UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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New insights in the disinfection of the root canal system using different research models

Novas abordagens na desinfecção do sistema de canais radiculares usando diferentes modelos de pesquisa

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“The only trust required is to know that when there is one ending there will be another beginning” Clarissa Pinkola Estés

ABSTRACT

"New insights in the disinfection of the root canal system using different research models"

The present studies aimed to evaluate the influence of irrigation on biofilm removal from simulated lateral morphological features and in the intratubular decontamination. An Optical Coherence Tomography analysis of biofilm removal from Polydimethylsiloxane (PDMS) root canal models with lateral morphological features, such as isthmus and lateral canal-like structures was described for the first time (first study). Sodium hypochlorite at various concentrations and in different flow rates was used for root canal irrigation followed by a final irrigation with a buffer at a high flow rate. In addition, irrigant velocity inside the root canal model using different flow rates was measured by Computational Fluid Dynamics and correlated with biofilm removal. The irrigant flow rate had more influence on biofilm removal than the irrigant concentration. The irrigant velocity influenced biofilm removal, since in areas with higher velocities, more biofilm was removed. A high flow rate was also related to higher irrigant velocity inside the model. In the second study, a comparison of four different irrigation protocols regarding their ability to remove biofilm from the PDMS root canal model with lateral morphological features as well as from human dentine tubules using Optical Coherence Tomography or Confocal Laser Scanning Microscopy as evaluation methods, respectively, was described. The irrigation protocols used included syringe irrigation with a buffer solution (control group), a modified salt solution called Risa, Sodium hypochlorite and Ultrasonic activation of the buffer solution, followed by a final irrigation at a higher flow rate using the buffer solution. The mechanical effect of syringe irrigation showed to be a relevant factor to be observed when studying biofilm removal. Ultrasonic activation of the irrigant showed to be effective when the contact surface biofilm-irrigant was smaller. In the dentinal tubule model, besides the antimicrobial efficacy, a recolonization analysis was performed five days after the treatment. The analysis showed that the post treatment remaining biofilm was able to regrow inside the dentine tubules in a five day period without any extra nutrition. The antibiofilm effect of the buffer solution, Risa and sodium hypochlorite was tested on a biofilm grown on dentine disks. The biofilm thickness reduction and biofilm viscoelastic properties of the post treatment remaining biofilm were evaluated, showing no differences among the studied substances. The third study describes the influence of the irrigant, flow rate, irrigant

refreshment and exposure time on biofilm removal from the root canal models with lateral morphological features by Optical Coherence Tomography. Sodium hypochlorite and demineralized water (control group) were used as irrigant solutions delivered in the root canal model with flow rates of 0.05 or 0.1 mL/s. Sodium hypochlorite and the higher flow rate presented greater biofilm removal from the isthmus like structures, whereas for the lateral canal, the flow rate had no influence. The samples were divided into different groups according to the irrigant solution refreshment number and that variable did not improve biofilm removal. Analysis of the sequential refreshments in the same biofilm showed a cumulative effect of irrigation on the biofilm removal.

Keywords: Biofilm, confocal laser scanning microscopy, isthmus, lateral canal, optical coherence tomography, polysaccharides, removal, RISA, sodium hypochlorite, ultrasound, velocity.

RESUMO

“Novos insights na desinfecção do sistema de canais radiculares usando diferentes modelos de pesquisa”

O objetivo do presente estudo foi investigar o efeito da irrigação na remoção de biofilme e na descontaminação intratubular. Para isso, foi descrita pela primeira vez uma análise por Tomografia de Coerência Óptica (OCT) da remoção de biofilme de modelos de canais radiculares com complexidades anatômicas, com estruturas semelhantes a istmos e a canais laterais (estudo1). A solução irrigadora hipoclorito de sódio foi utilizada em várias concentrações e em diferentes taxas de fluxo para irrigação do canal radicular, seguido de uma irrigação final com solução tampão em alta taxa de fluxo. Além disso, a velocidade do irrigante dentro do modelo de canal radicular foi medida em diferentes taxas de fluxo por meio de um Fluido Dinâmico Computadorizado e correlacionada com a remoção do biofilme. A taxa de fluxo de irrigante teve mais influência na remoção do biofilme do que a concentração de irrigante. A velocidade do irrigante influenciou a remoção do biofilme, pois em áreas com velocidades mais altas, mais biofilme foi removido. Uma alta taxa de fluxo também foi relacionada à maior velocidade de irrigação no interior do modelo. No segundo estudo, foi realizada uma comparação entre quatro diferentes protocolos de irrigação quanto à capacidade de remover biofilme do modelo de canal radicular com istmos e canais laterais, e de túbulos dentinários, por meio de OCT ou Microscopia Confocal de Varredura a Laser, respectivamente. Os protocolos de irrigação utilizados foram irrigação com seringa utilizando solução tampão (grupo controle), solução salina modificada chamada Risa, Hipoclorito de sódio e ativação ultrassônica da solução tampão, seguidos de irrigação final com alta taxa de fluxo utilizando a solução tampão. O efeito mecânico da irrigação com seringa mostrou-se um fator relevante a ser observado no estudo da remoção de biofilme. A ativação ultrassônica do irrigante mostrou-se eficaz quando em pequenas áreas de contato entre o biofilme e o irrigante. No modelo dos túbulos dentinários, além da eficácia antimicrobiana, uma análise de recolonização foi realizada cinco dias após o tratamento. Esta análise mostrou que o biofilme remanescente após o tratamento foi capaz de sobreviver e crescer novamente no interior dos túbulos dentinários, em um período de cinco dias sem qualquer nutrição extra. O efeito antibiofilme da solução tampão, Risa e Hipoclorito de sódio foi testado em um biofilme sobre discos de dentina. A redução da espessura do biofilme e as propriedades viscoelásticas do

biofilme remanescente após o tratamento foram avaliadas não mostrando diferença entre as substâncias estudadas. O terceiro artigo descreve a influência do irrigante, taxa de fluxo, renovação e tempo de exposição do irrigante na remoção de biofilme dos modelos de canal radicular por meio de OCT. Hipoclorito de sódio e água desmineralizada (grupo controle) foram utilizados como soluções irrigadoras levadas ao modelo de canal radicular com uma taxa de fluxo de 0,05 ou 0,1 mL/s. O hipoclorito de sódio e a maior taxa de fluxo apresentaram maior remoção de biofilme das estruturas semelhantes ao istmo, enquanto que, para o canal lateral a taxa de fluxo não teve influência. As amostras foram divididas em diferentes grupos de acordo com o número de renovações da solução irrigadora e essa variável não melhorou a remoção do biofilme. A análise de renovações sequenciais no mesmo biofilme mostrou um efeito cumulativo da irrigação na diminuição do biofilme.

Palavras-chave: biofilme, microscopia confocal de varredura a laser, istmo, canal lateral, tomografia de coerência óptica, polissacarídeos, remoção, RISA, hipoclorito de sódio, ultrassom, velocidade.

LIST OF ABBREVIATIONS, SIGNS AND SYMBOLS

0R	*Zero refreshment*
1R	*One refreshment*
2R	*Two-refreshments*
2D	*Two-dimensional*
A. naeslundii	*Actinomyces naeslundii*
ANOVA	*Analysis of Variance*
ATCC	*American type culture collection*
B	*Buffer solution*
BHI	*Brain Heart Infusion*
$CaCl_2$	*Calcium chloride*
CDFF	*Constant Depth Film Fermentor*
CFD	*Computational Fluid Dynamics*
CH	*Calcium hidroxyde*
CI	*Confidence Interval*
CLSM	*Confocal Laser Scanning Microscopy*
D	*Diameter*
EDTA	*Ethylenediaminetetraacetic acid*
E. faecalis	*Enterococcus faecalis*
EPS	*Extracellular polymeric substances*
E(t)	*Total stress exerted* by the biofilm
E	*Stress exerted by the biofilm*
f	*Effect size*
Fig.	*Figure*
G	*Gauge*
g	*Grams*
g/L	*Grams per liter*
IT	*Initial Treatment*
KCl	*Potassium chloride*
K_2HPO_4	*Dipotassium phosphate*
KH_2PO_4	*Monopotassium phosphate*
L	*Lenght*

LLCT	*Low Load Compression Testing*
MMS	*Modified salt solution*
µL	*Microliter*
µm	*Micrometer*
mL	*Milliliter*
mL/s	*Milliliter per second*
mL/h	*Milliliter per hour*
mm	*Millimeter*
mm^3	*Millimeter cubic*
min	*Minutes*
n	*Number of samples*
NaOCl	*Sodium hypochlorite*
OCT	*Optical Coherence Tomography*
P	*Probability*
Pa	*Pascal*
pH	*Potential hydrogen*
PI	*Iodide Propidium*
Px	*pixels*
PDMS	*PolyDimethylsiloxane*
PMNs	*Polimorphonuclear Neutrophilis*
r	*Irrigant velocity (log transformed)*
R	*Analysis after recolonization*
R	*Radius*
R	*Risa*
R	*Stress relaxation percentage*
rpm	*Rotations per minute*
RWS	*Reconstitutional human whole saliva*
s	*seconds*
S. oralis	*Streptococcus oralis*
SD	*Standard deviation*
US	*Ultrasonic activation*
W	*Wats*
WL	*Working length*

xg	*Centrifugal force*
ºC	*Degree Celsius*
τ_i	*Decay time*
%	*Percent*
<	*Less-than*
≥	*Greater than or equal to*

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1 INTRODUCTION

1 INTRODUCTION

Paths of infection in the root canal system: from caries to apical periodontitis

It is estimated that oral biofilms contain up to 19.000 species (Keijser *et al*. 2008) being a very diverse microbial community (Huse *et al*. 2012). These bacteria can be present on the tooth surface, periodontium, tongue and everywhere in the oral cavity. On the other hand, once the biologic protection such as enamel and cementum are preserved, in the endodontic space (pulp chamber and root canals) no bacteria can be found (Figdor & Sundqvist 2007).

An endodontic infection can be introduced for several reasons, including dentine exposure, dental trauma, periodontal disease and dental caries, the last representing the main cause (Kakehashi *et al.* 1965, Ferrari & Cai, 2010). Caries is a biofilm-induced disease that starts as a consequence of the metabolic activity of some bacterial species driven by a source of fermentable carbohydrates (Nyvad *et al*.2013, Pitts *et al*. 2017), causing a demineralization process. When this microbial irritation is maintained, the demineralization advances toward the dentine and, as a result of this aggression, the dentin-pulp complex responds with secondary and tertiary dentinogenesis (Duncan *et al*. 2019), in an attempt to avoid pulpal contamination. However, in the absence of caries removal, the inflammatory reaction started by the microbial infection reaches the pulpal connective tissue. Thenceforth, depending on the severity of the inflammation, the pulp can become reversible or irreversible inflamed. When a pulp exposure by caries occurs, an irreversible status of inflammation is reached, requiring a partial or total excision of the affected tissue (Ricucci *et al*. 2014).

During progression of pulp inflammation, a tissue expansion occurs, compromising the blood circulation. Thus, the catabolites produced inside the pulp cannot be drained, vessels are dilated, and since the pulp is surrounded by rigid walls, the connective tissue undergoes severe inflammation followed by necrosis (Ferrari & Cai, 2010, Siqueira 2011, Ricucci *et al*. 2014). Different from the vital pulp, in the necrotic tissue microorganisms can easily grow (Langeland 1987). First, bacteria are free in the tissue fluid, in a planktonic state, but they rapidly associate themselves to each other forming a biofilm that adheres to the root canal walls (Nair 1987, Molven *et al*. 1991, Siqueira *et al*. 2002). The microbial population

can invade the whole root canal system, including dentine tubules, isthmus, apical deltas, lateral and accessory canals, and all ramifications. This microbial encroachment causes an irritation of the periradicular tissues, which causes a recruitment of defence cells such as polymorphonuclear neutrophils (PMNs) and macrophages (Langeland, 1987, Ricucci & Siqueira, 2010a). These cells occupy the periodontal ligament space and, as the infection continues and increases and the bacterial toxic products are released, they recruit osteoclasts in order to reabsorb bone tissue increasing the periodontal space, forming the periapical lesion (Ferrari & Cai, 2010).

Once the periapical lesion is formed, its regression, healing, persistence and/or evolution will depend on the microbial control in the root canal system (Bystrom *et al*. 1987, Sjogren *et al*. 1990) and on the extraradicular biofilm presence (Ricucci & Siqueira 2008, 2010a). The broad identification of a periapical lesion is by the observation of a periapical radiolucency in a periapical radiography (Pak *et al*. 2012). The study of Pak et al. (2012), a systematic review and meta-analysis where 300,000 teeth from different studies were evaluated regardless the prevalence of periapical radiolucency and non-surgical endodontic treatment, showed an average of one lesion and two endodontic treatments per patient. In general, therefore, it seems that root canal infection and periapical disease are very prevalent, and greater efforts are needed to combat them.

Biofilm in the root canal system

Returning to the start of the endodontic infection with the process of necrosis, besides being a suitable place for microbial thriving, the necrotic root canal environment allows a biological selection that will dictate the type and course of the infection (Figdor & Sundqvist 2007). Primary root canal infections are mainly composed by anaerobic proteolytic bacteria that are able to survive with a limited amount of oxygen and nourish themselves with serum constituents, such as glycoproteins from the inflamed pulp and periapical tissues (Svensater & Bergenholtz 2004).

An endodontic treatment will cause an ecological disturbance in the existing root canal microbiota. Only the most resistant microorganisms will survive and adapt themselves to the stress generated by instrumentation, irrigation, intracanal medications and all procedures of the treatment (Chávez de Paz & Marsh, 2015).

If a polymicrobial flora characterizes an untreated root canal (equal proportion of Gram-positive and Gram-negative bacteria, dominated by obligated anaerobes), the literature is controversial regardless the microbiota in persistent endodontic infection. It was believed that one or a few bacterial species compose secondary infections (Molander *et al*. 1998, Sundqvist *et al*. 1998). On the other hand, the studies using molecular sequencing techniques found diverse microbiota in both primary and persistent infections (Hong *et al*. 2013, Sánchez-Sanhueza *et al*. 2018). These bacteria use different adaptive mechanisms of resistance to survive the environment ecological disturbances (Chávez de Paz *et al*. 2015). They are predominantly facultative or obligated anaerobic Gram-positive microorganisms (Figdor & Sundqvist 2007).

Łysakowska *et al*. (2016), using macromorphological, micromorphological and commercial biochemical tests examined the microbiota present in primary and secondary infections from root canals of 33 patients. In both primary and secondary infections, a great variety of bacterial species were found. However, there was greater diversity in the cultivable microbiota present in secondary infections. *E. faecalis* was found to be the most prevalent bacteria in both primary and secondary infections being also related to periapical radiolucency as well as *Actynomices ssp.* However, Sánchez-Sanhueza *et al*. (2018), using next-generation sequencing, showed low reports of *E. faecalis* and a high prevalence of Proteobacteria followed by Bacteroidetes in cases of filled root canals with apical periodontitis. Some patients presented a great amount of less often found phyla, such as, Actinobacteria or Tenericutes. The most abundant family of bacteria found was Pseudomonadaceae. These findings show the great variability in the microbiota present in the endodontic infections, which makes it very difficult to simulate a root canal biofilm in '*in vitro'* studies.

One of the main bacterial adaptative mechanisms of resistance is the biofilm mode of growth in the root canal system, which is the dominant microbial form of life in the endodontic environment (Chávez de Paz *et al*. 2015, Siqueira *et al*. 2010). Inside the biofilm, they are more resistant to the antimicrobial agents and procedures of the endodontic therapy, being able to survive in unfavourable environmental and nutritional conditions, which represents the greatest obstacle to the root canal treatment success (Baumgartner *et al*. 2008).

Biofilm formation starts with the adsorption of macromolecules from tissue fluids such as saliva onto a biomaterial and the adhesion in a substrate, in the case of the root canals, the dentine walls. The environmental conditions, bacteria type and substrate factors will influence

this very important stage of the biofilm proliferation. After this, bacteria will associate themselves by coaggregation and coadhesion. In coaggregation, distinct bacterial cells in suspension recognize each other and clump together, while coadhesion is the association between a bacterium already attached to a substrate with a suspended one. After this, bacteria start to produce the extracellular polymeric substance (EPS), and a biofilm expansion occurs (Baumgartner *et al*. 2008, Kishen & Haapasalo, 2015). The EPS is a matrix of biopolymers produced by the microorganisms, where they are embedded and protected from the environmental stresses. This matrix is able to mediate adhesion to surfaces, trap and concentrate essential nutrients and maintain bacteria cells in close proximity favouring intercellular interactions. The EPS is also responsible for the architecture of the biofilm, which will prevent the diffusion of antimicrobial agents to the resident bacteria (Flemming & Wingender 2010). Thus, different to the planktonic state of bacteria, the biofilm represents an extra obstacle to root canal disinfection.

Another significant aspect in endodontic disinfection is the anatomy of the root canal. It is called a "system" because it is not a unique root canal with a single apical foramen. The pulp space is divided into the pulp chamber, located within the anatomic dental crown, and the root canal, found inside the radicular portion of the tooth. This last part is often complex, comprising canals that divide and rejoin, isthmuses, fins, anastomosis, accessory and lateral canals, and apical deltas (Hargreaves & Cohen 2011, Versiani & Ordinola-Zapata, 2015), and root canal biofilm has been found in all of these areas (Nair *et al*. 2005, Riccuci & Siqueira, 2010a,b).

