information found in 7 electronic databases from January 2000 to May 2015. A manual search was performed on bibliography of articles collected on electronic databases. Authors were contacted to ask for references of more research not detected on the prior electronic and manual searches. *Results.* The literature search provided 62 titles and abstracts, from which 29 studies were related directly to the search theme. Considering all publications, 14 (48%) showed PDT to be more efficient in antimicrobial outcome than NaOCl (0.5–6% concentration) used alone and 2 (7%) revealed similar effects between them. Toluidine blue and methylene blue are the most used photosensitizers and most commonly laser has 660 nm of wavelength with a 400 nm diameter of intracanal fiber. *Conclusions.* PDT has been used without a well-defined protocol and still remains at an experimental stage waiting for further optimization. The level of evidence available in clinical studies to answer this question is low and at high risk of bias.

1. Introduction

The main goal of endodontic treatment is to prevent and, when required, to cure apical periodontitis and maintain or reestablish periapical tissue health [1]. To accomplish this objective, it is mandatory to control the microbial load inside the root canal system. The chances of a favourable outcome with endodontic treatment are significantly higher if infection is eradicated effectively by chemomechanical preparation before the root canal is obturated. However, if positive cultures can be obtained from the root canal at the time of root filling, there is a higher risk of treatment failure [2]. In an attempt to improve disinfection, an interappointment dressing has been advocated to diminish the percentage of root canals with no cultivable microorganisms in comparison to those only treated with chemomechanical preparation. Nevertheless, the two-visit treatment protocol did not improve the overall antimicrobial efficacy of the treatment [3]. Indeed, in all cases where viable microorganisms remain in the root canal system, the prognosis for repair is adversely affected [2, 3].

Presence of a *smear layer* after instrumentation reduces effectiveness of irrigants and temporary dressings in disinfecting dentinal tubules. Moreover, complexity of anatomy translated into root canal system with its isthmuses, ramifications, and fins [4] turns complete elimination of bacteria using instrumentation and irrigation into an almost impossible task. Besides, bacteria persisting in biofilms show diverse phenotypes when compared with planktonic cells, including increased resistance to antimicrobial agents [5]. It has been assessed that bacteria in biofilms are approximately 1000-fold less susceptible to effects of commonly used antimicrobial agents than their planktonic equivalents and are highly unaffected with phagocytosis by immune system [6]. There

are several mechanisms used by bacteria which allow them to adapt to the environment [7]. Biofilm formation [8], stress response [9], physiological adaptation [7], and the beginning of subpopulations of cells are among some of the adaptive mechanisms used by bacteria along with various systems involving the exchange of genetic material [10] between bacteria. These mechanisms can support bacterial survival under the limiting environments, such as that found in the root canal. One of the most relevant features of adaptation for oral bacteria is the adhesion to surfaces leading to the formation of plaque biofilms, which not only serves to aid in their retention but also results in increased survival rate [11]. Biofilms form when planktonic bacteria in a natural liquid phase are deposited on a surface containing an organic conditioning polymeric matrix or conditioning film [7]. In this dynamic process, several organisms coadhere to the surface [12] and grow with certain cells detaching from the biofilm over time. Biofilm formation in root canals, as postulated by Svensater and Bergenholtz [13], is probably initiated at the moment of the first invasion of the pulp chamber by planktonic oral organisms after some tissue breakdown.

Biofilm disruption and disinfection of root canals are the most critical steps during treatment of an infected root canal system, which are essential to avoid persistence of microbial infection and achieve endodontic success [14]. The mode of action and efficacy of a wide variety of cleaning, antimicrobial, and disinfecting agents such as NaOCl, chlorhexidine, ethylenediamine tetraacetic acid (EDTA), citric acid, hydrogen peroxide, halogens, and ozone have been investigated [15–18]. Disinfecting agents and antimicrobial medicinal products routinely used in endodontics can be inactivated by dentin, tissue fluids, and organic matter [6, 19]. Moreover, some microbial species, such as *Enterococcus faecalis* [20, 21] and *Candida albicans* [22, 23], show resistance to those agents and their efficacy is dependent on the concentration achieved and time of contact [24]. Most of these disinfectants with effective bactericidal activity are used at subtoxic level, but also at concentrations where toxicity is becoming a significant factor. Searching for new methods to provide extra disinfection for root canal system without cytotoxic effects and to improve treatment outcome, innovative techniques including various laser wavelengths [25], hydraulic [26], sonic, and ultrasonic irrigation [27–29], nanoparticles [30], inactivation of efflux pumps [31], and photodynamic therapy (PDT) has been proposed in literature.

PDT was discovered by chance at the very beginning of the twentieth century, when a combination of nontoxic dyes exposed to visible light resulted in microorganism cell death. As reviewed by Henderson and Dougherty in 1992 [32], Oscar Raab, a medical student working with Professor Herman Von Tappeiner in Munich, introduced the concept of microbial cell death induced by interaction of light and chemicals [32]. During the course of Raab's study, he demonstrated that the combination of light and dyes was much more effective in killing the microorganism *Paramecium*.

Those observations were repeated with a diversity of uni- and multicellular organisms. Succeeding work in this laboratory coined the term *photodynamic action* and demonstrated presence of oxygen as an essential requisite for photosensitization to occur. Years later, Dougherty and coworkers clinically tested PDT in cutaneous/subcutaneous malignant tumours. However, it was John Toth who renamed this therapy as PDT. Combined effect of three elements, *light*, *PS*, and *oxygen*, has been termed *photodynamic antimicrobial chemotherapy* by Wainwright [33] and also recognized as *antimicrobial photodynamic therapy* [34] and *photoactivated disinfection* [35].

PDT uses a nontoxic dye, known as photosensitizer (PS), on a target tissue, which is consequently irradiated with a suitable visible light of the appropriate wavelength to excite the PS molecule to the singlet state in presence of oxygen to produce reactive oxygen species (ROS) [36]. When PS absorbs light, this excited state may then undergo intersystem crossing to the slightly lower energy, but the longer lived, triple state can undergo two kinds of pathways known as Type I (reacting with the substrate) and Type II (reacting with molecular oxygen) photoprocesses. Both pathways require oxygen.

The *type 1 radical and reactive oxygen species* pathway comprises an *electron transfer step* between the triplet PS and a substrate with generation of radical species. The finalist is then intercepted by ground state molecular oxygen yielding a variety of oxidized products. The baseline PS has two electrons in opposite spins (singlet state) in the low energy molecular orbital. Subsequent to the absorption of light, one of these electrons is boosted into a high-energy orbital but keeps its spin (first excited singlet state). This is a short-lived time species, nanoseconds, and can lose its energy by emitting light (fluorescence) or by internal conversion into heat. Type 1 pathway frequently involves initial production of superoxide anion by electron transfer from the triplet PS to molecular oxygen (monovalent reduction) initiating radical-induced damage in biomolecules. Superoxide is not particularly reactive in biological systems and does not by itself cause much oxidative damage but can react with itself to produce hydrogen peroxide and oxygen, a reaction known as *dismutation* that can be catalyzed by the enzyme superoxide dismutase (SOD). The way of the electron relocation between the PS and the substrate is controlled by the relative redox potentials of the two species.

Type 2 pathway, *singlet oxygen*, involves an electronic *energy transfer process* from the triplet PS to a receptor, most frequently oxygen, which is a triplet in its ground state. The final compound is converted to a highly reactive species, the singlet oxygen ($^1O_2$). The excited singlet state PS may also undergo the process known as *intersystem crossing* whereby the spin of the excited electron inverts to form the relatively long-lived, in terms of microseconds, excited triplet state that has parallel electron spins. The long lifetime of the PS triplet state is explained by the fact that the loss of energy by emission of light (phosphorescence) is a *spin forbidden* process, as the PS would move directly from a triplet to a singlet state. Photosensitized processes of types 1 and 2 depend on the initial involvement of radical intermediates that are subsequently scavenged by oxygen or the generation of the highly cytotoxic singlet oxygen ($^1O_2$) by energy transfer from the photoexcited sensitizer. It is difficult to determine

without doubt which of these two mechanisms is more prevalent; both types of reactions can happen simultaneously and the ratio between them depends on three singular features: oxygen, substrate concentration, and PS type [37].

Hamblin and Hasan in 2004 [36] stated that antimicrobial PS can be divided into three categories: (I) those that strongly bind and penetrate the microorganisms (chlorin e6), (II) those that bind weakly as toluidine blue (TB) and methylene blue (MB), and (III) those that do not demonstrate binding at all such as rose bengal (RB). Understanding these mechanisms of action is essential because, in bacterial cells, outer membrane damage plays an imperative role, differently from mammalian cells, where the main targets for PDT are lysosomes, mitochondria, and plasma membranes [38]. Typically, neutral anionic or cationic PS molecules could powerfully destroy Gram-positive bacteria, whereas only cationic PS or strategies which attack the Gram-negative permeability barrier in combination with noncationic PS are able to kill multiple logs of Gram-negative species [39]. This difference in susceptibility between species in the two bacterial types is explained by their cell wall physiology. To understand better the PDT effect in those microorganisms, it is very important to analyse in detail the microbial cell walls. In *Gram-positive bacteria,* the cytoplasmic membrane is surround by a relatively porous peptidoglycan layer and lipoteichoic acid that allows the PS to cross. Different from this, the *Gram-negative bacteria* cell envelope consists of an inner and an outer membrane which are separated by a peptidoglycan layer. The outer membrane forms an effective permeability barrier between the cell and the environment and tends to restrict the binding and penetration of several PS. Fungi are provided with a thick cell wall that includes beta glucan and chitin offering a permeability barrier. In terms of PDT efficacy, in fungal wall, it was described as having an intermediate behavior between Gram-positive and Gram-negative bacteria [40]. On the basis of these considerations, it appears that Gram-negative bacteria represent the most challenging targets for any form of antimicrobial treatment. The mechanism of action of basic polymer PS conjugates is thought to be that of *self-promoted uptake pathway* [41]. In this method, cationic molecules first dislocate the divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), from their position on the outer membrane where they act as an anchor for the negatively charged lipopolysaccharides molecules [40, 41]. The debilitated outer membrane becomes slightly more permeable and allows even more of the cationic PS to gain access thus steadily increasing the disorganizations of the permeability barrier increasing PS uptake with each additional binding. It is thought that host cells only gradually take up cationic molecules by the process of endocytosis, while their uptake into bacteria is relatively fast [39]. Further important observation that has been made about these cationic antimicrobial PS concerns their selectivity for microbial cells compared to host mammalian cells [37]. These findings are relevant, because photoaction occurs in direct contact with membranes [42]. The PS efficiency in generating ROS within membranes is dependent on the intrinsic characteristics of the PS in aqueous solution as well as their partition in the membrane [42]. The early attack of singlet oxygen in membranes lipids is by the specific reaction with double bonds to form allylic hydroperoxides; the efficiency of this reaction is dependent on the lowest ionization potential of the olefins and also on the availability of allylic hydrogens [42]. *Photodynamic lipid peroxidation* is an oxidative degradation of cell membrane lipids, also known as *photoperoxidation*, and it has been related to several microbial cytotoxic effects, such as increased ion permeability, fluidity loss, inactivation of membrane proteins, and cross-linking, which disrupts the intracellular homeostasis. Consequently, necrosis is induced as a cell death process. A probable explanation is that PS bound to the membrane and generates most of the singlet oxygen, $^1O_2$, involved in photoperoxidation [43] highlighting the double selectivity (light and PS cellular localization) and the fact that it works in multiresistant strains and does not encourage resistance [42]. PDT's lethal action is based on photochemical production of ROS and not thermal and cavitation effects, as is the case with high power laser therapy [44]. One of several PDT's advantages clinically is the absence of thermal side effects in periradicular tissues [45] and this property of PDT aspect makes it highly effective in eradicating microorganisms such as bacteria [45], viruses [46], and fungi [47] without causing damage of adjacent tissues due to overheating [45].

In recent years, PDT has been applied in several areas, particularly in periodontology [48–50], in general dentistry [51] and also in endodontic field as an adjunct of classical irrigation solutions in root canal disinfection [52, 53]. These studies suggest PDT's potential as a therapeutic weapon, which aims to support endodontic antimicrobial treatment, especially enhancing irrigation solutions effect. The purpose of this narrative comprehensive literature review is to answer the focused question, "*Is antimicrobial PDT efficacy better than that of sodium hypochlorite's in root canal treatment?*" For this analysis of the literature, we selected and analysed 29 studies using antimicrobial PDT in endodontic field, highlighting methodologies used and their reported effectiveness and efficacy.

2. Materials and Methods

2.1. Criteria in Selection of Studies. For this comprehensive narrative literature review [54], eligibility criteria were (I) articles published in English language; (II) original papers; (III) experimental studies (*in vitro* and *ex vivo*); (IV) clinical studies (*in vivo*); and finally (V) scientific reports of PDT efficacy in root canal disinfection/asepsis. The exclusion criteria were (I) unpublished data, (II) conference papers, (III) historic reviews, (IV) letters to editor, and (V) papers due to PDT outcomes in other fields (outside of endodontics).

As a first step, the aim was to investigate the terms "Endodontic", "Photodynamic Therapy", and "Antimicrobial Disinfection". Briefly, we used PubMed to identify Medical Subject Headings (MeSH) terms corresponding to each term. Nevertheless, MeSH terms use is not common to all articles, making this search method infeasible. Then, exhaustive automated searches of Cochrane Collaboration, Evidence Based Dentistry (EBD), Journal of Evidence-Based Dental Practice (JEBDP), NHS Evidence, and PubMed (Figure 1) were performed from January 2000 up to and including May 2015

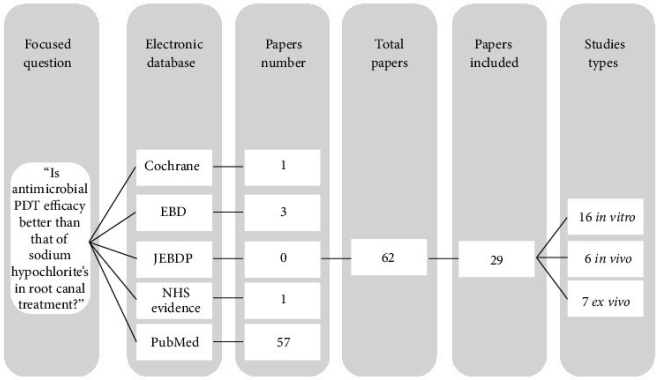

Figure 1: Identification of studies used in this narrative review.

using various combinations of the following key indexing terms: (a) *endodontic photodynamic therapy*; (b) *antimicrobial photodynamic therapy*; (c) *photo-activated disinfection*; (d) *light-activated disinfection*; (e) *laser-assisted photosensitization*; (f) *root canal disinfection*; and (g) *endodontic lasers*.