Important examples of these root canal anomalies are the isthmus and lateral canals. An isthmus is a narrow communication between two root canals, where biofilm, filling material, vital and necrotic pulp can be found (Weller *et al*. 1995, Vertucci 2005). An isthmus has a length of approximately 2,331mm but there is scarce information about its width. Degerness et al. (2010) reported a width of isthmusses (from mesial to distal direction) in maxillary molars ranging from 0.11 to 0.24mm. Because of the great variety of the size of an isthmus, Hsu & Kim (1997) classified the type of canal isthmuses in four categories where Type I is defined as two canals with no notable communication; Type II is a hair-thin connection between two canals; Type III is the hair-thin connection between three canals; Type IV is an isthmus with extended canals in the connection; and Type V is defined by a true connection or wide corridor of tissue between the two canals. A lateral canal (Figure 1) is a communication between the main root canal and the external root surface (AAE 2012). This

kind of accessory canal has a diameter ranging from 10 to 200 μm (Dammaschke *et al*. 2004), being not visible by periapical radiography, however its presence can be suspected by a lateral thickening in the periodontal ligament or lesion in the root lateral surface (Versiani & Ordinola-Zapata 2015). A lateral canal cannot be instrumented, and studies have found presence of necrotic pulp and biofilm after biomechanical preparation in isthmus (Versiani & Ordinola-Zapata 2015, Adcock *et al*. 2011). Thus, when bacteria are located in these anatomic complex areas, they are hard to eliminate. Since the instrumentation is not able to remove them, the role of irrigation is to debride these areas through a mechanical flushing action and a chemical effect (Gulabivala *et al*. 2005). After instrumentation and irrigation, the intracanal medication can also work in the prevention of a reinfection and in the killing of these remaining bacteria (Bystrom & Sundqvist, 1981).

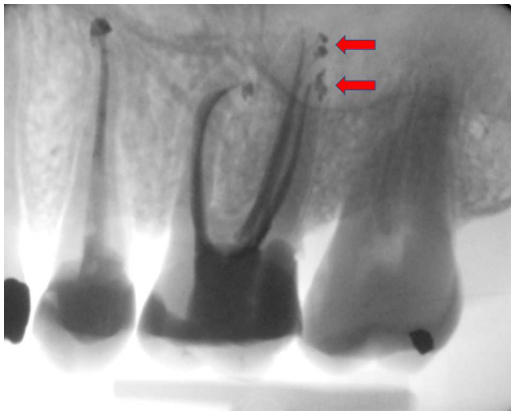


Figure 1 – Periapical radiography shows a maxillary first molar with root canal filling where it is possible to observe lateral canals in the palatal root, showing the complexity of the root canal system.

Acknowledgement: Periapical radiography kindly provided by Gianfranco Muñoz Reinoso.

Ecological Disturbances in the root canal system: Disinfection procedures

In the endodontic treatment, disinfection procedures consisting of root canal shaping, irrigation and intracanal medication (Bystrom *et al*. 1985), are the main cause of ecological disturbances in the root canal biofilm (Chávez de Paz & Marsh 2015). Since instrumentation mainly eliminates bacteria in the main root canal, in the problematic areas the ecological disturbances on biofilm are mostly caused by irrigation and intracanal medication (Shen *et al*. 2012). Several variables will influence the effectiveness of these disinfection procedures. For irrigation, these variables include the type and concentration of the irrigant solution and the irrigation regimen. Similarly, some features will influence the intracanal medication activity including the type of medication, mechanism of action and placement efficacy (Gulabivala &

Ng, 2015). In this section, the most used antimicrobial agents in the endodontic therapy will be presented together with the most used available methods for its delivery in the root canal system.

Intracanal Medication: Calcium hydroxide paste

Endodontic therapy may need to be performed in more than one appointment for a few reasons, including lack of time for finishing the complete treatment and on one hand the persistence of signs and symptoms or on the other hand when the root canal cannot be dried, mostly caused by persistence of infection. In these cases, an intracanal medication between appointments can be used to eliminate and degrade remaining bacteria and endotoxins after the first appointment (Xavier *et al.* 2013), besides serving as a chemo-physical barrier against recolonization by remaining bacteria and new invaders in the root canal system (Gulabilvala & Ng, 2015).

The most frequently used intracanal medication is the Calcium Hydroxide (CH) paste. This substance acts by ions diffusion in the dentine mass, releasing hydroxyl and calcium ions. The antibacterial activity of this medication is due to its high pH, able to alkalize the root canal system when used in the right conditions. The hydroxyl ions are related with the pH increase, acting in the bacterial cytoplasmic membrane (Ferrari & Cai 2010, Xavier *et al.* 2013). On the other hand, the calcium ions are related with CH mineralization, activating enzymes from the host tissue such as alkaline phosphatase (Bystrom *et al*. 1985).

There are different options for CH paste delivery, such as a syringe system, manual files, Lentulo spiral and automated NiTi files. All these methods are effective in filling the root canal with the medication, once an appropriate root canal preparation is performed (Simcock & Hicks 2006). However, an important aspect in the diffusion is the vehicle in which the CH paste is manipulated. Viscous and aqueous vehicles, such as distilled water, propylene glycol and polyethylene glycol, have a positive effect on dentin penetrability and must be seen as the vehicle of choice (Ferrari & Cai, 2010, Pereira *et al*. 2019). Added to it, some bacteria such as *Enterococcus faecalis,* can resist the CH paste because of their ability to deeply penetrate dentine tubules where they are not reached by the medication (Love 2001, Ferrari & Cai, 2010). Moreover, they have an inherent proton pump that makes this microorganism resistant to CH and its alkaline pH, by maintaining the homeostasis (Stuart *et al*. 2006), although, by direct contact, CH can kill this species (Ferreira et al., 2007). For this

reason, some studies are being performed evaluating different vehicles and the use of some additives in this medication, in order to improve its physical properties and antimicrobial action (Martinho *et al*.2017, Pereira *et al*. 2019). In addition, the application time of the CH paste will also influence its effectiveness against bacteria. The dentine's buffer effect that occurs in high pH situations can hinder CH antimicrobial activity. For this reason, this intracanal medication must be maintained in the root canal for a period of 7 to 14 days, to compensate this effect (Ferrari & Cai, 2010).

Martinho *et al*. (2017) compared *in vitro* the efficacy of CH pastes with saline, with 2% chlorhexidine gel and the 2% chlorhexidine gel alone, used for 7 and 14 days, in reducing bacteria and endotoxins from primary infected root canals. They found that all tested intracanal medications were able to reduce bacterial load both 7 and 14 days, with the chlorhexidine alone for seven days showing the lowest effectiveness. Because of the existing controversy in CH ability in improving or removing endotoxins from infected root canals, the study of Xavier *et al.* (2013) compared the removal of bacteria and endotoxins between the single-visit endodontic treatment and a two-visits with the use of CH paste between appointments, concluding that the use of an intracanal medication improved endotoxin reduction. Pereira *et al.* (2019) compared the antimicrobial ability against *E. faecalis*, intratubular penetrability, pH, calcium ions release and solubility of five different formulations of CH pastes. The tested pastes were CH with distilled water and propylene glycol as a vehicle, and chlorhexidine, propolis and camphorated paramonochlorophenol as additives. The authors found that the pastes that used propylene glycol as vehicle presented higher pH and calcium ions release in comparison with the paste with distilled water. All pastes showed great penetrability and antimicrobial effectiveness, reducing the amount of *E. faecalis* from the dentine tubules. However, bacteria inside a biofilm tend to be more resistant to an alkaline environment than in a planktonic state (Chávez de Paz *et al.* 2007). Zancan *et al.* (2016) evaluated the antimicrobial ability of different CH paste formulations against mono and dual-species biofilms in a seven days period, and found that it was an insufficient time for killing bacteria inside the biofilm. The addition of chlorhexidine to the CH paste improved the antimicrobial effectiveness against biofilm.

Another issue about this intracanal medication is that the presence of residual paste before root canal filling can disrupt the adhesion of endodontic sealers (Keles *et al*. 2014), which can lead to treatment failure (Ricucci & Langeland 1997). Activated irrigation by ultrasound, sonic and mechanical devices are being studied in order to improve CH removal.

Although no method has shown to be able to completely remove this medication from the root canal walls, irrigant activation methods are more effective than the conventional syringe irrigation (Donnermeyer *et al*. 2019, Marques-da-Silva et al. 2019).

Thus, the literature shows CH paste as a suitable option as intracanal medication when the endodontic treatment cannot be performed in a single visit, because of its high pH that improves bacteria and endotoxins elimination. However, because of the limited effectiveness against biofilms and the difficult removal of the paste from the root canal walls, its use is questioned and new vehicles for this paste need to be further investigated.

Irrigation in Endodontics

As discussed in the previous sections, the morphological complexity of the root canal system and the character of the biofilm infection are the most challenging issue in endodontic treatment (Nair *et al*. 2005, Zehnder 2006, Riccuci & Siqueira, 2010a,b, Hargreaves & Cohen 2011, Chávez de Paz *et al*. 2015, Versiani & Ordinola-Zapata, 2015). The contemporary instrumentation and irrigation methods are insufficient to control infection, mostly because of the inability in reaching all biofilms present in the endodontic space (Gulabivala *et al*. 2001, 2005). Moreover, the small size and volume of the pulp space are a physical limitation for the irrigation fluid dynamics (Gulabivala *et al*. 2010). Thus, irrigation must be further studied, analysing not only the irrigating solution and the delivery methods but also the flow-rate used during syringe irrigation, observing the chemical and mechanical action of this procedure.

Chemical Action of Irrigation

During biomechanical preparation, irrigation of the root canal system is performed by an antimicrobial solution, preferably with tissue dissolution ability (Haapasalo *et al*. 2010). Besides dissolution of organic matters and antimicrobial action, irrigants are used in the endodontic therapy as a lubricant for the instruments and to flush out instrumentation remnants debris, or the inorganic matters (Siqueira *et al*. 2000). After biomechanical preparation and before filling or placement of an intracanal medication, for the removal of the inorganic remnants (smear layer), the root canals must be irrigated with a chelating agent or acids, exposing collagen and opening the dentine tubules (Shen *et al*. 2012). Then, a final irrigation with the same solution used during instrumentation is performed. Since there are

well-established and smaller amounts of chelating/acid substances, in this section, the main irrigating solution used during the biomechanical preparation and final irrigation will be discussed.

Sodium hypochlorite

Sodium hypochlorite (NaOCl) is the most used irrigating solution in the endodontic treatment due to its effective antimicrobial activity and tissue dissolution ability, which allows organic matters dissolution including pulp tissue and biofilm (Naenni *et al*. 2004, Shen *et al.* 2012, Petridis *et al*. 2019a). Its action depends on its volume, concentration, exposure time, temperature, pH and the contact surface biofilm-irrigant. Furthermore, NaOCl has a low surface tension. Considering this, penetration in areas untouched by instrumentation remains challenging (Shen *et al*. 2012).

It seems to be logical that increasing NaOCl concentration would increase bacterial elimination, biofilm removal and tissue dissolution. However, especially in higher concentrations, NaOCl has toxical effects for the periapical tissues, and an extrusion of this irrigant during endodontic treatment can cause severe irritation (Hülsmann & Hahn, 2000). Thus, studies analysing different NaOCl concentrations regardless its antimicrobial and tissue dissolution effectiveness have been performed. Baumgartner & Cuenin (1992) evaluated the debridement capability of 0.5, 1, 2.5 and 5.25% NaOCl in instrumented and uninstrumented root canal surfaces. The authors found that 1; 2.5 and 5.25% NaOCl were able to completely remove pulpal remnants and pre-dentin from uninstrumented surfaces, whereas after using at 0.5% some fibrils were left on the surface. Siqueira *et al*. (2000) compared the antimicrobial activity of NaOCl in 1, 2.5 and 5.25% and found that all concentrations were able to reduce bacteria from the main root canal. In both papers, it was emphasized that besides the importance of the exposure time of the irrigant in the root canals, the volume and regular refreshments of the given solution can compensate the concentration (Baumgartner & Cuenin 1992, Siqueira *et al*. 2000). Moreover, the reaction between NaOCl and the organic matters inside root canals causes a reduction in the amount of available active chlorine (Baker 1947). Petridis *et al*. (2019b) evaluated, in a diffusion-dependent model, the antibiofilm ability of 2, 5 and 10% NaOCl. The authors observed that by increasing the concentration, the antibiofilm efficacy was enhanced. However, 10% NaOCl provoked great bubble formation, which can

improve biofilm displacement, and also induce stable bubbles that can contribute to biofilm removal.

The antimicrobial action performed by NaOCl is suggested to be due to the active chlorine present in the hypochlorous acid formed when NaOCl reacts with water. The active chlorine is an oxidizing agent able to disrupt the metabolic functions of the bacterial cells by an irreversible oxidation of sulfhydryl groups of essential enzymes (Siqueira *et al*. 2000). The "reservoir" of active available NaOCl solution will be influenced by its applied volume (Petridis *et al*. 2019a). Thus, it is preferred to use copious amounts of NaOCl than this solution at high concentrations (Zehnder 2006). The increase of volume and exposure time of NaOCl in an intermediate concentration was associated with greater biofilm disruption and dissolution, and EPS removal, proving that these two features influence NaOCl anti-biofilm ability (Petridis *et al*. 2019a). Also, it was suggested that an irrigant exchange, which means to perform NaOCl refreshments in the root canal, could improve its chemical efficacy by compensating this chemical instability caused by the reduction of active chlorine (Druttman & Stock 1989). However, the direct influence and frequency of renewing the solution in the root canals need further investigation.

Another important factor when analysing irrigation is that the surface contact and the substrate (pulp, biofilm) will influence its effectiveness (van der Sluis *et al*. 2015). The root canal space represents a limited contact surface between NaOCl and the organic matters and substrate, which means that the chemical effect of irrigation happens by diffusion of this biocide (van der Sluis *et al*. 2015, Petridis *et al*. 2019a). Root canal enlargement can improve the cleaning ability of NaOCl. However, overpreparation can weaken the tooth structure (Druttman & Stock 1989). Moreover, even in these cases, anatomic complex areas could still be difficult to reach by instrumentation and irrigation. The effect of the flow rate as a mechanical effect on biofilm removal is an important subject to be studied in order to improve NaOCl diffusion and contact during root canal disinfection (Moorer & Wesselink, 1982; Shen *et al*. 2012).

An ideal irrigation solution should have a broad antimicrobial spectrum of action and high effectiveness against anaerobic and facultative microorganisms, especially when they are organized in biofilms; should be able of inactivating endotoxins, dissolve pulp tissue; and prevent and dissolve smear layer formation. Although NaOCl has limitations and disadvantages, it presents more desirable conditions of an ideal irrigant, making it the most

suitable option to be used during endodontic treatment (Zendher 2006). The major limitations of this biocide can be compensated by the mechanical character of irrigation that will be discussed in the next section.

Mechanical Action of Irrigation

The debridement efficacy of irrigation depends on a chemical and mechanical action. The mechanical effects of irrigation are generated by the in and out flow of the irrigant (Siqueira *et al*. 2000) and by its activation by files, gutta-percha cones, sonic or ultrasonic activated inserts, and laser. Syringe irrigation is conventionally used and is performed by placing the needle as close as possible to the root end and then, delivering the irrigant in the root canal. The relation between the volume and the time in which the irrigant will be delivered will determine the flow-rate of the irrigant (Boutsioukis *et al*. 2007, van der Sluis *et al*. 2015).

Also, during irrigation, a flow pattern of the irrigant will be produced inside the root canal which will depend on the needle type used and its insertion depth. More precisely, the needle type will influence the jet formed at its outlet. Open-ended needles will form a relatively high-speed jet, and the flow extends along the longitudinal axis of the root canal, apically to their tip. In the closed-ended needles the jet of the irrigant is formed near the apical side of the outlet, and is directed toward the apex with a divergence of approximately 30º following a curved path around the tip. Besides, the needle diameter and the size of the apical preparation can also influence the flow pattern. Small-size flexible needles tend to present better results in the irrigant delivery because they can be placed closest to the root end, even in roots with curvature when it was enlarged to a 0.30 or 0.35 diameter (Boutsioukis *et al*. 2009, 2010a, b, c, van der Sluis *et al*. 2015). However, the many variables in syringe irrigation cause a lack on protocols standardization, leading to negative results in studies (Caputa *et al*. 2019).

Activation of the irrigant has been used during biomechanical preparation and final irrigation, in an attempt to ensure a complete debridement of the root canal system. Ultrasonic activation (US) has shown positive results in cleaning areas unreachable by instrumentation and has thus become the most used method for irrigant activation (van der Sluis *et al*. 2007, Adcock *et al*. 2011, Dutner *et al*. 2012). During US cavitation of the irrigant occurs leading to bubble implosion that produces a focus of energy (van der Sluis *et al*. 2007). When this

implosion occurs close to a wall it can generate a high-speed jet on its direction, which enhances the cleaning (Brennen 1995, Ohl & Wolfum 2003). Moreover, it produces a lateral flow component that improves cleaning in lateral anatomic complexities (Burleson *et al*. 2007, Al-Jadaa *et al*. 2009, de Gregório *et al*. 2009, Castelo-Baz *et al*. 2012).

Another important fact to be observed is the occurrence of wall shear stress during irrigant flow that consists of frictional forces between the flowing irrigant and a solid body, or between a moving solid body and a static irrigant (Mott 1999; Tilton 1999; White 1999). During US, the oscillatory shear stresses caused by oscillation of the insert can cause biofilm energy loss, leading to fatigue and failure of the biofilm (Guelon *et al*. 2011, van der Sluis *et al*. 2015). However, the influence of US on biofilm removal from areas of accessory canal anatomy needs further investigation.

Macedo *et al*. (2014) evaluated the removal of a biofilm- mimicking hydrogel from simulated lateral canal and isthmus by US with different irrigant solutions. The authors found that US improved the hydrogel removal from the lateral canal and isthmus models. However, the formation of stable bubbles inside the simulated structures may jeopardize cleaning. Robinson *et al*. (2018) evaluated the influence of some variables in US during removal of hydrogel from simulated lateral canal extensions in the same above-mentioned root canal model. Also, they measured the amount of cavitation and streaming generated with all different parameters. They concluded that cavitation and streaming play a significant role in the accessory canal anatomy cleaning.

The use of higher flow-rates during syringe irrigation and the activation of the irrigant are important because weak forces, such as low pressures and shear stress, can only cause an elastic deformation on biofilm that can be reverted after the stress removal (van der Sluis *et al*. 2015). US may be an effective tool for biofilm and debris removal from problematic areas of the root canal system. However, little is known about the antimicrobial ability of the US, and if it is really more effective than syringe irrigation in this sense (Caputa *et al*. 2019). Moreover, considering that, until now, NaOCl remains the most suitable irrigating solution, an association between this substance and the US or the performance of higher flow-rates during syringe irrigation appears as a solution for the root canal system disinfection problem.

AIM OF THE THESIS

The aim of this thesis was to investigate disinfection of the root canal system focussing on the lateral morphological features of the root canal and dentinal tubules and improving different research models used for *'in vitro'* studies on irrigation.

In Article 1, a new root canal biofilm model with lateral morphological features filled with biofilm was described and the effect of fluid flow on the biofilm was tested.

In Article 2, four different irrigation protocols on biofilm removal from a root canal model with lateral morphological features, on the antimicrobial activity and EPS removal from dentinal tubules were investigated. Besides, the recolonization ability of the biofilm in the dentinal tubules after irrigation was evaluated.

In Article 3, the influence of refreshments, exposure time, irrigant and flow-rate on biofilm removal from lateral morphological features were analyzed.

2 ARTICLES

2 ARTICLES

The articles presented in this thesis were written according to instructions and guidelines for article submission presented in International Endodontic Journal.

2.1 ARTICLE 1 - Title: Biofilm removal from an artificial isthmus and lateral canal during syringe irrigation at various flow rates: a combined experimental and Computational Fluid Dynamics approach.

Abstract

Aim: a. to quantify biofilm removal from an isthmus-like and a lateral-canal-like structure in an artificial root canal system during syringe irrigation with NaOCl at different concentrations delivered at various flow rates **b.** to simulate the irrigant flow in those areas using a computer model **c.** to examine whether biofilm removal is correlated to the irrigant velocity predicted by the computer model.

Methodology: Ninety-six artificial root canals with either an isthmus-like or a lateral-canal-like structure were used. A dual-species biofilm was formed in these structures using a constant depth film fermenter. Sodium hypochlorite at various concentrations (2, 5 and 10%) or adhesion buffer (control) was delivered for 30 s by a syringe and an open-ended needle at 0.033, 0.083, or 0.166 mL/s or passively deposited in the main root canal (phase 1). All specimens were subsequently rinsed for 30 s by adhesion buffer at 0.166 mL/s (phase 2). The biofilm was scanned by Optical Coherence Tomography before the experiments, after phase 1 and after phase 2 to determine the percentage of the remaining biofilm. Results were analyzed by two 3-way mixed-design ANOVAs (α=0.05). A Computational Fluid Dynamics model was used to simulate the irrigant flow inside the artificial root canal system.

Results: The flow rate during phase 1 and additional irrigation during phase 2 had a significant effect on the percentage of the remaining biofilm in the isthmus (P=0.004 and P<0.001). Additional irrigation during phase 2 also affected the remaining biofilm in the lateral canal significantly ($P\leq$0.007) but only when preceded by irrigation at medium or high flow rate during phase 1. The effect of NaOCl concentration was not significant (P>0.05). Irrigant velocity in the isthmus and lateral canal increased with increasing flow rate and it was substantially correlated to biofilm removal from those areas.

Conclusions: The irrigant flow rate affected biofilm removal more than NaOCl concentration. The computer model can predict biofilm removal from isthmus-like and lateral-canal-like structures.

Key Words: Biofilm; isthmus; lateral canal; sodium hypochlorite; velocity.

Introduction

Root canal irrigation is one of the most important steps during root canal treatment (Gulabivala *et al.* 2005, Zehnder 2006). Its primary aim in infected root canal systems is the elimination of microorganisms which often adhere to the root canal wall and form a biofilm (Ricucci *et al.* 2009). Biofilm located in remote areas such as isthmuses, fins, oval extensions and lateral canals and also in some parts of the main root canal is beyond the reach of instruments (Peters 2004, Gulabivala *et al.* 2005, Ricucci *et al.* 2013), so irrigants are expected to disrupt and remove it by a combination of chemical and mechanical effects (van der Sluis *et al.* 2015).

The chemical effect is mainly realized by sodium hypochlorite, which remains the most widely used primary irrigant (Zehnder 2006, Dutner *et al.* 2012). Its molecules and ions need to be transported to the areas of interest predominately by the bulk irrigant flow and, to a lesser extent, by diffusion (van der Sluis *et al.* 2015). Since the reactive components of NaOCl are rapidly consumed when in contact with bacteria, pulp tissue or dentine (Moorer & Wesselink 1982, Haapasalo *et al.* 2000, Portenier *et al.* 2002), frequent exchange is needed. The mechanical effect, on the other hand, is exerted by the direct effect of the irrigant flow on the biofilm residing on the root canal wall through shear stress (van der Sluis *et al.* 2015). Sodium hypochlorite penetration and exchange in the main root canal and the developed wall shear stress are strongly affected by the irrigant flow rate (Boutsioukis *et al.* 2009, Verhaagen *et al.* 2012, van der Sluis *et al.* 2015) but there is very little information concerning the effect of the flow rate on irrigant penetration in areas beyond the main root canal, such as isthmuses and lateral canals and on biofilm removal.

Earlier studies have also indicated that sodium hypochlorite concentration affects the disruption and removal of the biofilm following direct contact *in vitro* (Petridis *et al.* 2019b). Moreover, computer models and *in vitro* experiments have shown that sodium hypochlorite penetration into lateral canals is likely to be a diffusion-dominated process, therefore directly influenced by its concentration in the main root canal (Verhaagen *et al.* 2014). However, the effect of sodium hypochlorite concentration on biofilm removal from isthmuses and lateral canals remains largely unexplored.

While the chemical and mechanical effects of irrigation take place simultaneously, they are often evaluated in completely separate experiments (Zehnder 2006, Kishen & Haapasalo 2015, van der Sluis *et al.* 2015, Kishen *et al.* 2016). And although such simplified experiments can provide valuable data, they only allow a partial understanding of this process

as the potential interactions between the two effects are ignored. In order to evaluate these effects and their interactions *in vitro* it is necessary to mimic the geometry of the root canal system and to use a biofilm that represents the actual biofilms present in root canals *in vivo* (Swimberghe *et al.* 2019). A dense *in vitro* biofilm with realistic viscoelastic properties could indeed mimic the basal layer of the *in vivo* endodontic biofilm, which is particularly difficult to remove (He *et al.* 2013). Previous studies have established the relevance of a dual-species biofilm (S. *oralis* and A. *naeslundii*) grown in a Constant Depth Film Fermenter (CDFF) for such a purpose (Hope & Wilson 2006, Busanello *et al.* 2018, Petridis *et al.* 2019a,b) and have introduced a root canal model (Macedo *et al.* 2014) that can be adapted for biofilm evaluation. Due to the inevitable variability in the structure of the biofilm even when grown *in vitro* under strictly controlled conditions (Busscher *et al.* 2003*,* Busanello *et al.* 2018, Petridis *et al.* 2019a), a longitudinal evaluation method allowing measurements before and after each irrigation step, such as Optical Coherence Tomography (OCT), is indispensable for such experiments (Busanello *et al.* 2018, Petridis *et al.* 2019b, Swimberghe *et al.* 2019).

A detailed investigation of the flow developed in an isthmus and lateral canal could complement the information on biofilm removal and allow for potential correlations. Experimental high-resolution analysis of the flow inside these areas is technically challenging even when using an artificial root canal system without any biofilm (Verhaagen *et al.* 2014). In order to circumvent these problems, a validated Computational Fluid Dynamics (CFD) model has been used in earlier studies to investigate in detail the irrigant flow in the root canal system (Boutsioukis 2010a, Verhaagen *et al.* 2014). Therefore, taking into account all the aspects discussed above, the aims of this study were:

1. To quantify biofilm removal from an isthmus-like and a lateral-canal-like structure in an artificial root canal system during syringe irrigation with NaOCl at different concentrations delivered at various flow rates and to identify any interactions between these variables.
2. To simulate the irrigant flow in those areas using a CFD model (Boutsioukis *et al.* 2010a).
3. To examine whether biofilm removal in those areas is correlated to the irrigant velocity predicted by the CFD model.

Materials and methods

Sample size calculation

A sample size calculation on the number of biofilm specimens was conducted a priori using G*Power 3.9 (Faul *et al.* 2007), assuming a mixed-design ANOVA, a two-tailed probability of alpha-type error 0.05 and 80% power. A large effect size (f = 0.7) was assumed, given the preliminary nature of this study, which resulted in a minimum of 3 specimens in each of the 16 groups used to evaluate biofilm removal from the isthmus-like or lateral-canal-like structures.

Artificial root canals and inserts

Ninety-six transparent artificial root canals were produced by solidified Polydimethylsiloxane (PDMS) (Sylgard 184, Dow-Corning, Midland, MI) around a D-size finger spreader (Dentsply Maillefer, Ballaigues, Switzerland) as described previously (Macedo *et al.* 2014). The length of the root canals was 18 mm and the apical diameter was 0.35 mm with a taper of 6% (Briseno Marroquin 2001). Moulds were modified to create a cylindrical empty space (D= 5 mm, L=6 mm) in contact with the apical third of the root canal and centred at ~2 mm from the apical terminus. The base of the cylinder was tangent to the root canal wall (**Figure 1**). The moulds were filled with degassed PDMS which was cured at 60ºC for 1 hour. PDMS inserts fitting the cylindrical empty space were created by the same process using different moulds, which included either a thin metal strip (3×3×0.15 mm, volume=1.35 mm^3) in order to create an isthmus-like structure (n=48) or a small metal cylinder (D=0.25 mm, L= 3 mm, volume=0.15 mm^3) in order to create a lateral-canal-like structure (n=48).

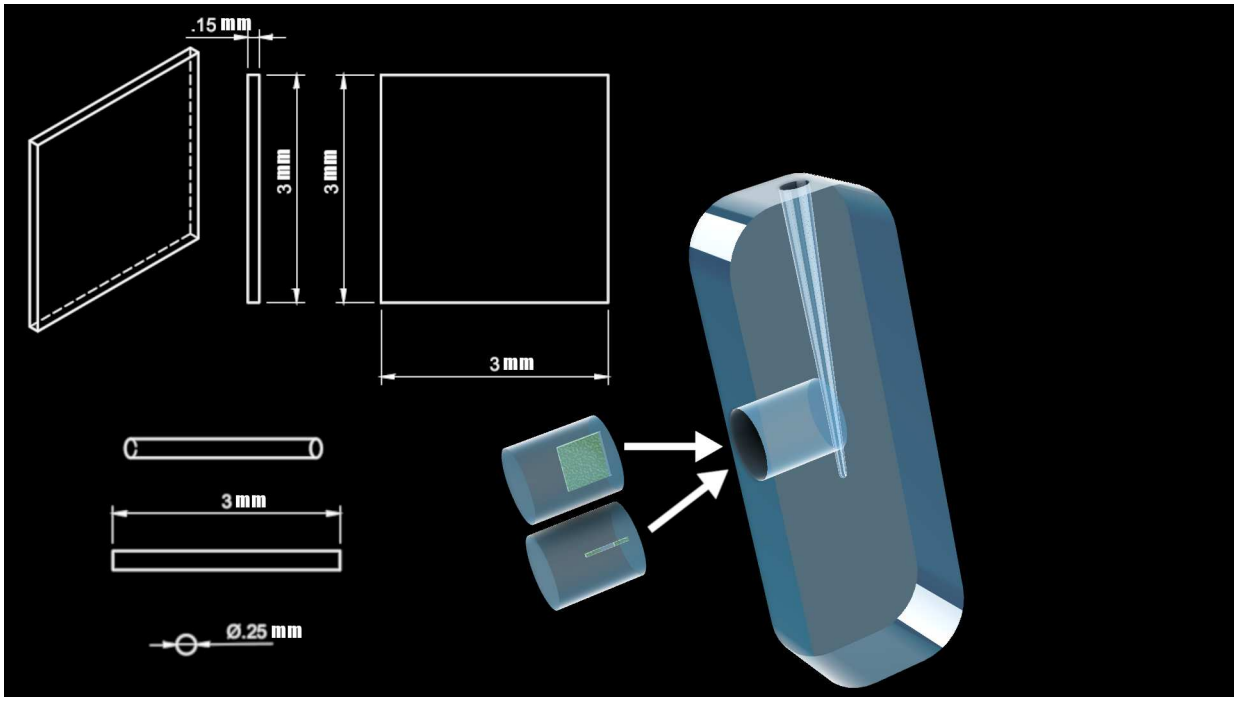


Figure 1. Schematic drawing of the artificial root canal and the isthmus-like and lateral-canal-like structures.

Bacterial strains and growth conditions

S. oralis J22 and *A. naeslundii* T14V-J1 were cultivated on blood agar plates and used to inoculate 10 mL of modified Brain Heart Infusion broth (37.0 g/L BHI, 1.0 g/L yeast extract, 0.02 g/L NaOH, 0.001 g/L Vitamin K1, 5 mg/L L-cysteine-HCl, pH 7.3) (BHI, Oxoid Ltd., Basingstoke, UK) cultured at 37°C for 24 h in ambient air for S. *oralis* J22 and 24 h in anaerobic conditions for A. *naeslundii* T14V-J1 (pre-culture). Pre-cultures were used to inoculate 200 mL of modified BHI, which was incubated at 37ºC for 16 h (main culture). Bacteria were harvested by centrifugation (6500×g), washed twice in sterile adhesion buffer (0.147 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.136 g/L KH_2PO_4, 3.728 g/L KCl, pH 6.8). To break bacterial chains, bacterial suspensions were sonicated intermittently in ice water for 3×10 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA). Bacteria were counted in a Bürker-Türk chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) and diluted in sterile adhesion buffer.

Biofilm formation

PDMS inserts with isthmus-like or lateral-canal-like structures were coated with reconstituted human whole saliva (RWS). RWS was obtained by dissolving freeze dried saliva in adhesion buffer at a concentration of 1.5 g/L, stirred for 2 hours and centrifuged at 10,000×g at 10ºC

for 5 minutes. Stimulated human whole saliva was collected in accordance with the guidelines of the Medical Ethical Committee of the University Medical Centre Groningen (approval letter 06-02-2009). Twenty volunteers chewed Parafilm© (Sigma-Aldrich, St Louis, Missouri, USA) and spat in ice cooled containers for 30 minutes. Collected saliva was pooled, centrifuged, stabilized by adding a protease blocker and freeze dried. The PDMS inserts were exposed to the RWS for 14 hours at 4ºC, under static conditions.

A CDFF was used to grow steady-state dense cell-rich biofilms similarly to previous studies (Bussanello *et al.* 2018, Petridis *et al.* 2019a,b). The CDFF consisted of a rotating turntable which held 15 sample holders in which biofilm could be formed under mechanical compaction by fixed scraper blades. The saliva-coated inserts were transferred to the sample holders on the turntable to ensure growth of biofilm with standardized structural properties. Dropwise inoculation of 100 mL of the dual-species bacterial suspension, containing S. *oralis* J22 ($6x10^8$ bacteria/mL) and A. *naeslundii* T14V-J1 ($2x10^8$ bacteria/mL), was introduced in the CDFF over 1 hour while the turntable rotated slowly at a constant speed (3 rpm). Next, rotation was stopped and bacteria were allowed to adhere for 30 min to the saliva-coated inserts. Rotation was then resumed and modified BHI was continuously supplied (45 mL/h) so that biofilms could develop during the next 96 hours at 37ºC. The fixed scraper blades applied the necessary pressure and distributed nutrients over the inserts for biofilm development.

Subsequently, the biofilm-filled inserts were removed from the CDFF and placed inside a jar containing adhesion buffer to prevent dehydration. Immediately before use they were removed for the jar and fitted in the cylindrical empty space of the artificial root canal creating a fluid-tight apically-sealed system. The biofilm was scanned by OCT prior to the irrigation experiments.

Irrigation procedures

Three different NaOCl solutions (2, 5 and 10%) were prepared from a 12-15% stock solution (Sigma-Aldrich, St Louis, Missouri, USA). Their concentration was verified by iodometric titration prior to the experiments. Adhesion buffer was used as a negative control (0% NaOCl). The PDMS inserts were randomly allocated to 4 groups with isthmus-like structures (n=12) and another 4 groups with lateral-canal-like structures (n=12) according to the irrigant flow rate. Each of these groups was further divided into 4 subgroups according to the NaOCl concentration used (n=3). Irrigation was carried out in two phases:

During phase 1 the specimens in each group were irrigated by a 5-mL syringe (Ultradent Products Inc, South Jordan, UT, USA) and a 30G open-ended needle (Navitip; Ultradent Products Inc) at a steady flow rate (0.033, 0.083 and 0.166 mL/s) for 30 s or the irrigant was passively deposited in the root canal and left undisturbed for 30 s in order to mimic a purely chemical effect (flow rate = 0 mL/s). The needle was initially placed at 2 mm from the apical endpoint of the root canal and it was also moved along the root canal during irrigation (between 1-5 mm short of the apical endpoint of the root canal). Within each group, the specimens were irrigated with either 2, 5, or 10% NaOCl or adhesion buffer, according to the subgroup allocation. Sodium thiosulfate 4.23% (Sigma-Aldrich) was subsequently deposited passively in all the root canals to neutralize any remaining NaOCl and the biofilm was scanned by OCT.