Titles and abstracts of all articles resulting from electronic search were screened independently and in duplicate by 2 reviewers. The review itself was performed to reject articles that did not meet inclusion criteria. Any disagreement between reviewers was solved via debate, although in specific cases of disagreement that were not resolved with discussion, opinion of a senior commentator was required. Hand searching of reference lists of original and reviewed articles that were found to be relevant was also performed.

In a second step, full-text copies of all remaining articles were obtained and further meticulous assessment was performed independently by each reviewer to determine whether or not they were eligible for this study based on the specific inclusion and exclusion criteria cited above and proven for agreement.

Quality evaluation of randomized clinical trials and observational studies was performed using STROBE [55] (strengthening the reporting of observational studies in epidemiology) and CONSORT [56] (consolidated standards of reporting trials) statement criteria, respectively.

3. Results

3.1. PDT Antimicrobial Efficacy in Included Studies. Literature search provided 62 titles and abstracts; from those, 29 studies concerned this theme: 16 were performed in *in vitro* conditions, 6 were *in vivo* studies, and the last 7 readings were *ex vivo*. From all 29 papers included in this review, 16 (55.2%) were *in vitro* studies (Table 1).

In data processing, authors classified all studies in three categories: *category I*, *in vitro*; *category II*, *in vivo*; and finally, *category III*, *ex vivo*, to describe and clarify studies' details. In category I, 16 *in vitro* studies, only 5 (31%) [57–61] reveal best antimicrobial PDT outcomes when compared with sodium hypochlorite (NaOCl) in range of 0.5 to 6%. Only one study performed by Nagayoshi et al. [62] reveals equal results between PDT and NaOCl; the remaining 10 (62.5%) studies [63–72] showed PDT outcomes unhelpful when compared with NaOCl as a classical irrigant solution, in concentration range of 0.5 to 6%. In category II, 6 (21%) papers [35, 58, 73–76] were analysed (Table 2).

All were performed in the human dentition, five [35, 58, 73, 74, 76] were performed in permanent dentition, and only one was achieved in deciduous teeth by Prabhakar et al. [75]. All studies in category II (100%) presented that PDT efficacy overthrew (0.5–2.5%) NaOCl. Considering tooth type and its influence in PDT efficacy outcomes, Garcez et al. group [58, 74] and Jurič et al. [76] tested only permanent uniradicular human teeth (incisors and canines) as samples. However, Prabhakar et al. [75] considered deciduous molars as a prerequisite for his study. Finally, Bonsor et al. [35, 73] used not only uniradicular but also permanent multiradicular teeth. In terms of endodontic diagnosis, Garcez et al. [58] in his first study used patients with necrotic pulps and periapical lesion; then, in 2010, his group [74] performed a second study to assess PDT efficacy in teeth with previous endodontic treatment, endodontic retreatment. Jurič et al. [76] in 2014 evaluated PDT antimicrobial outcomes efficacy applied also in endodontic retreatment. Both studies [74, 76] revealed PDT outcomes near 100% effective.

In category III (*ex vivo*), 7 (24%) papers [5, 77–82] were analysed (Table 3).

Based on this, 3 (43%) studies [5, 78, 79] revealed superior PDT outcomes compared to 0.5–6% of NaOCl and in one study by Xhevdet et al. group [81] showed 2.5% NaOCl

Table 1: *In vitro* studies compilation.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
				In vitro, 16 studies			
Seal et al. 2002 [63]	*Test groups:* Group #1: PDT with 20 combinations of 4 TBO concentrations and 5 laser energy doses ($n = 4$). TBO (12.5, 25, 50, and 100 mg mL^{-1}). Incubated for 30 s. Laser (60, 90, 120, 300, and 600 s). Energy dose (2.1, 3.2, 4.2, 10.5, and 21). Group #2: NaOCl ($n = 4$). *Control group:* Light source: Canals ($n = 4$) were filled with reduced transport fluid (RTF) for 30 s followed by application of various laser light doses (60, 90, 120, 300, or 600 s). TBO only: Canals ($n = 4$) were filled with TBO at various concentrations (12.5, 25, 50, or 100 mg mL^{-1}) and incubated for 30 s. No treatment: Canals ($n = 17$) were filled with RTF and incubated for 30 s.	3	*S. intermedius* (strain NS)	TBO [12.5, 25, 50, 100 μg mL^{-1}] Preincubation time (PIT): 30 s	Helium-neon [λ632.8 nm] Irradiation time (IT): 60 s, 90 s, 120 s, 300 s, and 600 s	Cell viability Colony-forming unit – CFU ($\log_{10}$)	PDT is bactericidal to *S. intermedius* biofilms in root canals but is not as effective as irrigation with 3% NaOCl.
				Sample: 35 root canals from human uniradicular teeth			
Silva Garcez et al. 2006 [57]	*Test groups:* Group #1: L[−] AZ[−] Group #2: L⁺ AZ[−] Group #3: L[−] AZ⁺ Group #4: L⁺ AZ⁺ Group #5: NaOCl *Control group:* Canals filled with BHI broth and incubated for 24 h.	0.5	*Enterococcus faecalis* (ATCC14894)	AZ paste [0.01%] PIT: 300 s Paste composition: urea peroxide 10%, detergent 15% (Tween 80) and vehicle 75% (carbowax).	GaAlAs diode [λ685 nm] IT: 180 s	Cell viability CFU ($\log_{10}$)	In root canals, PDT showed 99.2% *E. faecalis* reduction, whereas 0.5% NaOCl achieved 93.25%.
				Sample: 30 root canals from human uniradicular teeth (upper central incisors and upper canines)			
Garcez et al. 2007 [34]	*Test groups:* Group #1: PDT Group #2: RCT (root canal treatment) with NaOCl Group #3: Combined treatment (PDT + ET with NaOCl) *Control group:* Teeth with 3-day biofilms + BHI for 24 h	2.5	*Proteus mirabilis* (XEN44) *Pseudomonas aeruginosa* (XEN5)	PEI/e6 [NS] PIT: 600 s	MMOptics [λ660 nm] IT: NS	Bioluminescence imaging Cell viability CFU ($\log_{10}$)	NaOCl reduced bacteria by 90% while PDT alone reduced bioluminescence by 95%.
				Sample: 10 root canals from uniradicular human teeth (upper central incisors and upper canines)			

Table 1: Continued.

Study type	Groups	% NaOCl	Substracte	Photosensitizer	Laser	Parameters evaluated	Condusion
George and Kishen 2008 [59, 103] *In vitro* *Ex vivo*	*Test groups:* Group #1: RCT with NaOCl Group #2: PDT Group #3: RCT + PDT in an emulsion of H_2O_2 : triton-X100 in the ratio 75 : 24.5 : 0.5 Group #4: RCT + an emulsion of H_2O_2 : triton-X100 in the ratio 75 : 24.5 : 0.5 *Control group:* Root canal not subject to any treatment	IV: 1 EV: 5.2	*E. faecalis* (strain NS)	MB [1.5, 10, 15, 20, 25 *μ*M] PIT: 600 s (in the dark) Dark toxicity was evaluated Perfluorodecalydronaphthalene (oxygen carrier) H_2O_2 (oxider) Triton-X100 (nonionic detergent)	Power Technology Inc. [*λ*664 nm] IT: NS	CSLM Photooxidation activity Singlet oxygen generation Cell viability CFU (log_{10})	NaOCl showed no viable bacteria after 4 h, but 60% of the root canal shavings confirmed bacterial growth after 24 h. PDT alone or + NaOCl showed the absence of bacteria even after 24 h.
	Sample: *in vitro*: *E. faecalis* biofilms grown on a glass coverslip that was fixed covering a grove (6 mm diameter) made at the bottom part of a Petri dish *Ex vivo* (16–24 years): 30 root canals from human uniradicular teeth (anterior teeth)						
Meire et al. 2009 [64] *In vitro* *Ex vivo*	*Test groups:* Group #1: NdYAG laser ($n = 10$) Group #2: KTP laser ($n = 10$) Group #3: PDT ($n = 10$) Group #4: NaOCl ($n = 10$) *Control group:* Group #5: teeth with no treatment ($n = 20$) – positive control Group #6: uninoculated teeth ($n = 3$) – negative control	2.5	*E. faecalis* (ATCC10541)	TBO [12.7 mg mL^{-1}] PIT: 120 s	Dentotex [*λ*635 nm] IT: 150 s	Cell viability CFU (log_{10}) Solid phase cytometry Epifluorescence microscopy	PDT was less effective than NaOCl (15 min) in reducing *E. faecalis*, both in aqueous suspension and in the infected tooth model.
	Sample: 60 uniradicular human teeth						
Souza et al.2010 [65]	*Test groups:* Group #1: PDT with MB + NaOCl ($n = 16$) Group #2: PDT with TBO + NaOCl ($n = 10$) Group #3: PDT with MB + NaCl ($n = 16$) Group #4: PDT with TBO + NaCl ($n = 16$) *Control groups: Ø*	2.5	*E. faecalis* (MB35)	MB/TBO [15/15 *μ*g mL^{-1}] PIT: 120 s	MMOptics [*λ*660 nm] IT: 240 s	SEM Cell viability CFU (log_{10})	PDT did not significantly enhance disinfection after chemomechanical preparation using NaOCl as irrigant.
	Sample: 70 uniradicular human teeth						
Nagayoshi et al. 2011 [62]	*Test groups:* Group #1: 5 W, 30 s, PS (+) Group #2: 5 W, 60 s, PS (+) Group #3: 5 W, 120 s, PS (+) Group #4: 5 W, 120 s, PS (−) *Control groups:* Group #5: NaCl: negative control Group #6: NaOCl: positive control	2.5	*E. faecalis* (ATCC29212)	Indocyanine green [12. mg mL^{-1}] PIT: 60 s	P-Laser [*λ*805 nm] IT: 30, 60, 120 s	Cell viability CFU (log_{10}) Temperature	PDT had nearly the same antimicrobial effect as 2.5% NaOCl.
	Sample: *in vitro* model of apical periodontitis in resin blocks						

Table 1: Continued.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
Nunes et al. 2011 [66]	*Test groups*: Group #1: OF/IT90 ($n = 10$) Group #2: OF/IT180 ($n = 10$) Group #3: NOF/IT90 ($n = 10$) Group #4: NOF/IT180 ($n = 10$) *Control groups*: Group #5: untreated ($n = 10$) Group #6: NaOCl: positive control ($n = 10$)	1	*E. faecalis* (ATCC29212)	MB [100 μg mL^{-1}] PIT: 300 s	Thera Lase [λ660 nm] IT: 90, 180 s	Cell viability CFU ($\log_{10}$)	The highest percentage of *E. faecalis* reduction was achieved with NaOCl. The use of intracanal fiber during PDT does not reveal improvement.
				Sample: 60 uniradicular human teeth			
Poggio et al. 2011 [67]	*Test groups*: Group #1: PDT ($n = 10$) Group #2: PDT + NaOCl ($n = 10$) Group #3: TBO ($n = 10$) Group #4: PDT ($n = 10$) – more time than in group 1 *Control groups*: Group #5: NaOCl: positive control ($n = 10$)	0.5 5	*Streptococcus mutans* (CCUG35176) *E. faecalis* (ATCC19433) *Streptococcus sanguis* (CCUG17826)	TBO [100 μg mL^{-1}] PIT: 60 s	FotoSan [λ628 nm] IT: 30, 60 s	Cell viability	*In vitro* antimicrobial efficacy of 5% NaOCl is higher than PDT.
				Sample: 100 root canals from human uniradicular teeth			
Rios et al. 2011 [68]	*Test groups*: Group #1: NaOCl Group #2: TBO Group #3: Light Group #4: PDT Group #5: PDT + NaOCl *Control groups*: The experimental conditions were repeated seven independent times with 15 total experimental samples. Both negative (no growth) and positive (growth without any treatment) controls were done for each independent experiment.	6	*E. faecalis* (OG1X) A derivative of an oral isolate that has been shown to be cariogenic	TBO [NS] PIT: 30 s	FotoSan [λ628 nm] IT: 30 s	SEM Cell viability CFU ($\log_{10}$)	The bacterial survival rate of the NaOCl/PDT group (0.1%) was significantly lower than the NaOCl (0.66%) and PDT groups (2.9%).
				Sample: uniradicular human teeth (total number of teeth unknown)			
Cheng et al. 2012 [69]	*Test groups*: Group #1: Nd:YAG Group #2: Er:YAG/NaOCl/NS/DW Group #3: Er:YAG/NS/DW Group #4: Er,Cr:YSGG Group #5: PDT *Control groups*: Group #6: NaOCl: positive control Group #7: normal saline: negative control	5.25	*E. faecalis* (ATCC4083)	MB [50 μg mL^{-1}] PIT: 60 s	Nd:YAG [λ1064 nm] IT: 16 s Er:YAG [λ2940 nm] IT: 20 s Er,Cr:YSGG [λ2780 nm] IT: 4 s Lit-601 [λ660 nm] IT: 60 s	SEM Cell viability CFU ($\log_{10}$)	PDT was less effective than NaOCl at surface of the root and 100, 200, and 300 μm inside the dentinal tubule.
				Sample: 220 uniradicular human teeth			

Table 1: Continued.

Study type	Groups	% NaOCl	Substracte	Photosensitizer	Laser	Parameters evaluated	Conclusion
Vaziri et al. 2012 [70]	*Test groups*: Group #1: NaOCl ($n = 15$) Group #2: Laser + NaOCl ($n = 15$) Group #3: PDT ($n = 15$) Group #4: PDT + NaOCl ($n = 15$) Group #5: chlorhexidine ($n = 15$) *Control groups*: Group #6: no treatment: positive control Group #7: without inoculation of bacterium: negative control	2.5	*E. faecalis* (ATCC29212)	TBO [15 μg mL^{-1}] PIT: 300 s	FotoSan [λ625 nm] IT: 60 s	Cell viability CFU ($\log_{10}$)	NaOCl showed better results than PDT. However, PDT + NaOCl showed the best result.
	Sample: 90 root canals from 90 uniradicular human teeth						
Pileggi et al. 2013 [60]	*Test groups*: Group #1: PDT (Eosin-Y) with Light+ and L− Group #2: PDT (Rose bengal) with Light+ and L− Group #3: PDT (Curcumin) with Light+ and L− *Control groups*: Group #4: NaOCl positive control	3	*E. faecalis* (13573?)	Eosyn.-Y/RB/curcumin [50 μg mL^{-1}] PIT: 1800 s	Optilux 501 [λ380–500 nm] IT: 240 s	Cell viability CFU ($\log_{10}$)	In BS, PDT significantly reduced *E. faecalis* viability. For biofilm, PDT completely suppressed *E. faecalis*.
	Sample: *E. faecalis* 13573? culture collection of the University Hospitals of Geneva. CH was used for the inactivation assays because of its prominent role in endodontic infections						
Bumb et al. 2014 [61]	*Test groups*:Group #1: PDT (MB) with Light+ *Control groups*: Group #2: no treatment ($n = 10$) Positive control	3	*E. faecalis* (ATCC29212)	MB [25 mg mL^{-1}] PIT: 600 s	Diode laser [λ910 nm] IT: NS	SEM Cell viability CFU ($\log_{10}$)	Bacterial reduction in PDT group was 96.70%. PDT potential to be used as an adjunctive antimicrobial procedure.
	Sample: 20 uniradicular human teeth						

Table 1: Continued.