Phase 2 consisted of a 30 s final rinse with adhesion buffer delivered at 0.166 mL/s for all groups and subgroups using the same syringe irrigation protocol as in phase 1. Afterwards, the biofilm was scanned again by OCT.

Optical Coherence Tomography and image analysis

The biofilms in the isthmus-like and lateral-canal-like structures were scanned by an OCT scanner (Thorlabs, Newton, NJ, USA) three times (before irrigation, after phase 1 and after phase 2) using a 45-mm field of view and a refraction index of 1.33 to obtain real-time three-dimensional volumetric data on the amount of remaining biofilm. The scans were initially processed with ThorImage OCT software (Thorlabs) and pre- and post-irrigation scans were exported to the open-source image analysis software Fiji 1.50g (Schindelin *et al.* 2012). The scans were processed in order to segment biofilm from voids and the isthmus or lateral canal volume that was occupied by biofilm was calculated. The biofilm volumes after phase 1 and phase 2 were divided with the biofilm volume scan before irrigation to calculate the percentage of remaining biofilm in the isthmus or lateral canal after each irrigation phase.

Statistical analysis of biofilm removal

The effect of the flow rate and NaOCl concentration during phase 1 and the additional irrigation during phase 2 were analyzed separately for the isthmus-like and lateral-canal-like structures by two 3-way mixed-design ANOVAs. The percentage of the remaining biofilm was selected as the dependent variable. Normality was verified by the Shapiro-Wilk test and equality of error variances was assessed by Levene's test. The null hypotheses were that the flow rate and the NaOCl concentration during phase 1, and the additional irrigation during

phase 2 have no significant effect on the amount of remaining biofilm. Tukey's Honestly Significant Difference post-hoc test was employed for pair-wise comparisons. The alpha level was set to 0.05. Bonferroni correction for multiple comparisons was applied to this level where appropriate. Confidence intervals (95% CI) of the differences between groups were also calculated. Statistical analysis was performed using SPSS 23 (IBM Corp, Armonk, NY).

Computational Fluid Dynamics model

The irrigant flow inside the artificial root canal system without any biofilm was studied using a previously validated Computational Fluid Dynamics (CFD) model (Boutsioukis *et al.* 2010a,b, Verhaagen *et al.* 2014). The irrigant was delivered by a 30G flat open-ended needle (NaviTip; Ultradent) which was modelled as described previously (Boutsioukis *et al.* 2010b). Since the needle was moved along the root canal between ~1 and ~5 mm from the apical endpoint of the root canal during the biofilm removal experiments, five different cases were simulated as a preliminary step (needle tip at 1, 2, 3, 4, and 5 mm from the apical endpoint) using the highest irrigant flow rate (0.166 mL/s) to determine the optimum needle position regarding irrigant penetration into the isthmus and into the lateral canal. Subsequently, additional cases were simulated with the needle at the optimum position using the other two flow rates (0.083 mL/s and 0.033 mL/s). A hybrid mesh (1.4-4.3 million cells) was constructed and refined near the walls and in the isthmus/lateral canal. Grid-independence of the results was also verified. Sodium hypochlorite 5% [density = 1.090 g/cm^3 and viscosity = 1.11·10-3 Pa·s (Guerisoli *et al.* 1998)] was modelled as the irrigant in all cases. The commercial CFD solver Fluent 14.5 (ANSYS Inc., Canonsburg, Pennsylvania, USA) was used to set up and solve the problem (Boutsioukis *et al.* 2010a). Computations were carried out in a workstation with a 6-core Intel Xeon 3.2 GHz processor (Intel, Santa Clara, CA, USA) and 32 GB of RAM. The flow fields for the 6 simulated cases with the needle at the optimum position were compared in terms of time-averaged irrigant velocity and wall shear stress.

Correlation between biofilm removal and irrigant velocity

The percentage of the biofilm removed along a narrow plane in the middle of the isthmus-like (thickness = 3 voxels) and lateral-canal-like structure (thickness = 1 voxel) was calculated separately for each specimen based on the OCT scans. The results from the different irrigants were averaged in order to calculate the average percentage of biofilm removed for each flow rate since irrigant velocities calculated by the CFD model depended only on the density and

viscosity of the irrigant and there are only minor differences in these properties between the various irrigants (van der Sluis *et al.* 2010, Bukiet *et al.* 2013).

The spatial correlation between the average percentage of biofilm removed in the isthmus-like and lateral-canal-like structures and the log-transformed irrigant velocity calculated by the CFD model was examined using Pearson's correlation coefficient. The log-transformation was used because the velocity inside the isthmus-like and lateral-canal-like structures spanned several orders of magnitude and its distribution was highly skewed. In order to increase the signal-to-noise ratio of the OCT scans and to account for minor experimental misalignments, an interrogation window of 10×10 pixels was chosen; CFD data were downsampled accordingly. Data from the three different flow rates (0.033, 0.083, and 0.166 mL/s) were pooled to increase the sample size. Simple linear regression models were also fitted to the data.

Results

Remaining Biofilm

The mean biofilm volume in the isthmus-like structure was 0.809 ± 0.339 mm^3, so approximately 60% of the isthmus was occupied. The biofilm in the lateral-canal-like structure was organized in plugs (dense masses of biofilm) near the entrance and the distal part. The mean biofilm volume was 0.025 ± 0.009 mm^3 (approximately 17% of the total lateral canal volume). Irrigation generally resulted in a decrease in the biofilm volume after both phase 1 and phase 2 but an increase was also observed in some groups (Figures 2, 3).

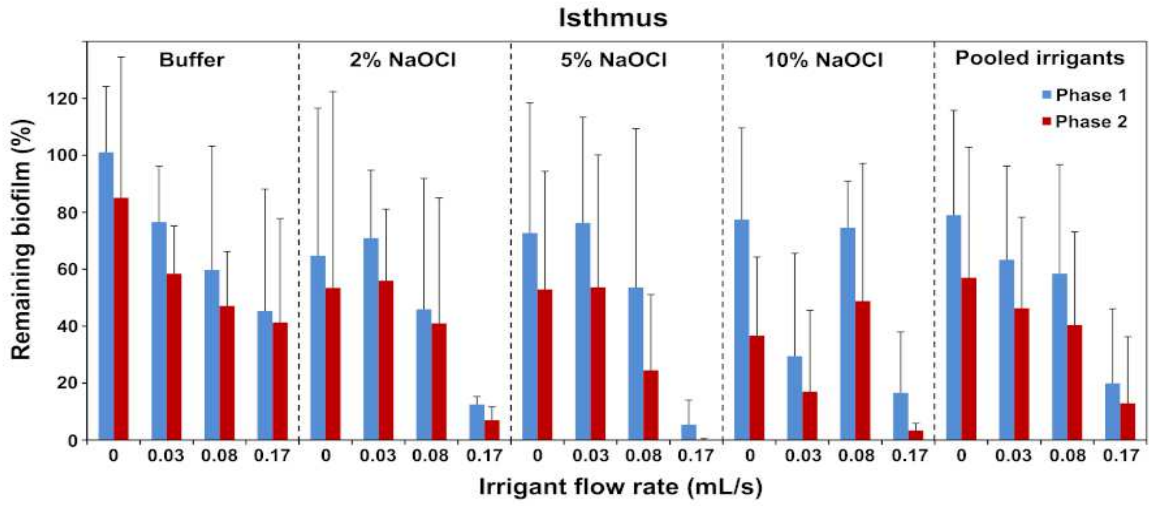


Figure 2. Biofilm remaining in the isthmus-like structure after phase 1 and phase 2 expressed as average percentage of the initial biofilm volume, for the various irrigants and flow rates. Error bars indicate standard deviation.

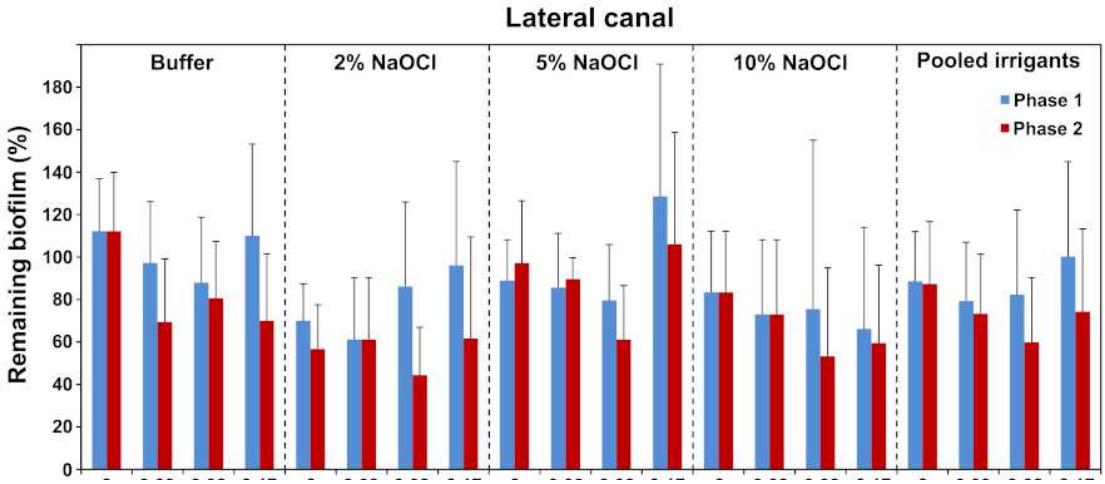


Figure 3. Biofilm remaining in the lateral-canal-like structure after phase 1 and phase 2 expressed as average percentage of the initial biofilm volume, for the various irrigants and flow rates. Error bars indicate standard deviation.

Regarding the isthmus, none of the interactions between the independent variables were significant, so the main effects were interpreted. The flow rate during phase 1 had a significant effect on the percentage of the remaining biofilm (P=0.004). Post-hoc comparisons showed that a flow rate of 0.166 mL/s resulted in significantly less biofilm than 0 and 0.033 mL/s (P=0.030; 95% CI: 15.12-88.03%, and P=0.036; 95% CI: 1.89-74.81%, respectively). No other significant differences were found among the various flow rates. Additional irrigation during phase 2 also reduced the amount of biofilm significantly, irrespective of other parameters (P<0.001; 95% CI: 8.5-23.5%). The effect of the NaOCl concentration was not significant (P=0.229).

In the lateral canal, the main effect of the NaOCl concentration was not significant (P=0.131) but the interaction between the phase and the flow rate was significant (P=0.005), so a simple-effects analysis took place for these two variables. The effect of the irrigation during phase 2 was significant only after irrigation at 0.083 or 0.166 mL/s during phase 1 (P=0.007; 95% CI: 7.5-37.4% and P=0.001; 95% CI: 12.8-39.1%), irrespective of the NaOCl concentration. The effect of irrigant flow rate was not significant for any of the two phases (*P*=0.478 and *P*=0.233). None of the other interactions between the independent variables were significant (P>0.1).

Irrigant flow

The optimum position of the needle was at 3 mm from the apical endpoint of the root canal in both the isthmus-like and lateral-canal-like structures, leading to the highest time-averaged

velocities (Figure 4). The irrigant velocity in these areas increased with increasing irrigant flow rate, which also led to higher wall shear stress. A comparison of the CFD results to the biofilm removal observed in the experiments showed that biofilm was more effectively removed in areas where the irrigant velocity was higher.

Correlation between biofilm removal and irrigant velocity

There was a substantial spatial correlation between the percentage of biofilm removed and the log-transformed irrigant velocity calculated by the CFD model in the isthmus-like (r=0.79) and in the lateral-canal-like structures (r=0.82) (Figure 5). The linear regression models indicated that 62.6% and 66.9% of the total variance in the biofilm removal in these areas could be explained by the irrigant velocity. Furthermore, there was a threshold velocity in the isthmus-like structure (~0.004 m/s) below which no biofilm removal was expected. No such threshold could be identified for the lateral-canal-like structures.

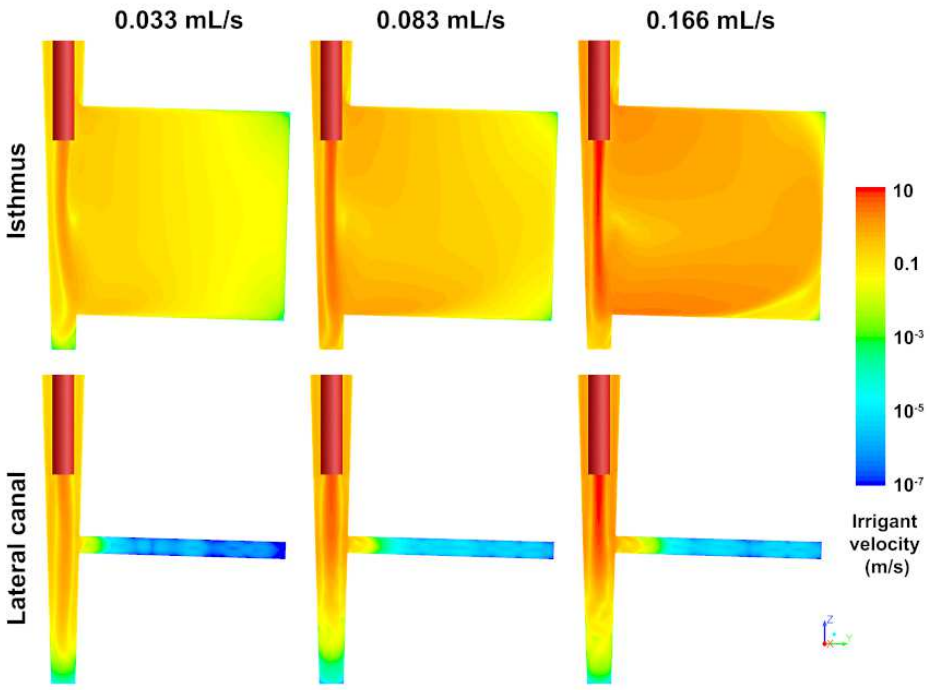
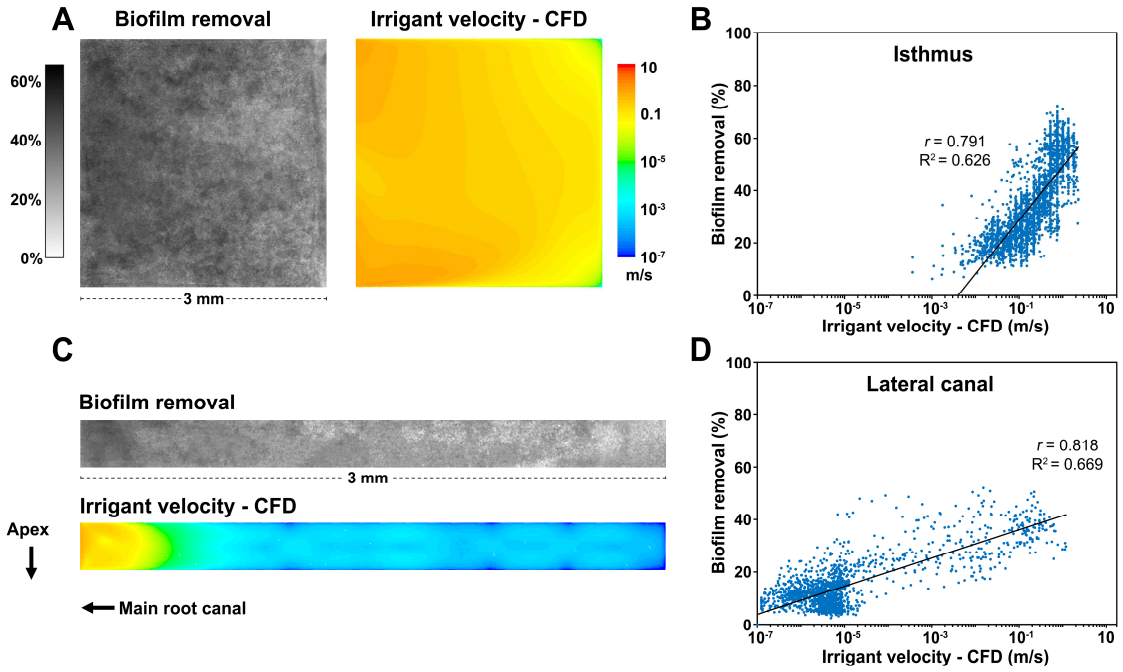


FIGURE 4. Contours of time-averaged irrigant velocity magnitude along the middle plane in the apical part of the main root canal and the isthmus/lateral canal for the three different irrigant flow rates simulated by the CFD model. A logarithmic scale has been used. The open-ended needle was inserted at 3 mm from WL and it is shown in red.



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IGURE 5. Average percentage of biofilm removed from the middle of the isthmus (A) and the lateral canal (C) during syringe irrigation at 0.083 mL/s for all irrigants tested (grayscale images), contours of time-averaged irrigant velocity calculated by the CFD model in the same area (colored images), and corresponding scatter plots of the pooled data from all three flow rates including linear regression lines (B, D). Each dot represents an area of 10×10 pixels inside the isthmus-like or lateral-canal-like structure.

Discussion

This study tested the hypothesis that the flow rate and NaOCl concentration may influence the removal of biofilm from isthmuses or lateral canals. The CDFF biofilm used is an *in vitro* biofilm model replicating dental plaque, especially regarding its physical properties. The densely formed biofilm with strong interactions between the microorganisms resembles the basal layer of root canal biofilm (Hope & Wilson 2006, Derlon *et al.* 2008, Ricucci & Siqueira 2010) and especially cell-rich biofilm formed under space limitations in isthmuses or lateral canals (Ricucci & Siqueira 2010, Busanello *et al.* 2018). Both of these types of biofilms are very challenging to remove (Ricucci & Siqueira 2010, He *et al.* 2013).

Artificial root canal systems made of a transparent material (PDMS) were used instead of extracted teeth similarly to a previous study (Macedo *et al.* 2014) to allow standardization of the geometry and repeated evaluation of the biofilm by OCT. The biofilm can adhere to

PDMS (Song *et al.* 2015) and the dual species biofilm of S. *oralis* and *A. naeslundii* produces a strong internal network which improves biofilm cohesion and adhesion (He *et al.* 2013, Busanello *et al.* 2018).

The main outcome measure was the remaining biofilm volume which was determined before and after each experiment in a non-intrusive way by OCT. This longitudinal evaluation method provided information about the whole isthmus-like and lateral-canal-like structures and allowed for each sample to be used as its own control, so individual variations in the biofilm structure and volume could be accounted for (Busanello *et al.* 2018). However, it was not possible to ascertain the condition of the bacteria in the remaining biofilm (intact or damaged). Other methods such as Confocal Laser Scanning Microscopy in combination with fluorescent staining could have provided such information (Swimberghe *et al.* 2019) but at the expense of the repeated measurements.

One of the aims of this study was to investigate potential interactions between the chemical and mechanical effects of irrigation, using the NaOCl concentration and the flow rate as proxies. Phase 1 was designed in order to resemble a final irrigation with NaOCl after instrumentation. No EDTA or other chelators were used in order to reduce the number of variables and to isolate the effect of NaOCl. The interaction was studied in a 4×4 design including the various NaOCl concentrations and flow rates. Phase 2 was added to evaluate the potential mechanical cleaning effect of an additional rinse with an inert irrigant (no chemical effect) delivered at high flow rate following pre-treatment of the biofilm with various combinations of chemical and mechanical effects during phase 1. It is noteworthy that such a rinse with an inert irrigant is not part of current clinical protocols; chemically-active irrigants are used instead.

The experiments demonstrated the very significant effect of flow rate during irrigation in phase 1 and the additional effect of a high-flow-rate final irrigation with an inert solution (buffer) regarding the isthmus. Therefore, a strong mechanical effect appeared to be important for the removal of the biofilm during both phases. In the lateral canal, only the last rinse with the buffer had a significant effect on biofilm removal and this effect appeared only when preceded by irrigation at medium or high flow rate during phase 1. Thus, there was a synergistic effect between a strong mechanical effect in phase 1 and the additional mechanical effect in phase 2.

NaOCl concentration did not affect the results significantly in either type of structure, which appears to be at variance with earlier studies (Macedo *et al.* 2010, Petridis *et al.* 2019b, Verhaagen *et al.* 2014). NaOCl is not consumed by PDMS, as is the case with dentine,

therefore the total amount of free chlorine can react with biofilm. Irrigant penetration in lateral canals is dominated by diffusion except for a small part near the entrance (extending inside the lateral canal approximately twice its diameter) where a convective flow can develop. Thus, the concentration of the irrigant in the main root canal should be directly related to its effect in the rest of the lateral canal (Verhaagen *et al.* 2014). However, the shorter application time (30 instead of 600 s), which hampered diffusion, could be an explanation for this difference. Another possible explanation is that the biofilm created in this study was mainly located near the entrance, an area that could be reached easily by the flow, and near the distal end, an area unlikely to be reached by either the flow or by diffusion within the time-frame of the experiment. The middle part of the lateral canal, where diffusion would dominate, was almost empty. In addition, only the remaining biofilm volume was evaluated and not the viability of the bacteria within that volume. Moreover, it should be emphasized, regarding both isthmuses and lateral canals, that this study aimed to detect only relative large effects (f=0.7) and the sample size was selected accordingly (n=3 per subgroup). Concentration may still have some effect on biofilm removal, although this effect is likely to be smaller than that of the irrigant flow rate.

Despite irrigation at high flow rate with NaOCl, which is a strong antimicrobial solution, an increase was observed in the biofilm volume in some of the groups. Similar sporadic findings have also been reported in earlier studies and they have been attributed to the reaction of the biofilm to chemical and mechanical stress (Busscher *et al.* 2003, Busanello *et al.* 2018, Petridis *et al.* 2019a). Volumetric biofilm expansion may also explain some of the variance within each group/subgroup and it would have remained undetected if the biofilm was only evaluated after irrigation and this highlights the importance of the repeated measurements allowed by OCT.

A substantial correlation was found between biofilm removal and irrigant velocity in the isthmus and lateral canal, a finding that validates the use of irrigant velocity as a predictor for biofilm removal. Still, only around 65% of the variance in biofilm removal could be explained by the velocity. This could be attributed to the fact that, apart from the flow, biofilm disruption and removal could also be mediated by the chemical effect of NaOCl to some extent but chemical interactions were not included in the computer model. Furthermore, the model assumed that the isthmus and lateral canal were free of biofilm because the detailed physical interaction between the irrigant and the biofilm exceeds current computer modelling capabilities. Therefore, the calculated velocity was a good approximation of the actual

velocity in relatively clean isthmuses/lateral canals but it deviated from the velocity when large amounts of biofilms were present.

Conclusions

The irrigant flow rate during phase 1 and additional irrigation with buffer at high flow rate during phase 2 had a significant effect on biofilm removal from the isthmus. Additional irrigation during phase 2 also affected biofilm removal from the lateral canal significantly but only when preceded by irrigation at medium or high flow rate during phase 1. NaOCl concentration did not affect biofilm removal significantly. Irrigant velocity and wall shear stress in the isthmus and lateral canal increased with increasing flow rate and the irrigant velocity was substantially correlated to biofilm removal from those areas.

References

1. Boutsioukis C, Lambrianidis T, Kastrinakis E (2009) Irrigant flow within a prepared root canal using different flow rates: a Computational Fluid Dynamics study. *International Endodontic Journal* **42**, 144-55.
2. Boutsioukis C, Verhaagen B, Kastrinakis E, Versluis M, van der Sluis LWM (2010a) Irrigant flow in the root canal: experimental validation of a Computational Fluid Dynamics model using high-speed imaging. *International Endodontic Journal* **43**, 393-407.
3. Boutsioukis C, Verhaagen B, Versluis M, Kastrinakis E, Wesselink PR, van der Sluis LWM (2010b) Evaluation of irrigant flow in the root canal using different needle types by an unsteady computational fluid dynamics model. *Journal of Endodontics* **36**, 87 -9.
4. Bukiet F, Soler T, Guivarch M, Camps J, Tassery H, Cuisinier F, Candoni N (2013) Factors affecting the viscosity of sodium hypochlorite and their effect on irrigant flow. *International Endodontic Journal* 2013 **46**, 954-61.
5. Busanello FH, Petridis X, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2018) Chemical biofilm removal capacity of endodontic irrigants as a function of biofilm structure: optical coherence tomography, confocal microscopy and viscoelasticity determination as integrated assessment tools. *International Endodontic Journal* **52**, 461-74.
6. Busscher HJ, Rustema-Abbink M, Bruinsma GM, de Jager M, Gottenbos B, van der Mei HC (2003) Non-contact removal of coadhering and non-coadhering bacterial pairs from pellicle surfaces by sonic brushing and de novo adhesion. *European Journal of Oral Sciences* **111**, 459-64.
7. Derlon N, Massė A, Escudié R, Bernet N, Paul E (2008) Stratification in the cohesion of biofilms grown under various environmental conditions. *Water Research* **42**, 2102–10.
8. Dutner J, Mines P, Anderson A (2012) Irrigation trends among American Association of Endodontists members: a web-based survey. *Journal of Endodontics* **38**, 37-40.
9. Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39**, 175-91.

10. Guerisoli DMZ, Silva RS, Pecora JD (1998) Evaluation of some physico-chemical properties of different concentrations of sodium hypochlorite solutions. *Brazilian Endodontic Journal* **3**, 21-3.
11. Gulabivala, K, Patel B, Evans G, Ng YL (2005) Effects of mechanical and chemical procedures on root canal surfaces. *Endodontic Topics* **10**, 103-22.
12. Haapasalo HK, Sirén EK, Waltimo TM, Ørstavik D, Haapasalo MP (2000) Inactivation of local root canal medicaments by dentine: an in vitro study. *International Endodontic Journal* **33**, 126-31.
13. He Y, Peterson BW, Jongsma MA, *et al.* (2013) Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms. *PLoS ONE* **8**, e63750.
14. Hope CK, Wilson M (2006) Biofilm structure and cell vitality in a laboratory model of subgingival plaque. *Journal of Microbiological Methods* **66**, 390-8.
15. Kishen A, Haapasalo M (2015) Laboratory models of biofilms: development and assessment. In: In: Chávez de Paz L, Sedgley CM, Kishen A. eds. *The root canal biofilm.* New York, NY, USA: Springer, pp. 127-54.
16. Kishen A, Peters OA, Zehnder M, Diogenes AR, Nair MK (2016) Advances in endodontics: Potential applications in clinical practice. *Journal of Conservative Dentistry* **19**,199-206.
17. Macedo RG, Wesselink PR, Zaccheo F, Fanali D, van der Sluis LWM (2010) Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. *International Endodontic Journal* **43**, 1108-15.
18. Macedo RG, Robinson JP, Verhaagen B, Walmsley AD, Versluis M, Cooper PR, van der Sluis LW (2014) A novel methodology providing insights into removal of biofilm-mimicking hydrogel from lateral morphological features of the root canal during irrigation procedures. *International Endodontic Journal* **47**, 1040-51.
19. Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15**, 187-96.
Peters OA (2004) Current challenges and concepts in the preparation of root canal systems: a review. *Journal of Endodontics* **30**, 559-67.
20. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Prashant SK, van der Sluis LWM (2019a) Factors affecting the chemical efficacy of 2% sodium hypochlorite against oral steady-state dual-species biofilms: exposure time and volume application. *International Endodontic Journal* **52**, 1182-95.

21. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019b) Chemical efficacy of several NaOCl concentrations on biofilms of different architecture: new insights on NaOCl working mechanisms. *International Endodontic Journal* **In press** (doi: 10.1111/iej.13198).
22. Portenier I, Haapasalo H, Orstavik D, Yamauchi M, Haapasalo M (2002) Inactivation of the antibacterial activity of iodine potassium iodide and chlorhexidine digluconate against Enterococcus faecalis by dentin, dentin matrix, type-I collagen, and heat-killed microbial whole cells. *Journal of Endodontics* **28**, 634-7.
23. Ricucci D, Loghin S, Siqueira Jr JF (2013) Exuberant biofilm infection in a lateral canal as the cause of short-term endodontic treatment failure: report of a case. *Journal of Endodontics* **39**, 712-8.
24. Ricucci D, Siqueira JF Jr (2010) Fate of the tissue in lateral canals and apical ramifications in response to pathologic conditions and treatment procedures. *Journal of Endodontics* **36,** 1-15.
25. Ricucci D, Siqueira JF, Bate AL, Pitt Ford, TR (2009) histologic investigation of root canal–treated teeth with apical periodontitis: a retrospective study from twenty-four patients. *Journal of Endodontics* **35**, 493-502.
26. Schindelin J, Arganda-Carreras I, Frise E, *et al.* (2002) Fiji - an Open Source platform for biological image analysis. *Nature Methods* **9**, 676-82.
27. Song F, Koo H, Ren D (2015) Effects of material properties on bacterial adhesion and biofilm formation. *Journal of Dental Research* **94**, 1027-34.
28. Swimberghe RCD, Coenye T, De Moor RJG, Meire MA (2019) Biofilm model systems for root canal disinfection: a literature review. *International Endodontic Journal* **52**, 604-28.
29. van der Sluis L, Boutsioukis C, Jiang LM, Macedo R, Verhaagen B, Versluis M (2015) Root canal irrigation. In: Chávez de Paz L, Sedgley CM, Kishen A. eds. *The root canal biofilm.* New York, NY, USA: Springer, pp. 259-302.
30. van der Sluis LWM, Voogels MPJM, Verhaagen B, Macedo R, Wesselink PR (2010) Study on the influence of refreshment / activation cycles and irrigants on mechanical cleaning efficiency during ultrasonic activation of the irrigant. *Journal of Endodontics* **36**, 737-40.
31. Verhaagen B, Boutsioukis C, Heijnen GL, van der Sluis, LWM, Versluis M (2012) Role of the confinement of a root canal on jet impingement during endodontic irrigation. *Experiments in Fluids* **53**, 1841-53.

32. Verhaagen B, Boutsioukis C, Sleutel CP, Kastrinakis E, van der Sluis L, Versluis M (2014) Irrigant transport into dental microchannels. *Microfluidics and Nanofluidics* **16,** 1165-77.

33. Zehnder M (2006) Root canal irrigants. *Journal of Endodontics* **32**, 389-98.

2.2 ARTICLE 2 - Title: Chemical and mechanical influence of root canal irrigation on biofilm removal from lateral morphological features of the root canal and dentinal tubules.

Abstract

Aim To investigate the chemical and mechanical impact of root canal irrigation on biofilm removal from lateral morphological features of the root canal and dentinal tubules.

Methodology Biofilm removal and analysis of viability and viscoelastic properties of post treatment remaining biofilm was studied using dentine discs, simulated root canal systems and dentine tubule models. Two chemically active irrigants (RISA, sodium hypochlorite (NaOCl), mechanically active buffer (ultrasound) and buffer as control were used in different experimental groups. Optical coherent tomography (OCT), confocal scanning laser microscopy (CSLM) and low load compression testing (LLCT) were used to analyze the biofilm. One- way analysis of variance (ANOVA) with Tukey-HSD *post-hoc* tests were used for LLCT analysis, a paired sample t test and one-way ANOVA test for the OCT analysis and Kruskal-Wallis tests and Wilcoxon ranked tests for the dentine model analysis.

Results For the dentine discs, no significant differences in the viscoelastic properties nor biofilm removal were detected. Ultrasound and high flow rate significantly influenced mechanical biofilm removal from lateral canal like structures. For biofilm removal from isthmus -like structures no significant differences were found between the groups. However, within-group analysis revealed significant differences between the different steps of the experiment, with the exception of NaOCl. In the dentine tubule model, NaOCl was the most chemically active, while RISA and NaOCl seem to stabilize the post treatment remaining biofilm.

Conclusions Within this experimental setup, the mechanical effect of syringe irrigation at low and high flow rate is important for biofilm removal. An extra irrigation phase of high flow was effective in biofilm removal after treatment. The mechanical effect of ultrasound could be effective when the surface contact biofilm-irrigant is small. The mechanical effect of ultrasound in cleaning an isthmus-like structure was not more effective than syringe irrigation with a low flow rate.

After the different irrigation procedures, biofilm was still left behind (post treatment remaining biofilm). This could regrow/survive in a period of five days in the root canals without extra nutrition. Both RISA and NaOCl seemed to have a stabilizing effect on the post treatment remaining biofilm.

Keywords: Biofilm; Polysaccharides; Confocal Laser Scanning Microscopy; Optical Coherence Tomography; RISA; Sodium hypochlorite; Ultrasound.

Introduction

Bacteria tend to grow in a biofilm formation, adhering to a surface or liquid interface and co-adhering to each other (Kolenbrander *et al*. 2010). In biofilms, microorganisms are protected by the extracellular polymeric substance (EPS) against chemical and mechanical stresses (Flemming *et al.* 2007) imposed by cleaning procedures and disinfectants (Stewart & Franklin 2008). The root canal wall is a surface where bacteria can adhere and biofilm can develop when the root canal system is infected (Chávez de Paz 2007, Ricucci *et al.* 2009). If the root canal is infected, biofilm will also be present in lateral morphological features such as lateral canals, fins, isthmuses and the dentinal tubules. The biofilm formation in these areas is a challenge to clean. These structures are not reached by instrumentation (Peters *et al.* 2001; Ricucci *et al.* 2013) and only limited accessible during irrigation (Verhaagen *et al*. 2014, Robinson *et al*. 2018). Therefore, it is also important to know the fate of the remaining biofilm after the mechanical or chemical challenges during root canal irrigation (post treatment remaining biofilm).

The aims of root canal irrigation are the chemical dissolution or disruption and the mechanical detachment of pulp tissue, dentine debris and smear layer (instrumentation products), microorganisms (planktonic or biofilm) and their products from the root canal wall and their removal from the root canal system. The chemically active sodium hypochlorite (NaOCl) is the most popular irrigation solution due to its bactericidal and antibiofilm effect and its capacity to inactivate endotoxins (Sarbinoff *et al.* 1983, Sprat *et al.* 2001, Silva *et al.* 2004, Tawakoli *et al.* 2017). Beside this, it has an excellent tissue dissolving ability (Abbott *et al.* 1991), being able to dissolve necrotic tissue (Naenni *et al.* 2004) and the organic compounds of smear layer (Tartari *et al.* 2016). However, NaOCl cannot dissolve inorganic tissue (Sen *et al.* 1995) and seems to have difficulty fully penetrating the biofilm (Van der Waal *et al*. 2014, 2016, Petridis *et al*. 2019a) especially when the biofilm is packed with bacteria (Busanello *et al*. 2019, Petridis *et al.* 2019a). *In vitro* investigations have shown that NaOCl has intratubular disinfection ability in root canal models in bovine and human teeth (Arias-Moliz *et al.* 2014, Baron *et al*. 2016, Morago *et al*. 2016, Giardino *et al*. 2018, Liu *et al*. 2019, Rodrigues *et al*. 2018, Giardino *et al.* 2019, Morago *et al.* 2019). However, it is not known whether NaOCl is effective in removing bacterial cell dense biofilm during irrigation from lateral morphological features in a root canal model.