Study type	Groups	% NaOCl	Substracte	Photosensitizer	Laser	Parameters evaluated	Conclusion
Gergova et al. 2015 [71]	*Test groups*:Group #1: lasers ($n = 40$) #1.1: Nd:YAG ($n = 20$) #1.2.: diode ($n = 20$) Group #2: PDT ($n = 60$) #2.1: FotoSan ($n = 20$) #2.2: without laser – dark control ($n = 20$) #2.3: without PS – light control ($n = 20$) Group #3: iontophoresis ($n = 120$) #3.1: Cupral ($n = 40$) #3.2: $Ca(OH)_2$ ($n = 40$) #3.3: I_2/KI, ($n = 40$) Group #4 ($n = 60$) #4.1: 2% Chx ($n = 20$) #4.2: 2.5% NaOCl ($n = 20$) #4.3: 30% H_2O_2 ($n = 20$) *Control groups*: Group #5: PBS ($n = 20$) Positive control	2.5	Two control strains from the American Type Culture Collection (ATCC): Methicillin sensitive *Staphylococcus aureus* (ATCC29213) *E. faecalis* (ATCC29212) Clinical isolates served as multidrug-resistant: *S. pyogenes* *S. intermedius* *E. coli K. pneumonia* *E. cloacae* *S. marcescens* *M. morganii* *P. aeruginosa* *A. baumannii* *C. albicans*	TBO [15 μg mL^{-1}] PIT: NS	FotoSan [λ625 nm] IT: 300 s	SEM Cell viability CFU ($\log_{10}$) X-ray laser particle sizer	2.5% NaOCl is the most satisfactory result; however, PDT with FotoSan, H_2O_2, and all tested types of iontrphoresis all showed strong disinfection potential without statistical significance.
	Sample: 300 uniradicular human teeth						
Wang et al. 2015 [72]	*Test groups*:Group #1: PDT ($n = 10$) Group #2: ultrasonic irrigation + NaOCl #2.1: US + 0.5% NaOCl ($n = 10$) #2.2: US + 1% NaOCl ($n = 10$) #2.3: US + 2% NaOCl ($n = 10$) #2.4: US + 2.5% NaOCl ($n = 10$) #2.5: US + 5.25% NaOCl ($n = 10$) Group #3: ultrasonic irrigation + PDT + NaOCl #3.1: US + PDT + 0.5% NaOCl ($n = 10$) #3.2: US + PDT + 1% NaOCl ($n = 10$) #3.3: US + PDT + 2% NaOCl ($n = 10$) #3.4: US + PDT + 2.5% NaOCl ($n = 10$) #3.5: US + PDT + 5.25% NaOCl ($n = 10$) *Control groups*: Group #4: ultrasonic irrigation with 0.9% NaCl ($n = 10$) Negative control	0.5 1 2 2.5 5.25	*E. faecalis* (ATCC33186)	MB [100 μM] PIT: 600 s	Diode laser [λ670 nm] IT: 300 s	SEM Cell viability CFU ($\log_{10}$)	PDT alone is less efficient than even the 0.5% NaOCl ultrasonic irrigation under the condition of this experiment.
	Sample: 120 intact bovine incisors						

Table 2: *In vivo* studies collection.

Study type	Groups	% NaOCl	Substracte	Photosensitizer	Laser	Parameters evaluated	Conclusion
In vivo, 6 studies							
Bonsor et al. 2006 [35, 73]. Private general dental practice in Scotland by the same operator, UK.	Group #1 (73% molars): Three samples ($n = 32$): (1.1) After gaining access to the root canal. (1.2) After apex location and PDT process carried out. (1.3) After completion of the canal preparation using citric acid and NaOCl. Group #2 (76% molars): Three samples ($n = 32$): (2.1) After gaining access to the root canal. (2.2) After conventional preparation using 20% citric acid and NaOCl. (2.3) After a subsequent PDT. *Control groups*: ∅ Random allocation? Yes	2.25	Human dentine of the canal's walls. No attempt was made to identify the specific bacterial flora during the culturing process.	TBO [12.7 mgL^{-1}] PIT: 60 s	SaveDent Diode laser [λ633 ± 2 nm] IT: 120 s	Scores for levels of infection	PDT showed best results (93%) when compared to conventional irrigants solutions like NaOCl and acid citric (76%).
Bonsor et al. 2006 [35, 73]. Private general dental practice in Scotland by the same operator, UK.	Sample (16–70 years): 64 root canals with closed apices randomly selected from uni- and multiradicular teeth of 14 healthier patients presented with symptoms of irreversible pulpitis or periradicular periodontitis Group #1: Three samples ($n = 30$) (1.1) After gaining access to the root canal. (1.2) After conventional endodontic therapy with NaOCl (1.3) After PDT. *Control groups*: ∅ Random allocation? Yes	2.25	Human dentine of the canal's walls. No attempt was made to identify the specific bacterial flora during the culturing process.	TBO [NS] PIT: 60 s	SaveDent Diode laser [λ633 ± 2 nm] IT: 60, 120 s	Scores for levels of infection	PDT showed best results when compared to conventional irrigant solutions.
Gutzer et al. 2008 [58]. Private dental practice in São Paulo by the same operator, Brazil.	Sample (16–70 years): 64 root canals with closed apices randomly selected from uni- and multiradicular teeth of 14 healthier patients presented with symptoms of irreversible pulpitis or periradicular periodontitis Group #1: Three samples ($n = 30$) (1.1) After gaining access to the root canal. (1.2) After conventional endodontic therapy with NaOCl. (1.3) After PDT. Group #2: Two samples after 1 week with $Ca(OH)_2$. (2.1) After 2nd conventional endodontic therapy with NaOCl. (2.2) After 2nd PDT. *Control groups*: ∅ Random allocation? Yes	2.5	Human dentine of the canal's walls. No attempt was made to identify the specific bacterial flora during the culturing process.	PEI:e6 [60 μmolL^{-1}] PIT: 120 s	MMOptics Diode laser [λ660 nm] IT: 240 s	Cell viability CFU (log_{10})	The use of PDT leads to a significant further reduction of bacterial load, and a second appointment PDT is even more effective than the first.
Sample (21–35 years): 20 selected cases of patients presenting with symptoms of irreversible pulpitis or periradicular periodontitis in anterior teeth (incisors and canines) selected at random							

Table 2: Continued.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
Garcez et al. 2010 [74]. Private dental practice in São Paulo by the same operator, Brazil.	Group #1: Three samples ($n = 30$) (1.1) After gaining access to the root canal. (1.2) After conventional endodontic therapy with NaOCl. (1.3) After PDT. *Control groups:* ø Random allocation? No	2.5	Biofilms At least 1 microorganism resistant to antibiotic medication.	PEI.e6 [≈39 $\mu g\ mL^{-1}$] PIT: 120 s	MMOptics Diode laser [A660 nm] IT: 240 s	Microbiological identification Antibiogram analyses Cell viability CFU (log_{10})	NaOCl reduced to 0.8 species per root canal. After PDT, microorganism growth was not detected on any of the samples.
	Sample (37–52 years): 30 anterior uniradicular human teeth with previous endodontic treatment from 21 patients without random allocation						
Prabhakar et al. 2013 [75]. Department of Pedodontics and Preventive Dentistry, Bapuji Dental College and Hospital, Davangere, Karnataka, India.	Group #1: Three samples ($n = 12$) (1.1) After gaining access to the root canal. (1.2) After conventional endodontic therapy with NaOCl (1.3) After PDT. *Control groups:* ø Random allocation? No	0.5	Culture samples	MB [50 $\mu g\ mL^{-1}$] PIT: 300 s	Silberauer low level laser Diode laser [A660 nm] IT: NS	Cell viability CFU (log_{10})	PDT showed best results than NaOCl.
	Sample (4–7 years): 12 human deciduous molars with caries lesions affecting the pulp and diagnosed as necrotic pulps (pulpectomies) from twelve children without random allocation						
Jurič et al. 2014 [76]	Group #1: Three samples ($n = 21$) (1.1) After gaining access to the root canal (initial) (1.2) After chemomechanical preparation (1.3) After chemomechanical preparation + PDT *Control groups:* ø Random allocation? Yes	2.5	Biofilms	Helbo blue PS [10 $mg\ mL^{-1}$] PIT: 120 s Phenothiazinium chloride	Helbo system Diode laser [A660 nm] IT: 60 s	Microbiological identification Cell viability CFU (log_{10})	Although endodontic re-treatment (ERT) alone produced a significant reduction in the number of bacteria species, the combination of ERT + PDT was statistically more effective.
	Sample (20–45 years): 21 anterior uniradicular human teeth (incisors or canines) with previous endodontic treatment from 21 patients with random allocation						

Table 3: *Ex vivo* studies compilation.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
Ex vivo, 7 studies							
Lim et al. 2009 [77]	*Experiment #1* *Test groups*:Group #1: laser ($n = 10$) Group #2: PDT + PS in water ($n = 10$) Group #3: NaOCl ($n = 10$) Group #4: PDT + PS in Mix ($n = 10$) *Control groups*:Group #5: no treatment ($n = 15$); positive control *Experiment #2* *Test groups*: Group #1: PDT + PS in water ($n = 6$) Group #2: PDT + PS in Mix ($n = 6$) Group #3: cleaning and shaping ($n = 6$) Group #4: PDT + PS in Mix + cleaning and shaping ($n = 6$) *Control groups*: Group #5: no treatment ($n = 6$); positive control	5.25	*E. faecalis* (ATCC29212)	MB [100 μM] PIT: NS Dissolved in water and MIX	Model PPM35 [1460 nm] IT: 1200 s	Cell viability CFU ($\log_{10}$)	NaOCl showed best results that conventional PDT.
Sample: 85 freshly extracted unirradicular human teeth							
Ng et al. 2011 [78]	*Test groups*:Group #1: chemomechanical debridement with NaOCl ($n = 26$) Group #2: PDT + chemomechanical debridement with NaOCl ($n = 26$) *Control groups*: Ø	6	Human intracanal dentinal shavings	MB [50 μg mL^{-1}] PIT: 300 s	BWTEK Inc. [1465 nm] IT: 150 s-break 150 s-150 s	DNA probes Cell viability CFU ($\log_{10}$)	PDT + NaOCl showed better results when compared to NaOCl alone.
Sample: 52 freshly extracted human teeth with pulpal necrosis (9 incisors, 5 canines, 12 premolars, and 26 molars)							

Table 3: Continued.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
Stojicic et al. 2013 [5]	*Test groups*:Group #1: 0.1% EDTA + 0.1% H_2O_2 (1 min) Group #2: 0.1% EDTA + 0.1% Chx (1 min) Group #3: MB 15 (PIT = 5 min) 1 min LASER Group #4: MB 100 (no PIT) 1 min LASER Group #5: MB 100 (PIT = 5 min) 1 min LASER Group #6: MB 100 (PIT = 5 min) + 0.1% EDTA + 0.1% H_2O_2 1 min LASER Group #7: MB 100 (PIT = 5 min) + 0.1% EDTA + 0.1% Chx 1 min LASER Group #8: 2% CHX 1 min Group #9: 1% NaOCl 1 min Group #10: 2% NaOCl 1 min *Control groups*: Group #11: 1 mL of sterile water for 6 min: positive control	1.0 2.0	*E. faecalis* (VP3-181, VP3-180, Gel 36, and Gel 32)	MB BS – [15 μmol L^{-1}] Bioelim – [100 μmol L^{-1}] PIT: 300 s	Twin Laser (MMOptics) [λ660 nm] IT: 30 s, 60 s, 180 s	Viability staining CLSM	Modified PDT killed 20 times more than conventional PDT and up to 8 times more than 2% CHX and 1% NaOCl.
Bago et al. 2013 [79]	Sample: Bacterial plaque from 3 adult volunteers used in 4 strains of *E. faecalis* (originally isolated from root canals of the teeth with periapical lesions) *Test groups*:Group #1: NaOCl ($n = 20$) Group #2: EndoActivator + NaOCl ($n = 20$) Group #3: Diode laser ($n = 20$) Group #4: PDT ($n = 20$) Group #5: PDT + 3D Endoprobe ($n = 20$) *Control groups*: Group #6: NaCl ($n = 10$): positive control	2.5	*E. faecalis* (ATCC29212)	Phenothiazine chloride/TBO [10 mg mL^{-1}]/[155 μg mL^{-1}] PIT: 60, 120 s	Helbo and Laser HF [λ660 nm] IT: 60 s The 2 lasers have the same wavelength.	SEM Cell viability CFU (log_{10}) PCR	PDT using both laser systems and the sonic activated NaOCl irrigation were significantly more effective than diode irradiation and single NaOCl.
Hecker et al. 2013 [80]	Sample: 120 uniradicular human teeth (mandibular incisors and maxillary second premolar extracted because of periodontal disease or extensive carious lesions without root caries or previous endodontic treatment) *Test groups*:Group #1: NaOCl (0.5%, 1.0% or 3.0%) for 30, 60, or 600 s ($n = 10$) Group #2: NaOCl (0.5%, 1.0% or 3.0%) for 30, 60, or 600 s + neutralizing solution ($n = 10$) Group #3: PDT ($n = 10$) *Control groups* Group #4: TBO (only) ($n = 10$) Group #5: laser (only) ($n = 10$) Group #6: apical section as sterile control: negative control Group #7: middle section to confirm successful infection: positive control	0.5 1.0 3.0	*E. faecalis* (ATCC29212)	TBO [NS] PIT: 60 s	Pact 200 system [λ635 nm] IT: 240, 360 s	Cell viability CFU (log_{10}) SEM	The antibacterial PDT system did not achieve sufficient disinfection when compared to NaOCl.
	Sample: roots of freshly extracted permanent bovine mandibular incisors (total number of teeth unknown)						

Table 3: Continued.