Modified Salt Solution (MSS) also called ‘RISA’ is a hypertonic salt solution with a high pH which can kill bacteria and inactivate and detach biofilm (van der Waal *et al*. 2011,

van der Waal *et al.* 2014, de Almeida *et al.* 2016). RISA was developed to be used as irrigant solution or intracanal medication during endodontic treatment with an antimicrobial action based on a multiple hurdle effect, using an osmotic effect and weak acid as hurdles (van der Waal *et al*. 2015, De Almeida *et al*. 2016). The intention of using RISA is to inactivate and remove biofilm from complex-shaped areas like the root canal system (van der Waal *et al.* 2017), which was actually illustrated in an *ex vivo* study (van der Waal *et al*. 2015). Also, in an *in situ* model, RISA was able to generate a porosity in the bacteria cell membranes and a shrinkage of the biofilm, due to its hypertonic nature. When compared to NaOCl in a narrow channel model, RISA presented greater biofilm diffusion (van der Waal *et al*. 2017). However, there is no information in the literature on the ability of RISA to remove biofilm from lateral morphological features in a root canal model or from dentinal tubules when used as root canal irrigant. Moreover, its influence on the viscoelastic properties on remaining biofilm have not been described.

To improve the mechanical and chemical effect of the irrigation procedure, ultrasound has been used (Verhaagen *et al*. 2014; Robinson *et al*. 2018). This results in a more effective mechanical removal of bacteria, dentine debris and organic tissues (van der Sluis *et al.* 2007). Furthermore, several studies have shown an increase of the chemical effect of NaOCl in combination with ultrasound (Sabins *et al.* 2003, van der Sluis *et al.* 2005, van der Sluis *et al.* 2007; Robinson *et al*. 2018). However, no study evaluated the typical mechanical efficacy of ultrasound in biofilm removal from lateral morphological features in a root canal model nor on the effect of bacterial activity in human dentinal tubules.

Biofilm is considered as a recalcitrant structure to remove (Peterson *et al.* 2015). In addition, the root canal anatomy is extremely complicated. Therefore, it is generally accepted that complete removal of biofilm from the root canal system is not possible. As a consequence, it is important to know how our treatment protocols influence the post treatment remaining biofilm. Comparing the composition of the remaining biofilm with the composition of the regrown remaining biofilm could give us more insight.

To test the above-mentioned topics, three experimental models were used to evaluate biofilm removal and bacterial activity in the root canal system. In the first model, biofilm removal from lateral morphological features (artificial lateral canal or isthmus like structures) was evaluated by optical coherent tomography (OCT) before and after irrigation procedures (Pereira *et al*. *in progress*). This longitudinal evaluation method provided information about the whole isthmus-like and lateral canal-like structures and allowed for each sample to be used as its own control. Thereby, individual variations in the biofilm structure and volume

could be accounted for (Busanello *et al.* 2019). In the second model, biofilm removal from dentinal tubules and bacterial activity in the dentinal tubules after irrigation procedures (Giardino *et al.* 2018) and after recolonization were evaluated. In the third model, the effect of the irrigants on the visco-elastic properties of the remaining biofilm was tested (Busanello *et al*. 2019).

The following aims were defined:

1) to evaluate biofilm removal from lateral canal and isthmus like structures and dentinal tubules after irrigation procedures with RISA, 2% NaOCl, buffer solution and ultrasonically activated buffer solution by OCT and CLSM;

2) to evaluate bacterial activity in the dentinal tubules directly and five days after irrigation procedures with RISA, 2% NaOCl, buffer solution and ultrasonically activated buffer solution by CLSM;

3) to evaluate biofilm removal from dentine discs after 30 seconds exposure to RISA, 2% NaOCl and buffer solution as well as the visco-elastic properties of the remaining biofilm with LLCT.

The null hypothesis was that there is no difference in the four irrigation protocols regarding the biofilm removal, inactivation and regrowth of bacteria in the biofilm, biofilm thickness reduction and viscoelastic properties of the remaining biofilm.

Materials and methods

Artificial root canal model and dentine discs with biofilm

Bacterial strains and growth conditions

The clinical isolates *Streptococcus. oralis* J22 and *Actinomyces naeslundii* T14V-J1 were grown as described previously (Busanello *et al.* 2019, Petridis *et al.* 2019). Briefly, the bacteria were streaked on blood agar plates and a single colony was used to inoculate 10 mL modified Brain Heart Infusion broth (BHI) (37.0 g/L BHI, 1.0 g/L yeast extract, 0.02 g/L NaOH, 0.001 g/L Vitamin K1, 5 mg/L L-cysteine-HCl, pH 7.3) (BHI, Oxoid Ltd., Basingstoke, UK). Subsequently, *S. oralis* were cultured at 37°C for 24 h in ambient air and *A. naeslundii* at 37°C for 24 h in an anaerobic chamber (pre-cultures). Pre-cultures were used to inoculate 250 mL modified BHI (1:20 dilution) and grown for 16 h (main cultures). Bacteria were harvested by centrifugal force (6350 xg) and washed twice in sterile adhesion

buffer (0.147 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.136 g/L KH_2PO_4, 3.728 g/L KCl, pH 6.8). The bacterial pellets were suspended in 10 mL sterile adhesion buffer and sonicated intermittently in ice-water for 3 × 10 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains. Subsequently, bacteria were counted using a Bürker-Türk counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) and both suspensions were diluted in adhesion buffer in order to prepare a dual-species bacterial suspension containing a concentration of 6×10^8 bacteria/mL for *S. oralis* and 2×10^8 bacteria/mL for *A. naeslundii*.

Preparation root canal model and dentine discs

Transparent PolyDiMethylSiloxane (PDMS) (PolyDiMethylSiloxane; Sylgard 184, Dow-Corning, Midland, MI) root canal models, with a small pocket (R= 2.5 mm) in the apical area perpendicular to the root canal were created using a D-size finger spreader (Dentsply Maillefer, Ballaigues, Switzerland) as described in Macedo *et al.* (2014) (Fig. 1). PDMS pocket inserts with anatomical features resembling an isthmus or lateral canal were created using molds consisting of a thin metal strip (width 3 mm, thickness 0.15 mm, length 3 mm, total volume 1.35 mm^3) or a small cylinder (length 3.0 mm and thickness 0.25 mm, total volume 0.29 mm^3) respectively, as described before (Fig. 1).

Dentine disks were prepared from the crown of freshly extracted human molars. Dentine cylinders were obtained using a diamond coated core drill (6 mm, CARAT N.V. Westerlo, NL). Next the cylinders were cut in 1.5 mm-thickness disks with the aid of a water-cooled diamond blade (IsoMet,Diamond Wafering blades 102 x 0.3 mm, Buehler, USA) mounted in a circular cutting machine. The dentine disks were treated 5 min with 17% EDTA, in a sonication bath to ensure removal of the smear layer.

Biofilm growth on dentine discs and in PDMS inserts

Biofilms with a structure mimicking oral biofilms were developed. For that purpose, steady-state biofilms were grown using a constant depth film fermenter (CDFF) in which a constant dropwise supply of nutrients combined with a repeated cycle of compression/scraping leads to a dental plaque-like bacterial dense biofilm (Kinniment *et al.* 1996, Rozenbaum *et al.* 2017). The PDMS inserts and the dentine disks were coated with whole human saliva. The saliva coating consisted of freeze dried whole human saliva pooled from at least 20 volunteers of both genders (saliva was collected in agreement with the guidelines set out by the Medical Ethical Committee of the University Medical Centre Groningen , Groningen, The

Netherlands, approval letter 06-02-2009) dissolved in 20 ml adhesion buffer (1.5 g/L), stirred for 2 hours and centrifuged at 10,000 xg, 10ºC for 5 minutes. Both the inserts and the dentine discs were exposed to the reconstituted saliva for 14 hours, at 4ºC, under static conditions. Subsequently, the inserts and dentine discs were transferred to the CDFF table at a depth of 250 μm, to ensure growth of biofilm with a standardized thickness. Dropwise application of 100 ml dual-species bacterial suspension over 1 h at a constant slow rotation of the CDFF table ensured inoculation of the inserts and dentine discs. Rotation was stopped and bacteria were allowed to adhere for 30 min to the saliva coated dentine discs and inserts. Subsequently rotation was resumed and a continuous supply of modified BHI (45 mL/h) was started so biofilms could develop over the next 96 h at 37ºC.

Evaluation of the chemical effect of the irrigants

In order to analyze the chemical effect of the irrigant solutions on the biofilm, 30 dentine disks with biofilm were used. The disks, submersed in buffer solution, were analyzed using OCT to measure the biofilm thickness pre-treatment. Subsequently, disks were randomly divided in the following three groups (n=10): Group 1: control (buffer solution) (B); Group 2: RISA (R); Group 3: 2% sodium hypochlorite solution (NaOCl).

With the aid of a pipette, 40 μL of each solution was gently dropped over the biofilm (no flow), after 30 seconds the disks were analyzed using OCT to obtain images post treatment and calculating biofilm thickness changes.

Low load compression testing (LLCT) and assessment of biofilm viscoelastic properties

Biofilms were compressed to a 20% deformation within 1 s, after which the deformation was held constant for 100 s (He *et al.* 2013). The relaxation was monitored over time and normalized over the cross-sectional area of the plunger to calculate the induced stress. The percentage change in induced stress occurring within 100 s from its initial value was termed the percentage stress relaxation (R). The stress relaxation curves for each biofilm were modelled using a generalized Maxwell model, in which E(t) represents the total stress exerted by the biofilm divided by the imposed strain and it is expressed as the sum of four Maxwell elements, with a spring constant E_i, and characteristic decay time, τ_i (Busanello *et al.* 2019). The relative importance of each element was expressed as the percentage of its spring constant to the sum of the spring constants of all elements at t=0. The elements derived were named corresponding with the outflow of water from the biofilm. Fast moving water or "free water" and relatively slow-moving water or "bound water" being the E_1 and E_2 elements,

extracellular polymeric substances being the E_3 and bacterial rearrangement being the E_4 (Busanello *et al,* 2019). Samples were submerged in adhesion buffer during measurements and due to the sensitivity of the weight and to the duration of the measurements (100 s), a correction for water evaporation was applied.

Irrigation protocols

After 96 h of biofilm formation, the inserts containing biofilm filled lateral canals or isthmuses were removed from the CDFF, carefully placed in the root canal model and analyzed using the OCT (Thorlabs Ganymade II, Newton, NJ, USA) to determine the biofilm volume present inside the lateral canal or isthmus prior to treatment. The experimental groups were established as a two-phase irrigation protocol. Phase one consisted of forty samples for either the lateral canal or isthmus structures and were randomly divided in 4 experimental groups (n=10): Group 1: control (buffer solution: 0.147 g/L $CaCl_2$, 43.5 g/L K_2HPO_4, 34.0 g/L KH_2PO_4, 3.728 g/L KCl, pH 6.8) (B); Group 2: RISA (R); Group 3: 2% sodium hypochlorite solution (NaOCl); Group 4: Ultrasonic Activated Irrigation (US) with a non-chemical active buffer solution. The irrigation process was performed using a 5mL irrigation syringe (Ultradent Products Inc., South Jordan, UT, USA) with a 30G irrigation needle (Endo-Eze - Ultradent Products Inc., South Jordan, UT, USA) at a flow rate of 0.05 mL/s. During irrigation, the needle was placed 2mm coronal from the apical endpoint of the root canal and the NaOCl solution was continuously released during 30s by in and outward movements of 5mm amplitude. The 2% NaOCl concentration was obtained from a standard solution of NaOCl 12-15% (Sigma-Aldrich, St Louis, Missouri, USA) by means of iodometric titration before every experiment was conducted. For group 4, the buffer solution in the root canals was 3 times ultrasonically activated for 20 seconds following a clinical protocol as described in (Duque *et al.* 2017). Prior to each activation, root canals were gently replenished with 0,5 mL of buffer solution. The US was performed with a 25 mm long, size 25 IrriSafe file (Satelec Acteon, Merignac, France), driven with a commercial endodontic ultrasound device (NSK Varios 350 Optic Complete System, Osaka, Japan). The instrument was centered as much as possible and up and down movements were made in coronal direction from 2 mm coronal from the apical end point.

Post treatment, the biofilm volume of all specimens was again analyzed using OCT. Phase two consisted of a final rinse with buffer at a high flow rate of 0,167 mL/s for 30 s (5 mL) to test its mechanical effect and the specimens were again analyzed using OCT.

Optical coherence tomography (OCT) and biofilm removal assessment

Image analysis for dentine discs was performed using 2D high resolution (5000x373 px) OCT scans (field of view 5 mm, refractive index 1.33) in order to determine the biofilm thickness. With the aid of Image J FIJI software program, a grey-value Otsu-threshold (Otsu 1979) was used to separate the biofilm from the background. Subsequently bottom and upper contour line of the biofilm was defined as those pixels in the image that have a grey value just higher than the grey-value threshold and were connected to a neighboring pixel. The mean biofilm thickness was calculated per vertical line scan (5000) based on the number of pixels between the bottom of the biofilm and the upper contour line. After this, the thickness reduction in biofilm height was calculated after treatment for each specimen.

Quantitative image analysis for the biofilm removal from the artificial isthmus and lateral canal structures was obtained by comparing pre, post and final images using Image J FIJI (National Institutes of Health, Bethesda, MD, USA) software program. By acquiring 3D scans, containing 750 slices of 750x373 px (field of view of 5.0 mm at refractive index 1.33), and analyzing them based on their greyscale composition, thresholding to only select the biofilm and filtering the background noise, the volume of residing biofilm inside the isthmus and lateral canal could be determined. Percentage removal was calculated by determining the difference in biofilm volume between pre-treatment and post treatment or final irrigation.

Dentine tubule model

Allocation and preparation of the specimen (human mandibular incisors) Eighty recently extracted human mandibular incisors were scanned and the root canal anatomy was defined using a desktop micro-focus computed tomographic scanner (SkyScan 1174v2; SkyScan, Kontich, Belgium). Thereafter, the teeth were equally distributed between four groups (n=20) based on their root canal anatomy (volume). Subsequently, the teeth were immersed for 12 hours in a 1% sodium hypochlorite solution (NaOCl) for surface disinfection. An access cavity was made and the root canals were irrigated and instrumented with Prodesign Logic rotary files 25.06 and 35.05 (Easy Equipamentos Odontológicos, Belo Horizonte, MG, Brazil). Working length was determined by measuring the length of a #15 K-file (Dentsply-Maillefer, Ballaigues, Switzerland), when just extruding through the apical foramen (visual observation by eye) and then subtracting 1 mm from the measurement obtained. Subsequently, the teeth were submitted to three ultrasonic baths of 10 minutes each with respectively 1% NaOCl, 17% EDTA (to remove smear layer) and saline solution to neutralize the NaOCl and EDTA (Marinho *et al*. 2015). Thereafter, the external surface of the roots was covered with two subsequent coatings of red nail polish (Colorama, Rio de Janeiro, RJ,

Brazil). The teeth were sterilized using an autoclave (Cristófoli, Campo Mourão, PR, Brazil) at 121ºC for 24 minutes, inserted in sterile BHI culture media (Brain Heart Infusion, Difco, Detroit, MI, USA) and submitted to an ultrasonic bath for 10 minutes for maximum penetration of the culture broth into the dentinal tubules. All experiments were conducted under aseptic conditions in a laminar flow chamber to prevent airborne bacterial contamination.

Contamination of the dentinal tubules

The bacterial reference strain *Enterococcus faecalis* (ATCC 29212) was used for the experiments. The colonial morphology and Gram stain was verified to confirm the strain's purity, which was repeated throughout the experiment. The microorganisms were cultivated in BHI, making a pre and a main culture before establishing an inoculum for sample contamination. Dilutions were made based on the absorbance value, obtained by SF325NM spectrophotometer (Bel Photonics do Brazil Ltda, Osasco, Brazil) at 540 nm, to a concentration of 3 x 108 CFU/mL. The root canals and dentine tubules were contaminated over a 5-day period, in BHI medium at 37°C, according to the protocol of Andrade *et al*. (2015) and the sequence of centrifugation steps of Ma *et al*. (2011).

Irrigation protocols

During the experimental procedures, the roots were placed in a sterilized metal device inside the laminar flow chamber. The samples were divided into four groups (n=20) according to the irrigant used: Group 1: control (buffer solution) (B), Group 2: RISA (R), Group 3: 2% NaOCl (NaOCl), Group 4: Ultrasonic Activated Irrigation of a buffer solution (US). In all groups the root canals were continuously irrigated with 1.5 mL of each irrigant for 30 sec (flow rate of 0.05 ml/s) with a disposable plastic syringe with attached to it a 25 mm stainless steel Endo-eze 30-gauge needle (Ultradent, South Jordan, UT, USA) positioned 2 mm short of working length (WL). In the US group, the buffer solution was 3 times ultrasonically activated for 20 sec following a clinical protocol as described in Duque *et al.* (2017). Prior to each activation, root canals were gently replenished with 0,5 mL of buffer solution. The US was performed with a 25 mm long, size 25 IrriSafe file (Satelec Acteon, Merignac, France), driven with a commercial endodontic ultrasound device (NSK Varios 350 Optic Complete System, Osaka, Japan). The instrument was centered as much as possible and up and down movements were made in coronal direction from 2 mm coronal from the apical end point.

After having performed the experiments, the root canals were irrigated with 1,5 mL of buffer solution for 30 seconds (flowrate of 0.05 ml/s). Ten teeth of each group were used for microbiological examination and the remaining teeth were used in the recolonization protocol.

Microbiological CLSM analysis

After irrigation, the roots were longitudinally sectioned using a circulating diamond blade saw (Erios, São Paulo, Brazil) under cooling in such a way that every section contained a buccal and a lingual part. Then, one half of each root was placed in a 24-well culture plate filled with 17% EDTA for 5 minutes and washed with 500 µL sterile saline to remove the smear layer resulting from the cutting. The specimens were stained for 20 min with 25 µL dye of the LIVE/DEAD® BacLightTM bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR, USA) in a dark environment staining the bacteria with intact cell membrane green and bacteria with compromised cell membrane red. Subsequently, excessive dye was removed and the specimens were stained with 25 µL of Calcofluor white M2R dye (Merck, Darmstadt, Germany) for 10 min to stain the polysaccharides in the matrix of the biofilm. The excessive dye was removed and the specimens were observed through the Leica TCS-SPE CLSM (Leica Microsystems GmbH, Mannheim, Germany). Eight images were obtained of each root, four of the cervical and four of the apical thirds. Of these four images, two were made of the buccal side of the specimen and two of the lingual side. Of these two images, one represented a scan of the superficial tubules and the other the deeper aspect of the tubules. The same procedure was followed for the apical third. Following this protocol, the dentinal tubules were clearly visible using a 40X objective, scanning with a resolution of 1024x1024 pixels and a spacing of 1 µm. The images (275µm x 275µm) were acquired with the Leica Application Suite-Advanced Fluorescence (LAS AF, Leica Mannheim, Germany) program (Fig. 1).

Recolonization

In 10 teeth from each group, selected based on an equal distribution of the root canal volume between the groups, the coronal opening and the apex were sealed with a temporary restorative material (Villevie®, Dentalville, Joinville, SC, Brazil) after the irrigation procedure. The teeth were placed in microtubes containing 1 mL of sterile BHI and incubated at 37ºC for 5 days to allow the remaining biofilm to reestablish itself. After this period, the teeth were sectioned, stained and analyzed using the CLSM as mentioned in the previous paragraph, verifying and comparing the recolonization ability of the given bacteria in the root canal system after each treatment.

Image analysis

After obtaining the CLSM images, ImageJ software (FIJI Image J v 1 .50g, National Institutes of Health, USA) was used to separate the bacterial contamination from the background fluorescence of the dentine, specifically developed for the intratubular protocol of contamination and CLSM analysis. Using this method, the image stacks were analyzed, evaluating the amount of green (bacteria with intact cell membrane), red (bacteria with compromised cell membrane) and blue (polysaccharides in matrix) present inside the dentinal tubules. By this analysis, the percentage of each component was obtained, resulting in the mean values.

Statistical analysis

The statistical analysis was performed using SPSS software. One- way analysis of variance (ANOVA) with Tukey *HSD post-hoc* tests were used for the LLCT analysis. For the OCT analysis (dentine discs, lateral canals and isthmus) a paired sample t test and one-way ANOVA test were performed. For the analysis of the results obtained from the dentine tubule model, Kruskal-Wallis tests were used for the comparisons of the different treatments, as were Wilcoxon ranked test was used to compare the positions within each group.

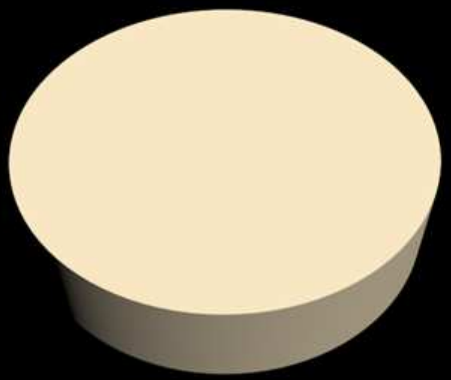
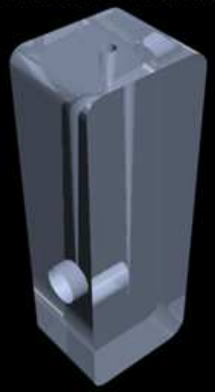
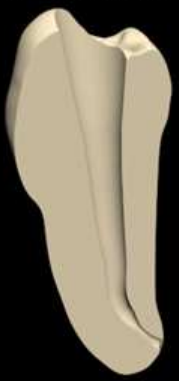
Dentin disks	Artificial root canal	Dentinal tubules
		
1. Chemical irrigants on biofilm: -Buffer -RISA -NaOCl 2. Determination Viscoelastic properties remaining biofilm	Irrigation protocols on isthmus / lateral canal -Buffer -RISA -NaOCl -US activated buffer	1. Irrigation protocols on tubules -Buffer -RISA -NaOCl -US activated buffer 2. Recolonization
Effect of irrigants determined with OCT and LLCT	Effect of irrigation protocol determined with OCT	Effect determined with bacterial staining (CLSM)
No difference between the irrigants	Lateral canal: only US effective in total treatment Isthmus: no difference between groups Final syringe irrigation with high flow rate is effective	NaOCl showed least viable bacteria US least non viable bacteria and polysaccharides Regrown biofilm after RISA had same composition

FIGURE 1 - Representation of PDMS root canal model, dentine discs and dentine tubules model and their respective irrigation protocols and analysis.

Results

Biofilm removal from dentine discs by OCT analysis

All data are presented in Figure 2. There was no significant difference between the groups. Within the groups, only NaOCl showed a statistically significant reduction in the biofilm thickness before and after 30 seconds of direct contact (14.20% removal: P=0.011). Figure 2 shows OCT images of biofilms pre and post direct contact with irrigation solutions.

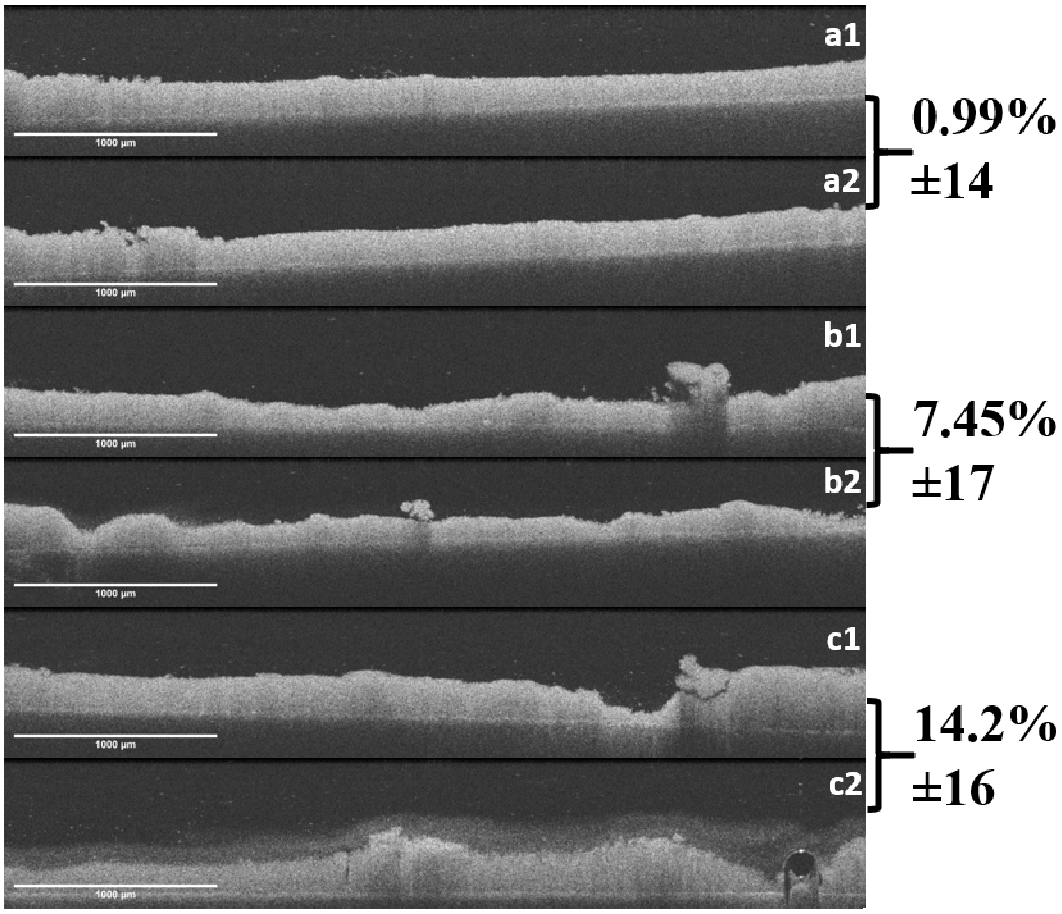


FIGURE 2 - OCT images of biofilm on the dentine discs pre (1) and post (2) direct contact with the irrigating solutions. a: control; b: R; c: 2% NaOCl.

Viscoelasticity of remaining biofilms on dentine discs after application of NaOCl, RISA and buffer:

All data are presented in Table 1. The results showed no significant difference between the three solutions.

Table 1 - Stiffness, relaxation and relative importance of the four Maxwell elements after irrigation with different solutions. Data refers to average±*SD* (n=5).

Groups	Stiffness (Pa)	Relaxation (%)	E1 (%)	E2 (%)	E3 (%)	E4 (%)
Buffer solution	1131±1084	70.1 ±15	42.2 ±17	22.6±6	4.3±4	30.7±17
R	1108±1541	66.7 ±18	28.4 ±17	30.7 ±12	6.7 ±7	34.1±19
2% NaOCl	436 ±253	63.4 ±17	35.3 ±14	18.1 ±13	10.1 ±7	36.3±17

Biofilm removal from lateral canal structures by OCT analysis

The data are presented in Figure 5. Between the different groups no significant difference could be found. Within the groups, only US showed significant difference in biofilm removal between the pre-treatment and final irrigation (26.18% removal:P=0) and post treatment and final irrigation (15.71% removal: P=0.008) (Fig. 3).

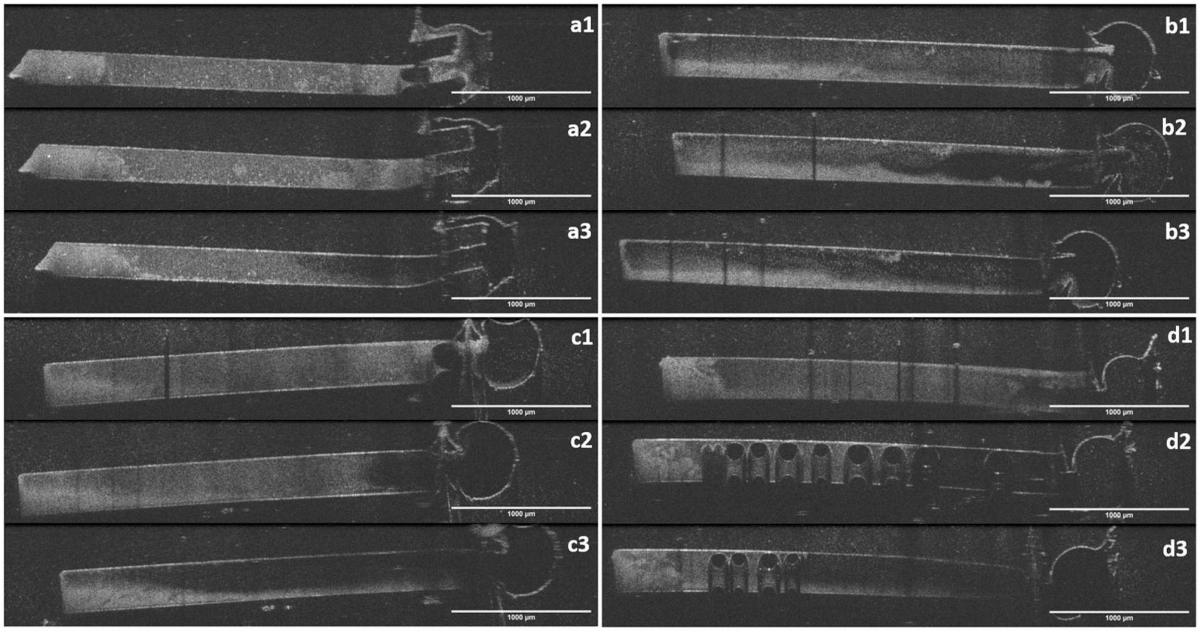


FIGURE 3 - Pre-treatment (1), post treatment (2) and after final irrigation (3) OCT images of biofilm removal in lateral canal model. a: control; b: RISA; c: 2% NaOCl; d: US.

Biofilm removal from isthmus structures by OCT analysis

The data are presented in Figure 5. Between the groups no significant differences could be found. Within the groups, all groups showed significant differences between the different steps of the experiment (pre-treatment, post treatment and final irrigation) with exception of NaOCl post-treatment and final irrigation (Fig. 4).

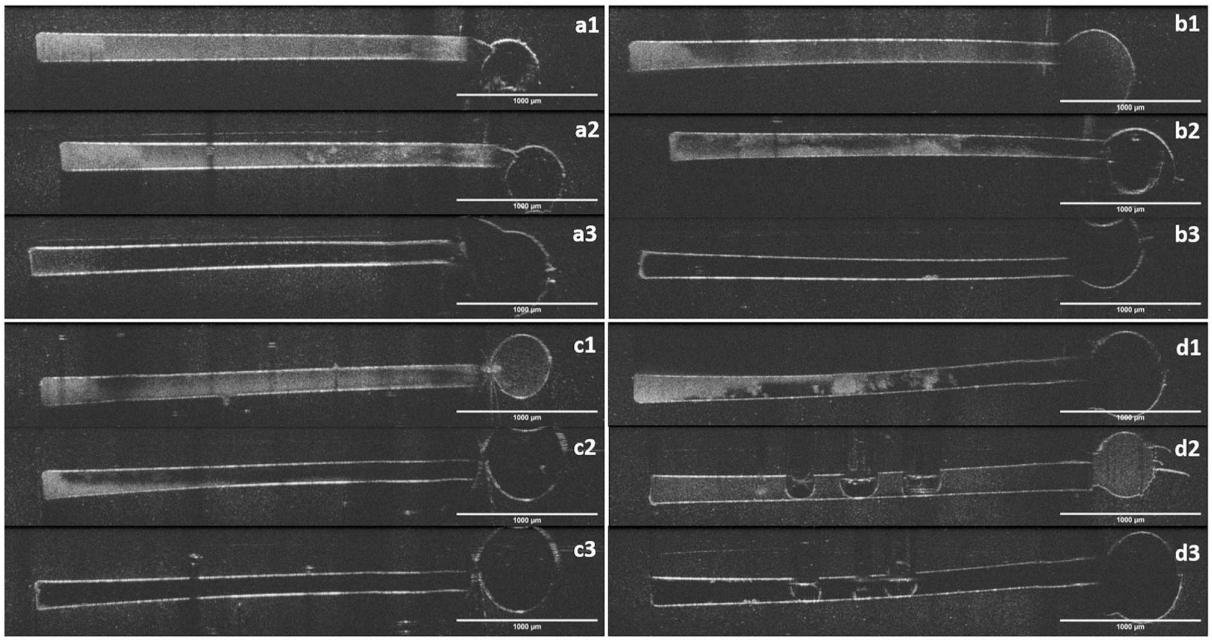


Figure 4 - Pre-treatment (1), post treatment (2) and after final irrigation (3) OCT images of biofilm removal in isthmus model. a: control; b: RISA; c: 2% NaOCl; d: US.

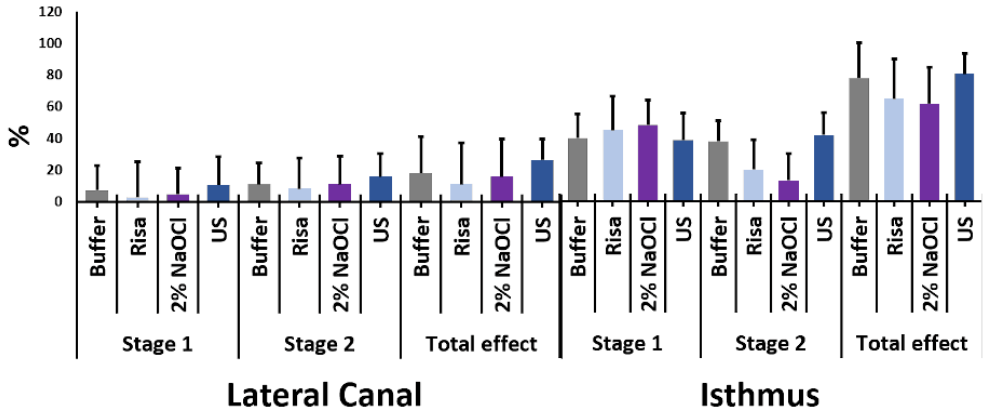


FIGURE 5 - Mean (±SD) percentage of biofilm removal between pre and post treatment, pre-treatment and final irrigation and post treatment and final irrigation for the lateral canal and isthmus structures.