Study type	Groups	% NaOCl	Substrate	Photosensitizer	Laser	Parameters evaluated	Conclusion
Mohammad et al. 2014 [82]	*Test groups*:Group #1: PDT with Aseptim Plus - LED disinfection system ($n = 10$) Group #2: PDT with diode laser Group #3: PUI + 17% EDTA + 2.6% NaOCl *Control groups* Group #4: no inoculation ($n = 2$) negative control Group #5: with inoculation ($n = 2$): positive control	2.6	*E. faecalis* *S. salivarius* (ATCC7073) *P. gingivalis* (ATCC 33277) *P. intermedia*	TBO [15 μg.mL^{-1}] PIT: 60 s	LED [λ635 nm] Diode laser [λ650 nm] $IT_{(LED)}$ (30,08): 120 s	SEM Scores for levels of infection (Bonsor et al. 2006 [35, 73])	The group treated with PUI + 2.5% NaOCl + 17% EDTA solution has the best results when compared to PDT with 2 different light sources.
	Sample: 30 roots obtained from 30 extracted human single and multirooted teeth						
Xhevdet et al. 2014 [81]	*Experiment #1* *E. faecalis* ($n = 78$) *Test groups*:Group #1: PDT (1 min) ($n = 13$) Group #2: PDT (3 min) ($n = 13$) Group #3: PDT (5 min) ($n = 13$) Group #4: NaOCl + PBS ($n = 13$) Group #5: NaOCl + 10 sec passive ultrasonic irrigation (PUI) ($n = 13$) *Control groups*: Group #6: no treatment ($n = 13$): positive control	2.5	*E. faecalis* (ATC29121)	Phenothiazine chloride [10 mg.mL^{-1}] PIT: 60 s	HELBO [λ660 nm] IT: 60, 180, 300 s	Flow cytometry SEM Cell viability CFU (10^{6}/μ)	Irrigation with NaOCl showed similar results to 5 min irradiation of PDT.
	Experiment #2 *C. albicans* ($n = 78$) *Test groups*: Group #1: PDT (1 min) ($n = 13$) Group #2: PDT (3 min) ($n = 13$) Group #3: PDT (5 min) ($n = 13$) Group #4: NaOCl + PBS ($n = 13$) Group #5: NaOCl + 10 sec PUI ($n = 13$) *Control groups*: Group #6: no treatment ($n = 13$): positive control		*Candida albicans* (ATCC60193)				
	Sample: 156 extracted unirradicular human teeth						

Table 4: PDT microbial reduction outcomes.

Author	Study type	Microorganisms	Efficacy (% or $\log_{10}$)
Seal et al. 2002 [63]	*In vitro*	*S. intermedius*	5 $\log_{10}$
Bonsor et al. 2006 [35, 73]	*In vivo*	Polymicrobial infected teeth	96.7
Bonsor et al. 2006 [35, 73]	*In vivo*	Polymicrobial infected teeth	91
Silva Garcez et al. 2006 [57]	*In vitro*	*E. faecalis*	99.2
Garcez et al. 2007 [34]	*In vitro*	*P. mirabilis* and *P. aeruginosa*	98
Garcez et al. 2008 [58]	*In vivo*	Polymicrobial human dentine of the canal's walls	99.9
George and Kishen 2008 [59, 103]	*In vitro/ex vivo*	*E. faecalis*	100
Lim et al. 2009 [77]	*Ex vivo*	*E. faecalis*	99.99
Meire et al. 2009 [64]	*In vitro/ex vivo*	*E. faecalis*	1–1.5 $\log_{10}$
Souza et al. 2010 [65]	*In vitro*	*E. faecalis*	99.48
Garcez et al. 2010 [74]	*In vivo*	Polymicrobial infected teeth	100
Nagayoshi et al. 2011 [62]	*In vitro*	*E. faecalis*	99.99
Ng et al. 2011 [78]	*Ex vivo*	Human intracanal dentinal shavings	70
Nunes et al. 2011 [66]	*In vitro*	*E. faecalis*	99.41
Poggio et al. 2011 [67]	*In vitro*	*S. mutans; E. faecalis*, and *S. sanguis*	91.49
Rios et al. 2011 [68]	*In vitro*	*E. faecalis*	99.9
Bago et al. 2013 [79]	*Ex vivo*	*E. faecalis*	99.99
Cheng et al. 2012 [69]	*In vitro*	*E. faecalis*	96.96
Pileggi et al. 2013 [60]	*In vitro*	*E. faecalis*	96.7
Stojicic et al. 2013 [5]	*Ex vivo*	*E. faecalis*	100
Vaziri et al. 2012 [70]	*In vitro*	*E. faecalis*	82.3%
Hecker et al. 2013 [80]	*Ex vivo*	*E. faecalis*	Not specified
Prabhakar et al. 2013 [75]	*In vivo*	Polymicrobial infected teeth	99.99
Bumb et al. 2014 [61]	*In vitro*	*E. faecalis*	96.7
Gergova et al. 2015 [71]	*In vitro*	*S. aureus; E. faecalis; S. pyogenes; S. intermedius; E. coli; K. pneumonia; E. cloacae; S. marcescens; M. morganii; P. aeruginosa; A. baumannii; C. albicans*	42–54
Jurič et al. 2014 [76]	*In vivo*	Polymicrobial infected teeth	100
Muhammad et al. 2014 [82]	*Ex vivo*	*E. faecalis; S. salivarius; P. gingivalis; P. intermedia*	Not specified
Xhevdet et al. 2014 [81]	*Ex vivo*	*E. faecalis* and *C. albicans*	71.59
Wang et al. 2015 [72]	*In vitro*	*E. faecalis*	5.20 $\log_{10}$

irrigation showed similar results to 5 min irradiation of PDT, 10 mg mL^{-1} phenothiazine chloride as PS irradiated with 660 nm light source.

Considering all 29 publications, 14 of them (48%) [5, 34, 35, 57–61, 73–76, 78, 79] showed best PDT antimicrobial outcome compared to (0.5–6%) NaOCl used alone; 2 (7%) [62, 81] papers reveal similar effects between them and the last 13 (45%) [63–72, 77, 80, 82] studies revealed supremacy of sodium hypochlorite (0.5–6%).

3.2. Antimicrobial PDT Outcomes. The present narrative literature review was based on hypothesis that antimicrobial PDT efficacy was better than sodium hypochlorite in root canal asepsis. Considering all studies chronologically organized in Table 4, 48% (14 papers) showed PDT is more efficient than NaOCl (0.5–6% concentration) used alone and 7% (2 papers) reveal similarity in antimicrobial outcome effects between them.

On the other hand, 45% (13 studies) of studies reveal supremacy of sodium hypochlorite. From all studies, it must be observed that 55.2% (16 studies) were conducted at *in vitro* conditions, revealing preferential experimental phase where PDT remains in the last two decades. This must be taken into consideration, when comparing with clinical PDT studies, in which evidence reveals unanimous evidence supremacy of PDT over NaOCl.

3.3. Evaluation Parameters. The 29 studies analysed for this review revealed assessment of antimicrobial PDT efficacy was done through several parameters, from microbiological evaluation (classical analysis) to recent advanced imaging approaches. At the beginning, bacteriological experimental *in vitro* studies presented results through colony-forming units (CFU). This approach overcomes limitation of direct microscopic counting of bacterial cells, where all cells, dead and live, are counted; CFU estimates only viable cells of each

group, before and after treatment, in planktonic suspensions and biofilms. Results are given as CFU/mL (colony-forming units per millilitre) for liquids. This approach was used in 24 studies (83%) [34, 57–66, 68–72, 74–81]; Bonsor et al. [35, 73] used bacterial load scores, instead of the usual CFU, to evaluate PDT antimicrobial efficacy in clinical studies. Muhammad et al. [82] in 2014 over an *ex vivo* study elected the same evaluation unit as in Bonsor et al. studies, repeating bacterial score, complemented with microbiological identification.

Scanning electron microscopic (SEM) *in vitro* investigations have demonstrated the penetration of bacteria up to 1000 μm into dentinal tubules and hence it is very difficult for normal irrigants to penetrate till this depth. NaOCl can penetrate in a range of 60–150 μm into dentinal tubules and of Nd:YAG laser at a range of 400–850 μm. *Enterococcus faecalis* is known to colonize dentinal tubules up to depth of 600–1000 μm and conventional irrigants cannot penetrate more than 100 μm [83]. With SEM, Bumb et al.'s [61] *in vitro* study revealed bacteria found till the depth of 980 μm (control group) and in PDT group achieved a depth of 890–900 μm free from microorganisms, which revealed PDT as a promising root canal disinfection approach. SEM is a remarkably versatile technique, which reproduces the exact morphology of structures, but as the main disadvantage of dehydration of the sample. It was used in 10 (34%) studies [61, 65, 68, 69, 71, 72, 79–82] and ESEM (environmental scanning electron microscope) [84] which allows preservation of the sample before and after light irradiation was not used in any study. CSLM was used only in one study of George and Kishen [59] showing capability of obtaining in-focus images from selected depths allowing three-dimensional reconstruction of topologically complex objects with a specific hardware analysis. The same study [59] also evaluated dark toxicity (detail described in photosensitizers subchapter) and ROS production. PDT antimicrobial killing can be mediated by type I and type II reactions, although singlet oxygen is the predominant chemical entity causing cell death. Analysis and quantification of singlet/reactive oxygen species detection seem to be an excellent methodology to quantify antimicrobial PDT outcomes. However, of all studies analysed, only George and Kishen [59] performed ROS quantification and state that the increased photooxidation potential and singlet oxygen generation were thought to have collectively contributed towards the biofilm matrix disruption [59] and bacterial inactivation.

3.4. Photosensitizers. Photosensitizers (PS), which were preferentially located at the bacterial cytoplasmic membrane, have been found to be very potent photoantimicrobial agents. One important exception is represented by acridines [36], such as proflavine or acridine orange, which mostly interpolate with DNA bases. Highest modifications of cell functions and morphology, triggered by photodynamic inactivation, are typically due to damaged membranous domains [36]. This pattern of photoinduced subcellular damage is in agreement with lack of mutagenic effects [85], as well as with absence of selection of photoresistant microbial strains even after several photosensitization treatments.

Methylene blue (MB), a well-established PS, has been used in PDT for targeting endodontic bacteria since 2007 [34] and remains as one of the most used; but the first PS used in endodontic field was toluidine blue (TBO) [63]. Hydrophilicity of MB, along with its low molecular weight and positive charge, allows it to cross outer membrane of Gram-negative bacteria through porin channels [33, 86]. MB predominantly interacts with anionic macromolecule lipopolysaccharide, resulting in generation of MB dimers, which participate in the photosensitization process. From all studies evaluated, 12 (41%) [35, 63–65, 67, 68, 70, 71, 73, 79, 80, 82] used TBO as PS, while 10 (34%) [5, 59, 61, 65, 66, 69, 75, 77, 78, 87] studies used MB. One study, elaborated by Souza et al. [65], used both TBO and MB as PS. The best antimicrobial PDT results were achieved with TBO and MB as PS in the same concentration, 15 μg mL^{-1} [5, 65, 70, 71, 82]. All concentration variations are studied first in preliminary findings to obtain fluorescence characteristics [45] in ultraviolet-visible absorption spectra on a diode-array spectrophotometer to understand absorption pattern and to establish final concentration. In designing criteria for definition of second generation PS, an essential feature has been evaluated, *dark toxicity* [88]. It is clearly desirable that PS has zero or very low cytotoxicity in total absence of light and this indicates antimicrobial PDT efficacy results strictly from combination between PS and light source. Reviewing literature in this aspect, only one study from George and Kishen [59] had this aspect in mind.

The period of intimate contact between PS and substrate without irradiation, known as preincubation time (PIT), diverges in terms of PS used. It is also important that PIT is fixed in total absence of light, even natural light [88]. The most used TBO PIT was 60 seconds (s) [35, 67, 73, 80, 82] from a range of 30–300 s (mean = 95.5 s) and MB PIT most used was 300 s [5, 66, 75, 78] from a range of 60–600 s (mean = 353.3 s).

3.5. Light. Phototherapy describes use of light in treatment of disease; photochemotherapy, on the other hand, involves a combination of administration of a photosensitizing agent followed by action of light on tissues in which the agent is located [89]. PDT kills microorganisms by combined action of visible light and a photosensitizing dye. From all 29 studies evaluated, laser wavelength gap referred to in literature was between 380 [60] and 910 nm [61] (mean = 650.8 nm), while most used light source was a diode laser of 660 nm [5, 34, 58, 65, 66, 69, 74–77, 79, 81] wavelength. Some orthodox photosensitizers have lost their proficiency because they needed specific light source for each one and combination between them triggers the costs. Several examples can illustrate this aspect: Azpaste (685 nm) [57]; indocyanine green (805 nm) [62]; eosin-Y, and curcumin (380–500 nm) [60] which make them, nowadays, outdated.

In terms of commercial light sources, there are three diode lasers that authors would like to remark: *Denfotex* of 635 nm (SaveDent; Denfotex, Inverkeithing, UK) [64, 90, 91], *Helbo* of 660 nm (Helbo Photodynamic Systems, Grieskirchen, Austria) [91], and *FotoSan* emitting in the red spectrum with a power peak at 628 nm (FotoSan; CMS Dental, Copenhagen, Denmark) [67, 68, 71]. Delivery of PDT treatment with Denfotex, according to the manufacturer's recommendations, includes TBO as PS at a concentration of 12.7 mg L^{-1}, applied

in 120 s as preincubation time (PIT); followed by an irradiation time (IT) of 150 s with a laser output power of 100 mW using the spherical tip. Helbo system advocates Helbo Endo Blue PS, a MB dye, at a concentration of 10 mg L^{-1} fully covering the root canal with a PIT of 180 s; after this time, according to the manufacturer's recommendations, excess PS dye should be removed and light source applied for an IT of 120 s and an output power of 75 mW with an attached 2D spot probe Helbo Photodynamic Systems. Meire et al. in 2012 [91] performed an *in vitro* study comparing Denfotex with Helbo. The same team [91] reported that log reduction with Helbo system was higher than with Denfotex; however, the best results were achieved with 2.5% NaOCl for 300 s. Several differences between the two systems were described and might account for the distinctive reduction outcomes in viable cells [91]. First, the PS dyes are chemically different; secondly, Helbo Blue PS is much more concentrated than Denfotex PS. Thirdly, following the PS application and the recommended PIT, the PS excess has to be removed with the Helbo system, dried canal [91], but not with the two other systems: Denfotex and FotoSan, where fiber is inserted in the liquid [67, 68, 71, 91]. In the three PDT systems, all probes are different. While the Helbo systems 2D spot probe is designed for two-dimensional exposure, Denfotex and FotoSan tips emit in three dimensions and this has strong implications for energy densities at the target. Also the lasers wavelengths are slightly different. It seems that there is also a clear reduction in light exposure as irradiation time (IT): Denfotex (150 s) [91], Helbo (120 s) [91], and FotoSan (30 s) [67, 68, 71].

FotoSan uses only TBO as a FotoSan PS, available in three types of viscosities (low, medium, and high), all at the same concentration (100 μg mL^{-1}) and the light source with an output power of 100 mW. FotoSan was evaluated in 3 (10.3%) studies [67, 68, 71], curiously all conducted in *in vitro* conditions with FotoSan protocol IT of 30 s.

Poggio et al. [67] tested 30 s and also 90 s of IT and declared that with the longer light exposure, it results in an increased percentage of bacterial reduction for different groups of *Enterococcus faecalis*, *Streptococcus mutans*, and *Streptococcus sanguis* strains. For this reason, this group admits that FotoSan needs to be applied into canal for at least 90 s, because 30 s of irradiation showed lower performance when compared to PDT with IT of 90 s, although the same group reveals that the best outcomes were achieved with PDT 30 s of IT combined with 5% NaOCl.

Irradiation time (IT) is an important issue to considerer and, in this parameter, PDT studies outcomes are very dissimilar with a range between 30 s [63, 68] and 1800 s [60]. Considering the most used wavelength of 660 nm, preference irradiation time is in the range between 30 s [5] and 1200 s [77] (mean = 223 s).

The last aspect considered in laser literature is the need for an intracanal fiber tip to spread light into root dentinal walls as well as within biofilms. From all studies analysed, only Nunes et al. [66] explored *in vitro* effectiveness of PDT with and without use of an intracanal optical fiber. Nunes et al. [66] concluded that, under experimental conditions, PDT was effective against *E. faecalis*, regardless of whether or not it is applied through an intracanal fiber. Considering the use of intracanal fiber, only 4 (13.8%) studies [63, 70, 75, 80] were not performed with intracanal fiber (Table 5).

Prabhakar et al. [75], in these particular conditions, revealed in a clinical study that antimicrobial PDT performance is better than 0.5% NaOCl. When PDT is implemented in planktonic suspensions established in multiwells, light source was applied 20 mm [60] away from well. Considering intracanal fiber, fiber tip diameter most used was 400 nm [59, 62, 64, 72, 77]. In terms of intracanal fiber location inside root, it varies from full working length (WL) [34, 58, 62, 64, 66, 71, 76, 79, 81, 82], the most prevalent, to WL-1 millimeters (mm) [57, 74], WL-2 mm [61, 68], WL-3 mm [67–69], and WL-4 mm [35, 73]. Contemplating the same device, intracanal fiber, in terms of applying movements to itself or inserting endodontic tip static inside root canal to improve the best light diffusion through root canal [66]. The former was applied in 5 studies [34, 58, 65, 66, 79] with spiral movements from apical to cervical and latter maintained static [64, 76, 77] inside root canal orifice [77] or at WL [64, 76].

3.6. Disinfection Protocol. In literature, when PDT studies are accomplished in teeth, the majority of them are performed in human single rooted tooth specimens with no evidence of caries or defects and radicular pathology. Considering tooth type, there is only one study performed in deciduous teeth [75]; the majority was achieved in permanent uniradicular human teeth. However, four studies used not only uniradicular but also multiradicular teeth [35, 73, 78, 82]. Besides, decayed teeth are also studied in deciduous [75] and permanent teeth [79].

Slaughterhouse bovine teeth [80] are convenient to use in antimicrobial PDT studies because of their match with human dentine; more precisely, their dentinal tubules are very similar to human teeth in quantity, size, diameter, morphology, and density. Moreover, bovine teeth [12] are simple to acquire and reduced size makes handling easier; in this term, they were used in 2 (7%) studies [72, 80]. Only one study, performed by Nagayoshi et al. [62], was executed in a resin block which attempts to mimic an *in vitro* model of apical periodontitis.

In the most common experimental model, dental specimens are decoronated to a standard length of 12 mm [67, 68, 78, 79] although gap value is very wide, from 8 [59, 77] to 15 mm [66, 81] or complete root canal length. Patency of apical foramina is established and then mechanical [35, 58, 61, 63–68, 70, 71, 73, 74, 76, 78, 79, 81, 82] instrumentation is performed using nickel-titanium rotary files, predominantly in a coronoapical (crown-down) technique [35, 58, 61, 63–68, 70, 71, 73, 74, 76, 78, 79, 81, 82] from canal orifice to apical third, until it reaches the value of master apical file (MAF) of K (Kerr) file 40 [58, 59, 68, 70]. However, other MAF have been described, such as 35 [57, 79, 81] and 30 [34].

In terms of irrigation with disinfecting agents, those are used for smear layer (SL) removal, lubrication, debris removal, and antimicrobial effects. SL is composed of organic and inorganic components like vital or necrotic pulp tissue, microorganisms, saliva, blood cells, and tooth structure. Among irrigation solutions, sodium hypochlorite (NaOCl)

Table 5: Studies compilation: laser, photosensitizer, and fiber applied.

Study type	Year	Author	Laser					Photosensitizer		PDT outcomes	
			Wavelength (nm)	Diameter of fiber (μm) Working length (WL) EL	Power of output (mW)	Power of density (mW/cm^2)	Energy fluence (J/cm^2)	Type	Concentration (μg/mL)	+	–
In vitro, 16 studies	2002	Seal et al. [63]	632.8	*Without fiber* Light at the orifice of the access cavity 365	35	—	42.9, 63.3, 85.7, 214.3, 428.6	TBO	12.5, 25, 50, 100		–
	2006	Silva Garcez et al. [57]	685	WL-1 mm Helicoidal movements, from apical to cervical 200	50	—	—	AZ paste	0.01% AZ paste	+	
	2007	Garcez et al. [34]	660	WL Spiral movements, from apical to cervical 400	40	—	5, 10, 20 e 40	PEI/e6	NS	+	
	2008	George and Kishen [59, 103]	664	NS NS 400 WL IV: static spherical tip in the centre of the liquid	30	—	63.69	MB	1, 5, 10, 15, 20, 25 μM	+	
	2009	Metre et al. [64]	635	EV: 70% of the light radially as a cylinder uniformly and 30% at the tip; moved up and down in the canal 300	100	—	—	TBO	12.7 mg mL^{-1}		–
		Souza et al. [65]	660	NS Spiral movements from apical to cervical 400	40	—	—	MB/TBO	15/15		–
	2011	Nagayoshi et al. [62]	805	WL NS *Without fiber* Handpiece placed in root canal orifice	5000	—	—	Indocyanine green	12. mg mL^{-1}		≈
	2011	Nunes et al. [66] Study with and without fiber	660	216 WL Spiral movements from apical to cervical 500	90	300	—	MB	100		–
		Poggio et al. [67]	628	WL-3 mm Endotip guide to the apical parts	—	—	—	TBO	100		–

Table 5: Continued.

Study type	Year	Author	Laser					Photosensitizer		PDT outcomes	
			Wavelength (nm)	Diameter of fiber (μm) Working length (WL) EL	Power of output (mW)	Power of density (mW/cm^2)	Energy fluence (J/cm^2)	Type	Concentration (μg/mL)	+	–
				NS							
		Rios et al. [68]	628	WL-2/3 mm	—	—	—	TBO	NS		–
				NS							
		Cheng et al. [69]		*Nd:YAG*							
			Nd:YAG [λ1064 nm]	200							
				WL-1							
				Spiral movement	—	—	—	MB	50		–
				Er:YAG							
				300							
			Er-YAG [λ2940 nm]	Orifice of root canal							
				NS							
				Er,Cr:YSGG							
	2012		*Er,Cr-YSGG* [λ2780 nm]	415							
				WL-1							
				NS							
				Diode							
			Diode [λ660 nm]	2000							
				WL-3 mm							
				NS							
		Vaziri et al. [70]	625	*Without fiber*	—	200	12	TBO	15		–
									[BS]		
									All 1 μM		
				10.4 mm				Eosin-Y	*Biofilms*		
		Pileggi et al. [60]	380–500	Light source 20 mm away from the bacteria	—	450	108	RB	Eosin-Y	+	
				NS				Curcumin	100 μM		
									RB/curcumin		
									10 μM		
				NS							
	2014	Bumb et al. [61]	910	WL-2 mm	1000	—	—	MB	25 mg mL^{-1}	+	
				Circular movements, from apical to cervical							
				200							
				WL							
	2015	Gergova et al. [71]	660	Helicoidal traction movements, from apical to cervical	100	—	—	TBO	0.1 mg mL^{-1}		–
				400							
	2015	Wang et al. [72]	670	NS	50	—	—	MB	60 μM		–
				NS							

Table 5: Continued.

Study type	Year	Author	Wavelength (nm)	Laser			Photosensitizer		PDT outcomes	
				Diameter of fiber (μm) Working length (WL) EL	Power of output (mW)	Power of density (mW/cm^2)	Energy fluence (J/cm^2)	Type	Concentration (μg/mL)	+	−
In vivo, 6 studies				Flexible hollow tube							
		Bonsor et al. [35, 73]	633	WL-4 mm	100	—	—	TBO	12.7 mg mL^{-1}	+	
				Moved up and down about 3 mm at 20 s							
	2006			Flexible emitter tip							
		Bonsor et al. [35, 73]	633	WL-4 mm	100	—	—	TBO	NS	+	
				Moved up and down about 3 mm at 20 s							
				300							
	2008	Garcez et al. [58]	660	WL	40	—	—	PEI/e6	60 μmol L^{-1}	+	
				Spiral movements, from apical to cervical							
				200							
	2010	Garcez et al. [74]	660	WL-1 mm	40	—	—	PEI/e6	≈19	+	
				Spiral movements							
		Prabhakar et al. [75] Deciduous teeth	660	*Without fiber*	30		8.6	MB	50	+	
	2013			450							
				WL	100	—	—	Phenothiazinium chloride		+	
	2014	Jurič et al. [76]	660	Static							
				400							
Ex vivo, 7 studies	2009	Lim et al. [77]	660	Root canal orifice	30	—	—	MB	100 μM		−
				Static							
				250							
		Ng et al. [78]	665	10 mm	—	100	30	MB	50 μg mL^{-1}	+	
				360°							
				NS							
				[BS]							
				Long optical fiber with a diameter 0.4 mm					[BS] 15 μmol L^{-1}		
	2011	Stojicic et al. [5]	660	*Biofilm*	40	—	—	MB	Biofilm	+	
				Conical frustum tip with the end diameter of 5 mm					100 μmol L^{-1}		
				NS							
				320							
		Bago et al. [79]	660	WL	100	—	—	Phenothiazine chloride/TBO	10 mg mL^{-1}/155	+	
				Spiral movements, from apical to cervical							

Table 5: Continued.

Study type	Year	Author	Laser					Photosensitizer		PDT outcomes	
			Wavelength (nm)	Diameter of fiber (μm) Working length (WL) EL	Power of output (mW)	Power of density (mW/cm^2)	Energy fluence (J/cm^2)	Type	Concentration (μg/mL)	+	−
	2013	Hecker et al. [80]	635	*Without fiber* 300 WL	200	—	—	TBO	NS		–
		Muhammad et al. [82]	650	Moved all along the canals NS WL	20	—	—	TBO	15 μg mL^{-1}		–
	2014	Xhevdet et al. [81]	660	Light at the tip and from the lateral sides	100	100	—	Phenothiazine chloride	10 mg mL^{-1}		≈

is the classical irrigant most used in endodontic therapy as a powerful antibacterial organic tissue dissolving agent.

NaOCl penetrates to a depth of approximately 130 μm [92] to 160 μm into dentinal tubules whereas tubular infection may occur closer to cementum-dentin junction (up to 1000 μm) [93]. Bumb et al. [61] demonstrated in scanning electron microscope (SEM) penetration up to 1000 μm into dentinal tubules of *E. faecalis* and compared penetrating power between a high power laser (Nd:YAG) that can go to a range of 400–850 μm and PDT group that reaches as deep as 890–900 μm.

Considering NaOCl as an unquestionable irrigation solution, its universal effective minimal concentration remains unclear. Apart from various outcomes reported by previous studies on comparative effectiveness of hypochlorite at different concentrations, it is regularly accepted that effectiveness of NaOCl is proportional to its concentration [24, 72, 94]. In antimicrobial PDT studies, NaOCl concentration range is between 0.5 [57, 67, 75, 80] and 6% [68, 78] and mainstream of studies used 2.5% NaOCl concentration [34, 58, 62, 64, 65, 70, 71, 74, 76, 79, 81]. Due to the fact that NaOCl has an influence upon only organic components of SL, it should be used with demineralizing agents, which can remove inorganic component of smear layer. Concerning SL elimination, only 3 readings [35, 70, 73] reported citric acid as a SL deletion, one at 10% [70] and two at 20% from the same author, Bonsor et al. [35, 73]. But the most popular SL removal is by far 17% ethylenediamine tetraacetic acid (EDTA) [34, 57–59, 61, 63, 65–69, 71, 74, 76–78, 81, 82].

3.7. Microorganisms. Reviewing literature on use of several microorganisms in PDT studies, authors could not evaluate *in vivo* studies in those terms, because no attempt was made to identify bacterial flora during culture process [35, 58, 73, 75] in four of six studies. Only Garcez et al., 2010 [74], and Jurič et al., 2014 [76], established microbiological identification.

Among all studies, we analysed 23 studies (all *in vitro* and *ex vivo*), and from those, 20 (87%) elected *Enterococcus faecalis* as substract to quantify antimicrobial PDT effectiveness. *E. faecalis* is a Gram-positive facultative anaerobe commonly detected in asymptomatic, persistent endodontic infections. Its prevalence in such infections ranges from 24% to 77% [95]. This finding can be explained by various survival and virulence factors [95] expressed by *E. faecalis*, including its ability to compete with other microorganisms, invade dentinal tubules, and resist nutritional deprivation.

E. faecalis was used not only in planktonic suspensions, but also in form of biofilms and the most common strain selected was ATCC29212. However, biofilm maturation time did not follow a linear pattern; besides, a huge discrepancy exists. Some authors used young biofilms with range of 2 [60, 68], 4 [60], and 7 days [81, 82] very distinct from mature biofilms performed with biofilms of 21 [61, 66], 28 [5, 69, 72, 77], and 70 days [59]. According to Kishen and Haapasalo 2010 [12], a mature biofilm is considered when maturation period is equal to or higher than 21 days and only 7 (30%) studies [5, 59, 61, 66, 69, 72, 77] respected this mature biofilm criteria. Apart from *E. faecalis*, other microorganisms were reviewed. Of note, in literature, the first PDT *in vitro* study was performed by Seal et al. 2002 [63] in root canals infected with *Streptococcus intermedius* (Gram-positive facultative anaerobe) biofilm with 2 days of maturation using TBO as PS and a helium-neon laser as light source.

4. Discussion

PDT, a technique with potentially significant antimicrobial properties, is a fairly recent approach in endodontic disinfection protocols. While the oral applications of PDT have been extensively tested, variations in study type and design limits the ability to synthesize or pool the available quantitative data, thereby permitting a formal meta-analysis and a systematic review.

Furthermore, many of the studies quantitatively measuring the degree of bacterial kill fail to report baseline bacterial counts or concentrations, thus limiting the ability to assess the bactericidal efficacy of PDT. Considering this apparent variation in reporting results among the studies analysed, it is difficult to provide a definitive assessment of the research question posed in this review. It is important to mention that PDT efficacy is shown in CFU or in percentage and logarithm (in form of $\log_{10}$); nonetheless, authors state this is pointless without the perception of the initial concentration. As an example, if we have an initial sample from a root canal of 10^7 microorganisms and if after PDT approach we had 10^5, statistically, 99% were killed, but there are still 100000 microorganisms left inside the root canal. Considering the variation in units at outcomes, the final results analysis is difficult.

Even though PDT has significant advantages (cited in Section 1), potential adverse events as tooth discoloration have been reported previously in root canal treatment when MB and TBO were used as PS [96]. It is also important that future clinical studies clearly report adverse events associated with PDT so that an estimation of the benefit-to-risk ratio from the use of PDT is feasible. Nonetheless, there were no adverse effects mentioned in the included studies of the current review.

PDT outcomes in literature have been reported by the dual combination of PS and a visible light source in the presence of oxygen; however, recently, Lins de Sousa et al. [97] analysed that twice-daily blue light of 420 nm, energy density of 72 Jcm^{-2}, and irradiation time of 776 s without PS are a promising approach in the inhibition of five days' *Streptococcus mutans* matrix-rich biofilm development. It has remarkably inhibited the production of insoluble EPS, which is responsible for the scaffold of the extracellular biofilm matrix. The authors suggest that this evidence is very important to improve standardization in PDT procedures in the total absence of light as the evaluation of PS dark toxicity in some studies reviewed did not address this important issue.

In the literature, residual systemic photosensitization has also been reported as a potentially adverse event associated with the use of intravenous PS [98]; but this effect appears to not be associated with oral applications of PDT [99]. The role of PDT in root canal disinfection has been tested using several combinations of PS and light sources and has shown divergent results and these studies have revealed several limitations associated with antimicrobial PDT. For successful

PDT to affect significant reduction or eradication of microorganisms, a PS is required which will show enough affinity for microorganisms without catalyzing photodamage to host tissues, a light source at a wavelength that can penetrate tissues (630–700 nm), and sufficient oxygenation to produce a level of reactive oxygen species (ROS) necessary to induce photodynamic lipid peroxidation and, as a consequence, necrosis and cell death. If there is photodamage to both tissues and microorganisms, efficacy will be suboptimal.

Microorganisms in the root canal flora and their growth mode were found to influence their susceptibility to PDT in a dose-dependent manner [100] and biofilms can be difficult to eradicate not only because of their effect as barriers to PS uptake, but also their ability to diffuse or attenuate light in the root canal dentinal tubules. Even dentin, dentin matrix, pulp tissue, bacterial lipopolysaccharides, and bovine serum albumin were found to significantly decrease PDT antimicrobial efficacy [101] and, as a consequence, an effort to enhance the PDT by nanoparticle-based technology appears promising [102]. Other strategies include the use of a PS solvent [103], efflux pump inhibitors [100], or photoactivated functionalized chitosan nanoparticles for disinfection and stabilization of the dentin matrix [104]. Because the application of PDT for additional reduction of the microbial load of root canal systems seems promising, it would be beneficial to identify the ideal combination of PS and light wavelength in preclinical studies and conduct future randomized controlled trials to test the effect of PDT on root canal disinfection in various indications.

5. Conclusion

PDT has been used thus far without a consensus-based, well-defined protocol, and therefore still remains at an experimental stage waiting for further optimization. Limited clinical information is currently available on the use of PDT in root canal disinfection. Currently, the level of evidence of available clinical studies to answer this question is low. Nevertheless, the results of this review suggest, based primarily on available *in vivo* studies, that PDT could perform well as an antimicrobial adjuvant. PDT appears to be a promising antimicrobial platform so further studies are warranted to optimize protocols using standardized laser and PS parameters to assess the PDT efficacy. Therefore, within the limits of the present review, one may conclude that the efficacy of PDT remains questionable, but promising. It is further suggested that an additional potential benefit from the use of PDT in root canal disinfection may exist where highly resistant bacteria are present in the root canal space, thus affecting the treatment prognosis. Further research is necessary to establish the appropriate PDT parameters allowing adequate antimicrobial action without harmful host side effects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Antimicrobial Photodynamic Therapy against Endodontic *Enterococcus faecalis* and *Candida albicans* Mono and Mixed Biofilms in the Presence of Photosensitizers: A Comparative Study with Classical Endodontic Irrigants

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Endodontic biofilms eradication from the infected root canal system remains as the primary focus in endodontic field. In this study, it was assessed the efficacy of antimicrobial Photodynamic Therapy (aPDT) with the Zn(II)chlorin e6 methyl ester (Zn(II)e_6Me) activated by red light against monospecies and mixed biofilms of *Enterococcus faecalis* and *Candida albicans*. The results were compared with the ones obtained with Rose Bengal (RB), Toluidine Blue-O (TBO), the synthetic tetracationic porphyrin (TMPyP) as well as classical endodontic irrigants (3% NaOCl, 17% EDTA and 2% CHX). The antimicrobial efficacy of aPDT toward monospecies and mixed biofilms was quantified resorting to safranin red method. The changes of biofilm organization and of cellular ultrastructure were evaluated through several microscopy techniques (light, laser confocal and transmission electron microscopy). Zn(II)e_6Me once activated with light for 60 or 90 s was able to remove around 60% of the biofilm's biomass. It was more efficient than TBO and RB and showed similar efficiency to TMPyP and classical irrigants, CHX and EDTA. As desirable in a PS, Zn(II)e_6Me in the dark showed smaller activity than TMPyP. Only NaOCl revealed higher efficiency, with 70–90% of the biofilm's biomass removal. The organization of biofilms and the normal microbial cell ultrastructure were extensively damaged by the presence of Zn(II)e_6Me. aPDT with Zn(II)e_6Me showed to be an efficient antimicrobial strategy deserving further studies leading to a future clinical usage in endodontic disinfection.

Keywords: antimicrobial photodynamic therapy, endodontic biofilms, chlorin e_6, *Enterococcus faecalis*, *Candida albicans*

INTRODUCTION

Apical periodontitis is an inflammatory reaction of periradicular tissues caused by a microbial infection in the root canal system (Siqueira et al., 2000; Nair, 2006). Microbial biofilms are considered the major cause for primary and secondary root canal infection and the success of endodontic treatment relies on the effective eradication of such biofilms (Nair, 2006). Conventionally, this is accomplished by chemo-mechanical disruption with instruments and antimicrobial chemicals used topically inside root canals. However, current treatment strategies are insufficient to reduce microrganisms inside root canals below detection limits before permanent root filling. This is mandatory to achieve optimal healing conditions for the periapical tissues (Sjögren et al., 1997). Therefore, advanced disinfection approaches are required to effectively eradicate biofilms and increase the endodontic treatment success rate.

It is widely accepted that the main reason for endodontic treatment failure is the insufficient root canal microrganisms eradication (Siqueira et al., 2000). As residual species are not reachable to the host's immune system, propagation and re-colonization is highly possible, endorsing microbial spread inside root canal system, which leads to endodontic infections. In 1965, apical periodontitis was recognized as a microbial mediated infection, which was later reinforced by ultrastructural microscopic techniques, revealing bacteria organized as biofilms within the infected root canals (Nair, 1987). Moreover, histopathological studies have also contributed to the concept that apical periodontitis is indeed a microbial biofilm-mediated disease (Carr et al., 2009; Ricucci et al., 2009; Ricucci and Siqueira, 2010).

As described by Donlan and Costerton (2002), a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are imbedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transfer. In addition, it is accepted that microbial cells comprising the biofilm are more resistant than the planktonic counterparts (Donlan and Costerton, 2002) and multispecies or mixed biofilms are more resistant to drugs than monomicrobial biofilms (Costerton et al., 1999). As such, polymicrobial biofilms diseases are associated with worse clinical outcomes than monomicrobial infections for decades (McKenzie, 2008). Although the endodontic biofilm is constituted by multiple microrganisms (Tan et al., 2015), most of *in vitro* studies have been made in monospecies biofilms of bacteria or combined with *C. albicans* (Sabino et al., 2015).

The problem of endodontic biofilms eradication from the infected root canal system remains as the primary focus in endodontic field. In recent years, photodynamic therapy (PDT) has been applied with success in several types of cancers (Ochsner, 1997; Gomes et al., 2015), age-related macular degeneration (Kawczyk-Krupka et al., 2015) and also in the photoinactivation of several microrganisms (Almeida et al., 2014), called antimicrobial photodynamic therapy (aPDT). In the endodontic field, aPDT has emerged as an optional extra to classical irrigation solutions in root canal asepsis (Bonsor et al., 2006a,b) such as sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX) and ethylenediamine tetraacetic acid (EDTA). The NaOCl solution is the most widely used in endodontic treatment (Siqueira et al., 2007; Mohammadi, 2008; Vaziri et al., 2012; Wang et al., 2015) albeit with some degree of toxicity. To avoid this toxicity, other root canal asepsis approaches with lower or insignificant toxicity should be implemented. Therefore, aPDT has emerged with promising experimental results, anticipating a possible new era in endodontic disinfection (Siddiqui et al., 2013; Chrepa et al., 2014).

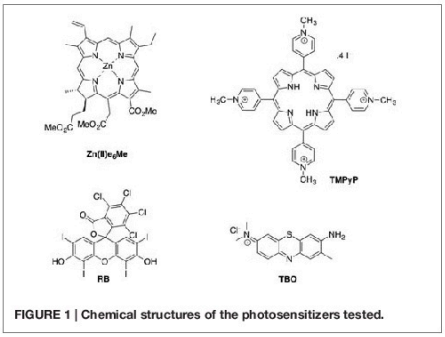

FIGURE 1 | Chemical structures of the photosensitizers tested.

Photodynamic therapy involves the combination of a non-toxic photosensitizer (PS) with a harmless visible light source in the presence of oxygen. After being excited by light, the PS releases its energy or electrons to molecular oxygen producing highly reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), which induce microrganism's injury and death, ideally with no host cell damage. Also, it has been indicated as bearing a strong potential in the fight against antimicrobial resistance (Hamblin and Hasan, 2004; Tavares et al., 2010; Costa et al., 2011). aPDT has also been studied as an auspicious approach to eradicate oral pathogenic microrganisms that cause, not only endodontic diseases, but also periodontitis, peri-implantitis, caries lesions and mucositis (Diogo et al., 2015).

In this study, we analyzed the aPDT efficacy against monospecies and mixed biofilms of *E. faecalis* and *C. albicans* using the following PSs: toluidine blue (TBO), rose bengal (RB), a synthetic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) and Zn(II)chlorin e_6 methyl ester (Zn(II)e_6Me) obtained from chlorophyll a (**Figure 1**). The antimicrobial results obtained by aPDT approach were compared with the ones achieved with three endodontic classical irrigants 3% NaOCl, 2% CHX, and 17% EDTA toward *in vitro* biofilms.

MATERIALS AND METHODS

Strains and Media

The strain of *C. albicans* (YP0037) used in this study was obtained from the Pathogenic Yeast Collection of FMUC, University

of Coimbra. *E. faecalis* (ATCC29212) was purchased from the American Type Culture Collection (ATCC). Microrganisms were stored at −80°C in 25% glycerol. When needed, pre-cultures were prepared by defrozen microbial cells in appropriate media, brain-heart infusion (BHI) for *E. faecalis* and YPD (0.5% yeast extract, 1% bacto-peptone, and 2% glucose) for *C. albicans*. For *C. albicans* growth it was used YPD broth. *E. faecalis* growth and biofilm formation was obtained in BHI liquid medium (Difco, Detroit, MI, USA). *C. albicans* biofilms and mixed biofilms consisting of *C. albicans* with *E. faecalis* were obtained in RPMI 1640 (Roswell Park Memorial Institute) medium (R8755, Sigma–Aldrich®).

In vitro Biofilm Formation

For *E. faecalis in vitro* biofilm formation, bacterial cells were previously grown in 4 mL of BHI overnight, at 37°C. These cells were harvested by centrifugation (Biofuge Fresco, Heraeus, UK), at 16,000 *g* during 5 min at 4°C, and washed twice in sterile BHI. A bacterial cell suspension with a density of 1.5×10^8 cells/mL (0.5 McF of McFarland scale) was obtained; 200 μL of this suspension was pipetted to each well of sterile 96-well polystyrene microtiter plates (Nunc F, Nalgene, Denmark). These plates were covered and sealed with parafilm, and incubated during 48 h at 37°C without agitation.

For the preparation of *C. albicans* biofilm, a loopful of cells from the solid stock cultures was used to inoculate 20 mL of YPD and incubated overnight in an orbital shaker (120 rpm) at 30°C. The cells were harvested by centrifugation (16,000 *g* for 5 min at 4°C), and washed twice with phosphate buffered saline (PBS). The final pellet was resuspended in pre-warmed RPMI-1640 at 37°C. The resulting cell suspension was diluted in RPMI to obtain a final suspension with a cell density of 1.0×10^6 cells/mL. This was used to prepare *C. albicans* biofilms in 96-well polystyrene microtiter plate (Nunc F, Nalgene, Denmark). For that 200 μL of *C. albicans* suspension was pipetted to the plate wells. After sealing with parafilm, they were left to incubate during 48 h at 37°C, without agitation.

For the mixed biofilm of *E. faecalis* and *C. albicans,* the two microbial species were pre-grown overnight and prepared as described for the monospecies biofilms except that they were resuspended in 37°C pre-warmed RPMI-1640 (R8755, Sigma–Aldrich®). The two microbe suspensions at a concentration of 1.0×10^6 cells/mL were mixed in pre-warmed RPMI-1640 in 1:1 ratio and incubated at 37°C, during 48 h to allow biofilm formation.

Biofilms Eradication with Classical Irrigants

The classical irrigants tested were 3% NaOCl, 17% EDTA, and 2% CHX (CanalPro™- endodontic irrigating solutions, Coltene). The biofilms were exposed to the irrigants for 60 and 90 s. A longer period of irrigation (30 min) was also tested because some authors defend that a continuous irrigation and time are important factors for the efficacy of classical irrigating solutions (Bystrom and Sundqvist, 1985; Haapasalo et al., 2010). After each period, the supernatants were removed and the chemical reactions were stopped using adequate inhibitors: 200 μL of sodium thiosulfate (S7026, Sigma–Aldrich) was added to the NaOCl treated group; 3% Tween 80 (T2575, Sigma–Aldrich) was used to neutralize CHX. Finally, 200 μL sterile distilled water was applied to dilute the 17% EDTA. Controls were made without the irrigants, in which the stop solutions were added, proving that these stop solutions, especially 3% Tween 80, did not interfered with the biofilm biomass quantification.

Photodynamic Inactivation of Biofilms

In the aPDT experiments all the PSs tested (TBO, RB, TMPyP, and Zn(II)e_6Me) were used at the same concentration (0.1 mg/mL). This concentration was chosen based on market formulation FotoSan agent®, in which the active substance is TBO at 0.1 mg/mL. This formulation is available in the dentistry market with a light source device (FotoSan®: 630, CMS Dental A/S, Glyngore, Roslev, Denmark) (Rios et al., 2011). The cationic porphyrin TMPyP and the modified chlorophyll, Zn(II)e_6Me, were synthetized and isolated according to the literature (Carvalho et al., 2010; Menezes et al., 2014). Their ^{1}H NMR and UV–vis spectra were consistent with literature data and their purity was confirmed by thin layer chromatography and ^{1}H NMR (data not shown). TBO and RB used were purchased from Sigma Aldrich (T3260 and 330000-1G, respectively). Stock solutions (10 mg/mL) of each porphyrinic derivative (TMPyP and Zn(II)e_6Me) were prepared in dimethyl sulfoxide (DMSO). For biological assays, the stock solutions of photosensitizers were diluted to the final concentrations in PBS.

The irradiations of the PSs in the aPDT experiments were performed in the presence of adequate light emitting diode (LED) source setup to comply with the 96-well plates used in this study (**Figure 2**). The LED sources were built at request by the Telecommunications Institute – Informatics, Electronics and Telecommunications Engineering Department of the University of Aveiro, Portugal. RB was irradiated with a green LED with a wavelength peak centered at 557 nm, made with gallium

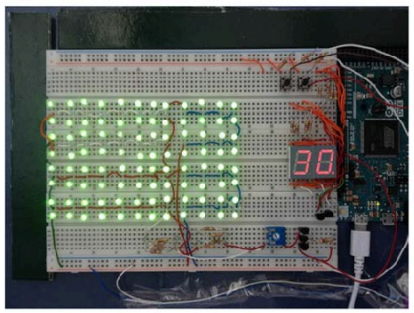

FIGURE 2 | Model of the experimental light emitting diode (LED) sources [green light diode (GaP); 557 nm of wavelength].

phosphide pure (GaP), an output of 62.5 mW, continuous waves, density power of 42 mW.cm^{-2}, energy fluence of 3780 J.cm^{-2}, voltage of 2.5 V. TBO, TMPyP, and Zn(II)e_6Me were irradiated with a red LED device with a wavelength peak centered at 627 nm, a gallium arsenide phosphide on gallium phosphide (GaAsP/GaP), with an output power of 75 mW, continuous waves, density power of 35 mW.cm^{-2}, energy fluence of 3150 J.cm^{-2} and a voltage of 2.5 V.

The experimental methodology included a pre-incubation period of the biofilms with the PSs, in total absence of light, for 15 min, to allow the entrance of PSs into the cells (Diogo et al., 2015). After that, light activation was performed for 60 or 90 s. Also, in each assay it were included controls in which the PSs were not added, to study the impact of irradiation in the biofilms.

Biofilm Biomass Quantification

The biofilm biomass was quantified using the safranin red (SR) assay (Kueng et al., 1989). After each experiment, 200 μL of methanol was added to each well of the 96-well plate. After 15 min, the content of each well was aspirated and let to dry. After drying, 0.1% SR solution was added and incubated for 20 min. The resulting solution was removed with a Pasteur pipette and two washes were made with distilled water. Two hundred microliter of acetic acid 33% (v/v) was added and 20 min after the absorbance was measured at 590 nm on a microplate reader (SpectraMAX Gemini XM, Molecular Devices, USA). The results were expressed as a percentage of biofilm removal when compared with the biomass quantified before irradiation or before exposure to the irrigants.

Microscopic Study of the Biofilms

E. faecalis and *C. albicans* were grown and prepared as described above. One mL of the final suspensions was added to sterile 12-well polystyrene microtiter plates with glass coverslips (CBAD00120RA1#1.5, ThermoScientific-Menzel) coated with poly-D-lysine (Sigma-Aldrich®, P1149). After seeding, the entire microtiter plate was covered and sealed with Parafilm®and incubated for 48 h at 37°C without agitation. For confocal fluorescence microscopy, fresh cultures of biofilms were used. *E. faecalis* was stained with Syto 13 Green Fluorescent Nucleic Acid Stain (ThermoFisher Scientific®). *C. albicans* in monospecies biofilm was probed with polyclonal primary antibody Acris Antibodies Gmbh®RGTX40096 with anti-rabbit secondary antibody Alexa Fluor®594 (Invitrogen®, RA21207). Images were obtained with a Carl Zeiss Cell Observer Spinning Disk with Alpha Plan-Apochromat objective, at a magnification of 100×. For light microscopy, it was used an Olympus BX-40 microscope at 400× total magnification. Images were recorded on an Olympus C-200 digital camera.

For transmission electronic microscopy (TEM), samples of 48 h-biofilms were fixed with 2.5% glutaraldehyde in 0.5 M sodium cacodylate buffer (pH 7.2) for 2 h. Post-fixation was performed using 1% osmium tetroxide for 1 h. The samples were then rinsed with the same buffer, and dehydrated in a graded ethanol series (30 to 100%). Then, they were impregnated and embedded in Epoxy resin (Fluka Analytical). Ultrathin sections (~70 nm) were mounted on copper grids (300 mesh) and stained with uranyl acetate 2% (15 min) and 0.2% lead citrate (10 min). Observations were carried out on a FEI-Tecnai G2 Spirit Bio Twin transmission electron microscope at 100 kV.

Statistical Analysis

Data were analyzed using Prism (version 5) software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences between groups were assessed with the independent samples student's *t*-test or Mann–Whitney test and a significance level of 0.05 was assumed.

RESULTS

Biofilm Removal

Before initiating the comparative study of the efficacy of aPDT and classical irrigants in the clearance of biofilms, it was important to verify if the PSs selected, TBO, RB, TMPyP, and Zn(II)e_6Me, had the ability to disturb the biofilms in the dark (i.e., in the absence of light activation) at the same concentration used in the aPDT studies. It was clearly desirable that the PSs had zero or very low activity in total absence of light indicating that aPDT efficacy resulted strictly from the ROS generated by PS light activation. The results obtained from the biofilm biomass analysis, using the SR assay, showed that upon 15 min of exposing the biofilms to the different PSs, in the dark, there was a decrease of the biofilms biomass in values ranging from 5.7 to 16.6% (**Table 1**). Following a pre-incubation period in the dark with the PSs, each preparation was irradiated with the appropriate LED light. Thus, TBO, TMPyP, and Zn(II)e_6Me were irradiated with a wavelength of 627 nm while 557 nm was used for RB. Three periods of irradiation were tested, 60, 90 s and 30 min. Since there were no differences between the 90 s and the 30 min periods, this longer period was abandoned (data not shown). Also, the controls of the impact of light irradiation in the biofilms during the 60 or 90 s of irradiation showed no damage of the biofilm, as assessed by the SR assay and by microscopic observation of the biofilm morphology (results not shown).

The results summarized in **Figure 3** (upper left panel) showed that Zn(II)e_6Me, is more effective in the removal of *E. faecalis* biofilm than the other PSs used, in both irradiation periods (60 and 90 s) ($P = 0.0079$). Similar reduction values of *E. faecalis* biofilm were obtained using TMPyP and RB (**Figure 3**; upper left panel). For *C. albicans* biofilm, Zn(II)e_6Me and TMPyP

TABLE 1 | Photosensitizers (PSs) effect in biofilm biomass(1) in the total absence of light during an incubation period of 15 min.

Biofilm	TBO (%)	TMPyP (%)	Zn(II)e_6Me (%)	RB (%)
E. faecalis	15.2	13.1	12.7	14.7
C. albicans	16.6	15.5	8.6	10.0
Mixed	12.4	10.0	5.7	7.5

(1) *The quantification of biofilm biomass was obtained with the SR assay and results are expressed as a percentage of biofilm removal when compared to the control biofilm biomass (n = 3).*

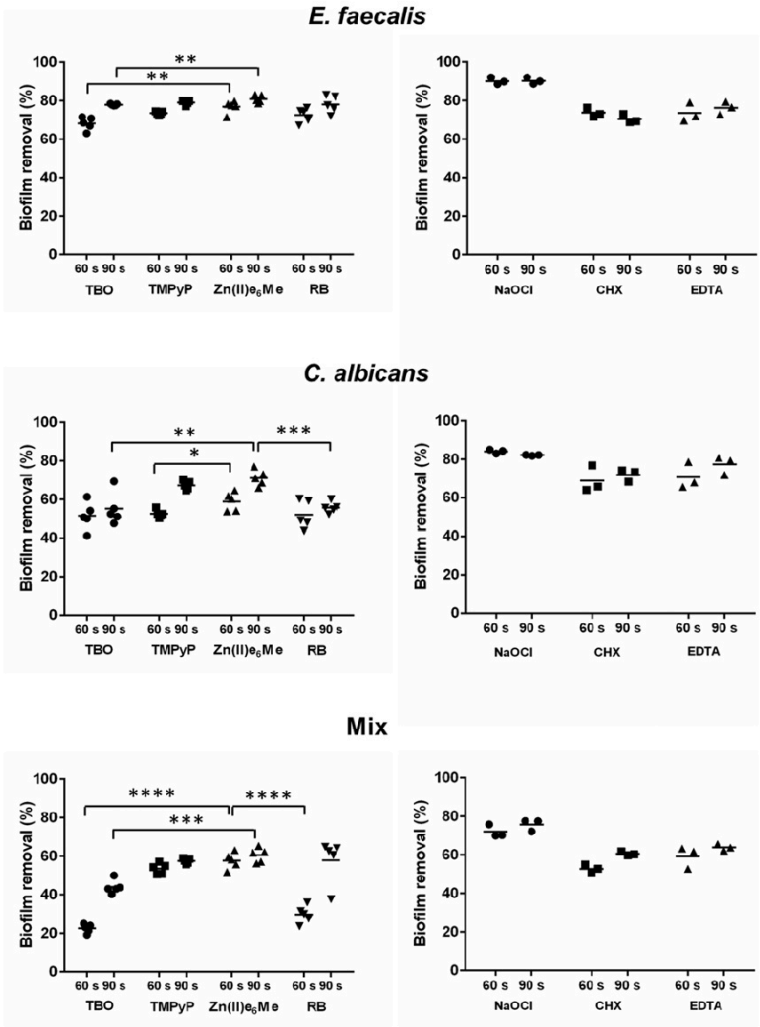

FIGURE 3 | Biofilm biomass removal upon antimicrobial photodynamic therapy (PDT) and treatment with classical irrigants. Monospecies biofilms and mixed biofilms of *E. faecalis* and of *C. albicans* were treated by a PDT using several photosensitizers (left panels) and classical irrigants (right panels). The quantification of biofilm biomass was performed with the safranin red (SR) assay. The percentage of biofilm biomass loss was calculated in relation to untreated biofilm. Differences were analyzed by Student's *t*-test using Prism software and considered significant at *P*-values of < 0.05. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

had similar efficacies in decreasing biofilm biomass upon 90 s of irradiation. However, a shorter period of irradiation, 60 s, revealed significant differences between the efficacies of both dyes in the capacity to remove biofilm biomass (**Figure 3**; middle left panel). Otherwise, Zn(II)e_6Me was much more effective than the other PSs used, TBO ($P = 0.0317$), and RB ($P = 0.0079$) after 90 s of irradiation and at the same concentration.

The mixed biofilm seems to be less susceptible to aPDT than the monospecies biofilm, especially when using TBO as PS, ($P = 0.0013$), (**Figure 3**; lower panel). In this mixed community of *E. faecalis* and *C. albicans*, Zn(II)e_6Me demonstrated to be the most efficient PS removing 58.98% of biofilm biomass ($P < 0.001$). When compared to classical irrigants, Zn(II)e_6Me was not as effective as NaOCl, the treatment that causes the higher damage, regardless of the type of biofilm (**Figure 3**; right panels). In fact, Zn(II)e_6Me (with an irradiation period of 90 s) was more effective in removing *E. faecalis* biofilm than EDTA or CHX (**Table 2** and **Figure 3**; upper panel). Zn(II)e_6Me reveal the same effect of CHX or EDTA treatment toward *C. albicans* biofilms (90 s) and toward mixed biofilms, either with 60 s or with 90 s of irradiation (**Table 2** and **Figure 3**; middle and lower panels).

TABLE 2 | Statistical analysis[(1)] of the efficiency of Zn(II)e_6Me against microbial biofilms in comparison with classical irrigants in the clearance of *E. faecalis*, *C. albicans*, and mixed biofilms.

	E. faecalis		*C. albicans*		Mix	
	60 s	**90 s**	**60 s**	**90 s**	**60 s**	**90 s**
Zn(II)e_6Me vs. NaOCl	***	***	***	**	**	**
Zn(II)e_6Me vs. CHX	ns	***	ns	ns	ns	Ns
Zn(II)e_6Me vs. EDTA	ns	*	*	ns	ns	ns

[(1)]*Differences were analyzed by Student's t-test using Prism software and considered significant at P-values of < 0.05. *P < 0.05; **P < 0.01; ***P < 0.001, ns: no significant difference.*

Biofilms Disturbance by aPDT in the Presence of Zn(II)e_6Me and NaOCl

The study of biofilms morphology was performed after 48 h of biofilm maturation, because 48 h- and 72 h-biofilms had similar morphologies. The changes observed in the biofilm organization developed for 48 h when treated with Zn(II)e_6Me and NaOCl (the classical irrigant with the best outcome) were compared with the untreated biofilms (control).

Zn(II)e_6Me eliminated most of the *E. faecalis* (**Figures 4A,B**) but *C. albicans* preparations retained some hyphae and yeast cells (**Figures 4D,E**). Otherwise, NaOCl eliminated all the cells adhered to the glass slide, either in *E. faecalis* or *C. albicans* biofilms (**Figures 4C,F**).

Using light microscopy, it was observed that while NaOCl lead to an almost complete loss of living cells (**Figure 5C**), aPDT with Zn(II)e_6Me resulted in a mixed biofilm with less *E. faecalis* cells and less *C. albicans* hypha, with a predominance of pear shaped cells (**Figure 5B**), when compared with the morphology of the untreated mixed biofilm (**Figure 5A**).

Ultrastructure Modification of Microbial Cells in Biofilms

The different morphological aspects observed in the fluorescence confocal microscopy lead us to study the ultrastructural changes using TEM. After several attempts, it was realized that the remainings of the biofilms (either monospecies or mixed biofilm) treated with NaOCl were so drastically damaged that no signs of cells were observed in the epoxy resin blocks sections (data not shown). The ultrastructural modification of bacterial and fungal cells were studied in biofilms exposed to Zn(II)e_6Me with an activation period of 90 s. In *E. faecalis* monospecies biofilm it was observed the existence of cell wall "ghosts", i.e., bacterial cell wall forming a structure with typical morphology of *E. faecalis*, without its intracellular content (**Figures 6A,D–F**). The complexity of the cellular ultrastructure of *C. albicans*, a eukaryote, allowed the observation of induced modifications. Most of the yeast cells showed an atypical irregular cell wall thickness and the cytoplasmic membrane integrity was lost, with cell membrane invaginations (**Figure 6G**), caused by 90 s of Zn(II)e_6Me-aPDT treatment. The cell membrane was damaged and the cell wall surface was rougher (**Figure 6H**) than in control cells (**Figure 6B**). Abnormal intracellular membrane arrangements probably corresponding to endoplasmic reticulum (ER) whorls (**Figure 6I**) were also observed. Some *C. albicans* cells exhibited big vacuoles with electrodense materials (**Figure 6J**).

A general view of the mixed biofilms showed *E. faecalis* cell wall structures devoid of the intracellular content and irregular *C. albicans* cell walls (**Figure 6K**). In fact, in mixed biofilms microbial cells ultrastructural modifications were similar to those observed in monospecies biofilms, including invaginations of the cell membrane found in *C. albicans* cells (**Figure 6L**). Additionally, in mixed biofilms, *C. albicans* cells showed persistent extracellular vesicles, at the surface of the cell wall, with different sizes and shapes (**Figure 6M**). In **Figure 6N** it is also possible to observe several ultrastructural features of mixed biofilms treated with Zn(II)e_6Me by PDT: in the extracellular matrix, besides the spread of electrodense materials typical of a biofilm matrix, fragments of membranes or of fibrous materials were also observed, which were not observed in *E. faecalis*–*C. albicans* mixed biofilms untreated (**Figure 6C**); also, the cytoplasm of *E. faecalis* showed electrodense agglomerates and some fungal cells showed a twisted irregular shape.

DISCUSSION

The aim of this work was to compare the efficacy of Zn(II)e_6Me to disturb *in vitro* models of endodontic biofilms comparatively with three other PSs, TBO, RB, and TMPyP, and also with endodontic classical irrigants. For this, monospecies biofilms of *E. faecalis* and of *C. albicans* were used, together with a mixed biofilm model with both microrganisms. The main conclusion is that Zn(II)e_6Me had a better antimicrobial efficacy than the clinically used PSs, TBO, and RB. Although the efficacy of

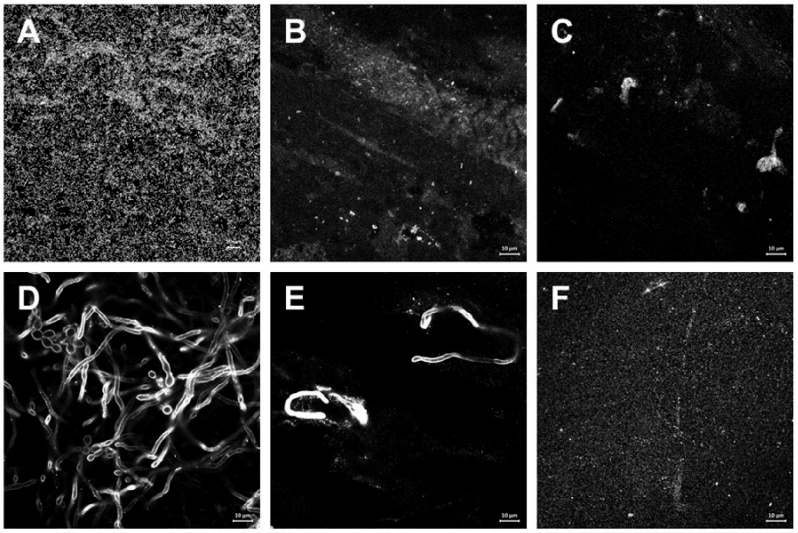

FIGURE 4 | Effect of aPDT with Zn(II)e_6Me as photosensitizer compared with the classical irrigant NaOCl in the morphology of monospecies biofilms. Endodontic *in vitro* 48 h-biofilms of *E. faecalis* and *C. albicans* were obtained and treated as described under Materials and Methods. **(A–C)** *E. faecalis* was stained with Syto 13 Green Fluorescent Nucleic Acid Stain. **(D–F)** *C. albicans* was probed with polyclonal primary antibody and with anti-rabbit secondary antibody Alexafluor®594. Representative images of biofilms untreated **(A,D)**, treated with Zn(II)e_6Me as PS activated for 90 s **(B,E)**, and treated with NaOCl **(C,F)**, were obtained with a Carl Zeiss Cell Observer Spinning Disk with Alpha Plan-Apochromat objective (100×).

Zn(II)e_6Me and TMPyP is similar, one of the main advantage of using Zn(II)e_6Me is its availability from natural sources, associated to a lower toxicity in the total absence of light. It also presented the same antimicrobial potential than the clinically used classical irrigants, CHX and EDTA. It is worth mentioning that TBO (Seal et al., 2002; Bergmans et al., 2008; Rios et al., 2011) is available in the market under the name of Fotosan® agent (Gambarini et al., 2011; Rios et al., 2011) and RB has been widely studied (Shrestha et al., 2012, 2014; Persadmehr et al., 2014). As expected, 3% NaOCl had the best final outcomes. In fact, NaOCl at different concentrations, is considered an excellent irrigant solution in endodontics (Jeansonne and White, 1994; Siqueira et al., 2007; Mohammadi, 2008), nevertheless it also displays high toxicity levels toward the host tissues (Estrela et al.,

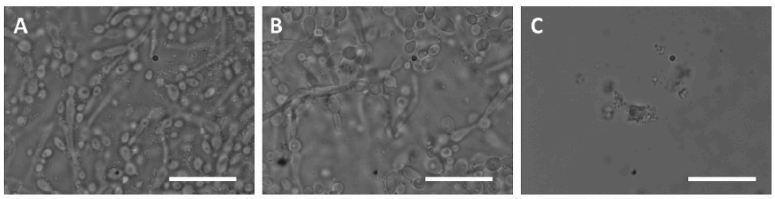

FIGURE 5 | Effect of PDT with Zn(II)e_6Me as photosensitizer compared with the classical irrigant NaOCl in the morphology of mixed biofilm. The images **(A)** untreated controls, **(B)** Zn(II)e_6Me activated during 90 s, and **(C)** with NaOCl, were obtained with using an Olympus BX-40 microscope at 1000× total magnification. Images were recorded at different time periods on an Olympus C-200 digital camera. Bars: 20 μm.

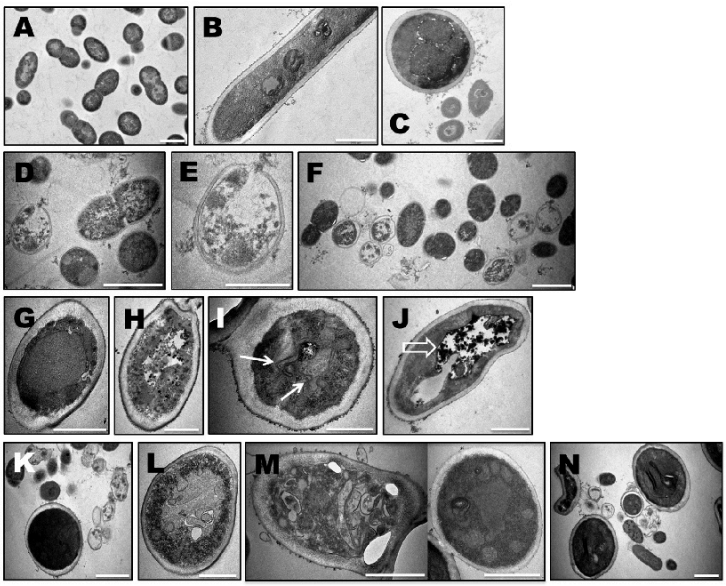

FIGURE 6 | Ultrastructural modification of microbial cells upon antimicrobial photodynamic therapy (aPDT) with Zn(II)e_6Me as photosensitizer. 48 h-biofilms of *E. faecalis*, *C. albicans* and mixed biofilms with both microrganisms untreated **(A–C)** and treated with aPDT with Zn(II)e_6Me as photosensitizer (*E. faecalis* biofilm: **D–F**), (*C. albicans* biofilm: **G–J**), (mixed biofilms: **K–N**). Solid arrows indicate peculiar intracellular membrane arrangements probably corresponding to endoplasmic reticulum whorls and open arrows depicted large vacuoles with electrodense materials. Bars, 1000 nm, except for E and I, 500 nm.

2002; Önçağ et al., 2003; Trevino et al., 2011; Wang et al., 2015).

The antimicrobial effect of aPDT is dependent both on the cellular localization of the PS, which may be determined by its physicochemical properties (Castano et al., 2004) and on the diffusion of singlet oxygen that should be sufficient to inactivate microrganisms structures and biomolecules. There have been several reports on the use of aPDT to kill both yeast and bacteria, however, fungi are much more complex targets than bacteria. Nevertheless, similarities with mammalian cells should be considered and this may indicate the use of cationic PSs, rather than their anionic counterparts, since the latter exhibit facile uptake by mammalian cells (Bonnett, 1995). The biochemical and functional effects of photosensitization include peroxidation of lipids, resulting in cell membranes disruption, lysosomes and mitochondria lysis and consequently autophagy (Schuck et al., 2014). The phenothiaziniums, such as TBO and MB, are known to target plasma membrane of yeast and bacteria (de Melo et al., 2013; Baltazar et al., 2015); TBO was described as increasing cell wall permeability (Wainwright et al., 1997), whereas MB produces bacterial DNA damage (Menezes et al., 1990).

The use of cholorophylls in endodontic root canal treatment was previously described (Mohammadi et al., 2013). There are evidencies showing that clorophyll present in green tea can be used in endodontic root canal treatment due to its antibacterial effects (Horiba et al., 1991). In this work, we describe Zn(II)e_6Me, obtained from the natural chlorophyll a, as a encouraging PS candidate displaying consistent antimicrobial outcomes. The ultrastructural study of microbial cells upon aPDT demonstrated that using Zn(II)e_6Me as PS, results in the irreversible damage of *E. faecalis* cells (mono and dual-species biofilms), displaying 'cell ghosts', empty of its cellular content but with almost intact cell walls. The presence of these inactive "ghost" cells was corroborated by the biomass loss assessed by SR assay. Before, it was described that *E. faecalis* elimination with aPDT resulted in bleb formations suggestive of damage of membrane components (López-Jiménez et al., 2015), shrunken, bacterial cell diameter reduction, rough and fractured appearance of the bacterial cells (Cheng et al., 2012). It was also described the presence of bacterial cell membrane shriveling and alterations including loss of cocci or bacilli shape, grooves on the cell surface and draining of the intracellular

components (Garcez et al., 2013). According to our observations, *E. faecalis* cell wall destruction was sporadic and not a massive one, which appear to indicate that the induced damage was directed to proteins and/or lipids of the cytoplasmic membrane, resulting in the leakage of cellular contents, as described by others (Girotti, 2001). It is also known that the extension of biochemical changes induced by aPDT is dependent on the PS nature and on the irradiation period (Dai et al., 2009). In this study the aPDT proceeded during a short period (60 or 90 s), which can justify the punctual cell wall destruction in *E. faecalis*.

In *C. albicans* cells in monospecies biofilms and in *C. albicans* cells in mixed biofilms, it was noticed several changes in the cellular organization, with a cytoplasmic membrane disruption, vacuoles morphology and organelles damage including signals of autophagy (e.g., ER whorls, and organelles inclusion in vacuoles) as described by others (Prates et al., 2011; Schuck et al., 2014). The intracellular damage induced by aPDT with $Zn(II)e_6Me$ is probably dependent on the entry of this PS, since this feature is crucial for aPDT efficacy and outcomes (Hamblin and Hasan, 2004; Baltazar et al., 2015). This lead us to speculate that the pre-incubation period of biofilms with PSs during 15 min in total absence of light, before the short irradiation period (60 and 90 s) most certainly contributed for the interaction between the PS and the cell. This would lead to intracellular PS distribution (due to its hydrophobic nature), impacting in the genesis of the intracellular damage observed. The questions raised by these observations highlight the importance of future further studies to unravel the intracellular distribution of $Zn(II)e_6Me$. The $Zn(II)e_6Me$ antimicrobial potential, that we showed by quantification of biofilm biomass loss and by a microscopic study of the biofilm morphology and of the cellular ultrastructure, leads to the importance of defining the mechanism by which this modified chlorophyll affects the endodontic biofilms.

Based on this, further research will be mandatory to improve the antimicrobial efficacy of aPDT in the root canal system, such as the ones recently published (Tennert et al., 2015; Cieplik et al., 2016) using human tooth models, ultimately leading to an optimization of light delivery and new PS formulations.

AUTHOR CONTRIBUTIONS

PD, IM, JS, FC, and TG were responsible for the conception and design of the study, and for the analysis and interpretation of data; PD, CF, FC, and MM did most of the lab work and analysis of data; PD and TG did most of the manuscript writings; MF, MN, MU, and KdO extracted, modified and analyzed two of the PDT compounds; all the authors contributed equally to the revision of the manuscript and approved the final version to be submitted.

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