Results from dentine tubule model

Initial treatment

The data are presented in Table:2. The NaOCl group showed a significant smaller percentage of biovolume of viable bacteria than the other groups, 16,1% versus control 31,7% (p=0,000); R 37,7% (p=0,000) and US 53,1% (p= 0,000). US resulted in a significantly higher percentage of biovolume of viable bacteria than the control (p=0,000) and R (p=0,027). For US, the percentage of biovolume of non-viable bacteria was significantly lower than the NaOCl group and the control, for both: p=0,000.

US showed a significantly lower percentage of biovolume of polysaccharides in the matrix than NaOCl 11,7% versus 27,0% (p= 0,000) and R (20,2%, p=0,001). The control group (15,3%) showed a statistically significant lower percentage of biovolume of polysaccharides in the matrix than NaOCl (p= 0,001).

Analysis after recolonization

The data are presented in Table 2: The control presented 20,1% biovolume of viable bacteria, which is significantly less than US (28,6%, p=0,044) and R (35,9%, p=0,000). This last group also had a significantly higher percentage of biovolume of viable bacteria than NaOCl (23,9%, p=0,000). US displayed a significantly higher percentage of biovolume of non-viable bacteria (57,2%) compared to the control (45,2%, p=0,012), R (42,0%, p=0,001) and NaOCl

(42,8%, p=0,003). US (11,7%) significantly showed the lowest percentage of biovolume of polysaccharides in the matrix compared to the control (29,4%, p=0,000), NaOCl (20,7%, p=0,003) and R (19,5%, p=0,010). Moreover, the control showed the highest percentage biovolume of polysaccharides in the matrix, being statistically significant with NaOCl (p=0,001) and R (p=0,001).

Initial treatment versus recolonization

In the control and US, the percentage of biovolume of viable bacteria in the tubules reduced after recolonization respectively from 31,7% to 20,2% (p=0,000) and 53,1% to 28,6% (p=0,002). The percentage of biovolume of non-viable bacteria was statistically significant different for US (32,9% versus 57,2%, p=0,000). A significant difference in the percentage of biovolume of polysaccharides in the matrix was found only in the control were initial treatment (15,3%) showed a lower percentage than after recolonization (29,4%, p=0,000) Figure 6.

Table 2 – Mean, SD and P values of the percentage of biovolume microorganisms with intact cell wall (LIVE), compromised cell wall (DEAD) and polysaccharides (EPS) after Initial Treatment (IT) and after Recolonization (R) for all groups ($p<0.05$).

	Buffer		**R**		**NaOCl**		**US**	
	IT	**R**	**IT**	**R**	**IT**	**R**	**IT**	**R**
LIVE	31.7 ±25.2^{A}	20.1 ±17.9^{ac}	37,7 ±21,3^{A}	35.9 ±21.4^{b}	16,1 ±17,3^{B}	23,9 ±24,1^{c}	53,1 ±28,2^{C}	28,6 ±19,2^{bc}
DEAD	48.4 ± 28.1^{AB}	45.2 ±23.9^{a}	42,0 ±19,8^{AB}	42,0 ±25,4^{a}	47,4 ±26,4^{A}	42,8 ±28,0^{a}	32,9 ±25,0^{B}	57,1 ±23,8^{b}
EPS	15,3 ±15,2^{AB}	29,3 ±14,3^{a}	20,2 ±11,2^{BC}	19,5 ±13,6^{b}	27,0 ±21,0^{C}	20,7 ±17,2^{b}	11,6 ±14,9^{A}	11,7 ±12,4^{c}

Different Capital and lowercase letters in columns indicate statistically significant intergroup after initial treatment and after recolonization, respectively ($p<0.05$).

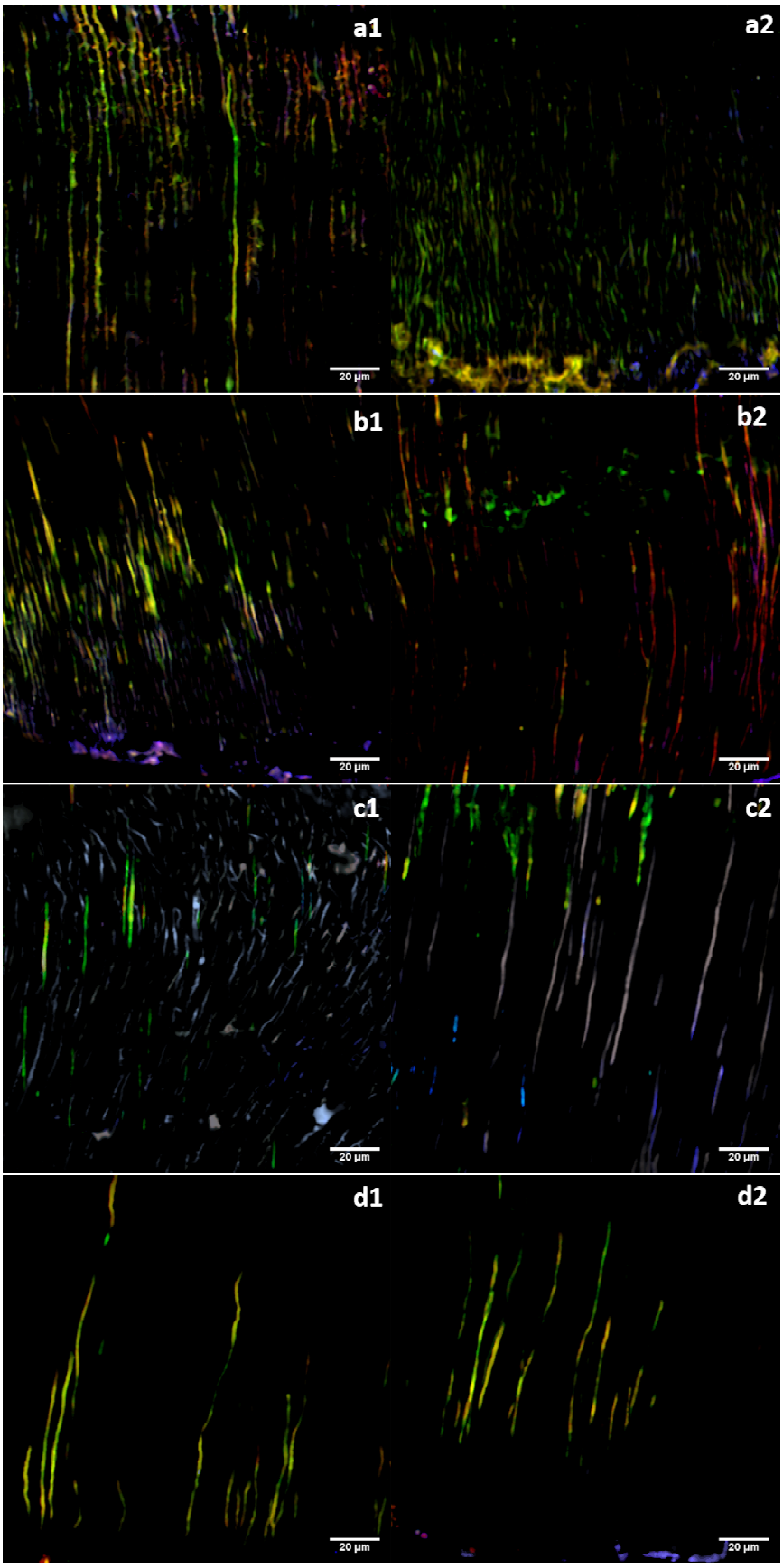


FIGURE 6 - CLSM images of the analysis after the initial treatment (1) and after recolonization (2). a: control; b: RISA; c: 2% NaOCl; d: US.

Discussion

Root canal model with lateral morphological features

In the root canal model with lateral morphological features, the outcome measure is biofilm removal evaluated by OCT. OCT is a -non-invasive- imaging method enabling multiple assessments on the same biofilm samples, biofilm height determination and illustration of the biofilm structure (Wagner & Horn 2017, Busanello *et al*. 2019). This implies that each biofilm is its own control which is important in biofilm research because biofilm growth is difficult to standardize notwithstanding standardized laboratory procedures (Swimberghe *et al.* 2019). However, OCT does not test bacterial vitality and therefore the dentine tubule model was included in this study. Furthermore, dentinal tubules can be seen as lateral morphological features with a smaller diameter than the lateral canal in the root canal model.

For the root canal model, a dual species in vitro biofilm (*S. oralis* J22 and *A. naeslundi*) was used instead of a hydrogel in Macedo *et al.* (2014) and Robinson *et al.* (2018) thus approaching more closely the clinical situation. Biofilms are not as homogeneous in its structure as the hydrogel and also have preferential growth in some locations and corners in the lateral canal and the isthmus like structures. In the biofilm used in this study, bacteria improve biofilm cohesion and adhesion to the PDMS through collateral bonds (He *et al.* 2013, Busanello *et al.* 2019). In an earlier study using a similar model, biofilm removal highly correlated with the irrigant flow pattern and streaming velocities (Pereira *et al*. in progress). Moreover, as the employed biofilm adheres firmly to PDMS (Song *et al*. 2015), it will be more difficult to remove as it would be in the clinical situation. The bacteria in the artificial biofilm are often found in the root canal (Chávez de Paz *et al*. 2003) and the viscoelastic properties of this biofilm resemble close to those of an *in vivo* oral biofilm (He *et al.* 2013). In addition, this root canal model is a closed system hampering biofilm removal by a steady state fluid flow which would be possible in an open system, thus mimicking the clinical circumstances. Biofilm removal from a closed system model is more difficult than a model which consists of an artificial isthmus between two canals, resembling an open system.

Firstly, this could explain why less biofilm is removed from lateral morphological features of the root canal compared to hydrogel in Macedo *et al.* (2014) and Robinson *et al.* (2018). Secondly, it could clarify why ultrasonic activation of a non-chemical active buffer did not remove more biofilm from an isthmus like structure than syringe irrigation with buffer, whilst the irrigation time of the ultrasonic group was even longer. In contrast, previous studies have shown that ultrasound was effective in cleaning isthmus like structures. This can partly be

explained by the fact that the different studies used different biofilms and the structure of the biofilm determines the outcome of a study (Busanello *et al.* 2019). Furthermore, in this study only the mechanical aspect of ultrasound was examined, using a buffer as irrigant. It is known that ultrasound improves the chemical effect of NaOCl (synergistic effect) (Moorer & Wesselink 1982).

Interestingly, for *the isthmus like structure*, the chemical effect of NaOCl and RISA was comparable to the mechanical effect of a low flow during syringe irrigation with buffer. Additionally, the mechanical effect of a high flow with buffer in phase 2 removed even more biofilm than the experimental group in phase 1 indicating the importance of a high flow rate in removing biofilm from lateral morphological features. This finding is supported by an earlier study (Pereira *et al*. *in progress*). The penetration of irrigant depends on the anatomy of the root canal (Gulabivala *et al*. 2005) and the isthmus allows a steady jet of irrigant solution, promoting a slow and steady biofilm removal that does not occur in the lateral canals, resulting in more biofilm removal (Jiang *et al*. 2010, Verhaagen *et al.* 2014). The relatively short irrigant application time (30 seconds) and realistic but rather small surface contact irrigant-biofilm compared to other *in vitro* studies probably have influenced the results. Time is an important factor and could improve the chemical action of the irrigants (Petridis *et al.* 2019b).

However, in *lateral canal like structures*, hydrogel detaches more in fragments (Macedo *et al.* 2014, Robinson *et al.* 2018). Here, the mechanical effect of the lateral streaming produced by ultrasound has a significantly better effect than the chemical effect of NaOCl and RISA and the mechanical effect of syringe irrigation with buffer at low flow rate. Ultrasonic activation of the irrigant produces an oscillatory component near the file and a steady component further away (Verhaagen *et al.* 2014) besides, irrigant flow can be guided because the flow is strongest in the direction of the amplitude of the file (Jiang *et al*. 2010). Interestingly, the mechanical effect of a final flow with a high flow rate using syringe irrigation in phase 2 removed even significantly more biofilm than the experimental group in phase 1. Probably, the mechanical effect of the lateral streaming produced by ultrasound removed biofilm from the lateral canal close to the main canal and the high flow rate of the final flow was needed to remove the deeper layers of the biofilm probably already loosened by the ultrasound. It has been shown that after around 20 seconds a plateau effect is reached for the effectivity of ultrasound in hydrogel removal from lateral canal like structures (Macedo *et al.* 2014). This plateau effect could also account for biofilm removal form lateral canal like structures.

Dentine tubule model

In the dentine tubule model, bacterial activity and the presence of matrix material (polysaccharides) in the post-treatment remaining and regrown biofilms were examined by CLSM. In this model *Enterococcus faecalis* was used because its ability to deeply penetrate the dentinal tubules and adhere to collagen, which is its virulence factor (Love *et al*. 2001). Besides, these bacteria can achieve a viable but not culturable state, activating a starvation response under stress conditions (Heim *et al.* 2002), being suitable to regrow. After irrigation with NaOCl, significantly less viable bacteria were seen compared to RISA and buffer, the last two not being significantly different from each other. The effect of NaOCl corresponds to earlier studies (Arias-Moliz *et al*. 2014, Rodrigues *et al.* 2018). In contrast to earlier studies (van der Waal *et al*. 2015, 2017, de Almeida *et al.* 2016) RISA was not more effective than the control. In this study the exposure time was shorter than in the other studies (30 seconds instead of 2 minutes and 1 hour) and biofilms used were different, all possible influencing the outcome. Perhaps RISA needs more time for effective diffusion in this model.

Ultrasonic activation of a buffer resulted in a significantly higher percentage viable bacteria than the other groups and a significantly lower percentage of non-viable bacteria than the NaOCl group and the control. Cells with compromised cell walls do not account for the structure of the biofilm because the bond with the surrounding biofilm structure is weaker and are therefore probably more prone to be removed by the mechanical effect of a strong focused irrigant flow (Petridis *et al*. 2019b). Furthermore, the ultrasound group showed a significantly lower percentage of polysaccharides in the matrix than NaOCl and RISA. Probably matrix polysaccharides, in contrast to viable bacteria, are easier to remove by focused fluid flow. In the root canal model, ultrasound was more effective than the other groups in removing biofilm structure from lateral canal like structures. This could be in line with the removal of polysaccharides from the dentinal tubules.

It was possible to regrow biofilm from post-treatment remaining biofilm even without extra nutrition. Intriguingly, the composition in relation to (non) viable bacteria and polysaccharides in the matrix of the regrown post-treatment remaining biofilm after five days seems to be unchanged after irrigation with the chemically active RISA and almost unchanged after irrigation with the chemically active NaOCl. This was in contrast to the composition of the regrown post-treatment remaining biofilm after irrigation with the non-chemical active buffer applied by syringe or ultrasound. The former being the most ‘active’ biofilm with significantly more matrix material and the latter having less viable cells than in the post-treatment remaining biofilm. This could imply that ultrasonic activation of the buffer may have a prolonged effect

over time. Chemical agents can alter the mechanical properties of the EPS, which may be explained by an influence of these agents on the EPS network formation (Körstgens 2001). This alteration can directly influence the removal of the biofilm if the matrix is stabilized like for chlorhexidine (Brindle *et al.* 2011). Such an effect has also been described for NaOCl (Busanello *et al.* 2019). The effect of RISA is partly based on an osmotic action which could have resulted in a more or less 'stabilized' biofilm.

In the dentine tubule model the percentage of polysaccharides in the matrix was also analyzed, differing from most other studies evaluating intratubular decontamination (Arias-Moliz *et al.* 2014, Arias *et al.* 2016). A significant increase in the matrix production was seen in the post-treatment remaining biofilm after irrigation with NaOCl and in the post-treatment remaining biofilm after buffer irrigation, which is for both probably related to the stress of the environmental conditions. For the former an indication for the highest chemical stress (lowest amount of viable cells) and for the latter an indication for the most viable remaining biofilm reacting to environmental stress (no nutrition). The matrix plays an important role in the protection mechanism of bacteria in the biofilm against chemical and mechanical stresses (Flemming & Wingender, 2010) and consequently also play a crucial role in the survival of *E. faecalis* (Lei *et al.* 2016). As a matter of fact, quantification of matrix material is a relevant approach in the search for what really happens with biofilm structure when it is in contact with the irrigating solutions in dentinal tubules (Lei *et al.* 2016). Moreover, in the start of the attachment and colonization on a substrate by the planktonic cells (Cerca *et al.* 2005), the matrix can digest enzymes surrounding bacteria, making the contact between the microorganisms and the antibacterial agents more difficult (Bowen & Koo, 2011). In the presence of matrix material, E. faecalis can, even in the non-culturable state, survive in the dentinal tubules (Trevors 2011, Lei *et al.* 2016), emphasizing the importance of matrix material.

Dentine discs

In the comparison between the groups, RISA and NaOCl did not remove significant more biofilm than the control. Within the groups only NaOCl removed significant more biofilm compared to the initial biofilm. This is in line with the outcomes of the root canal model. The viscoelastic properties of the remaining biofilm after contact with RISA or NaOCl were not significantly different from the control indicating diffusion into the remaining biofilm was not such that it could alter the structure of the biofilm. This indicates that within the 30 seconds the chemical effect of the irrigants is not more pronounced than the control. Perhaps more time or a bigger contact surface irrigant-biofilm was needed to diffuse in this bacterial dense biofilm used.

The *E. Faecalis* biofilm, used in the dentine tubule model, was not used for above mentioned studies because it has been used in another laboratory.

Trends combining outcome different models

Some interesting correlating trends were identified. When the contact surface irrigant-biofilm is relatively big (isthmus) and when the chemical effect of the irrigants was examined on biofilm grown on dentine discs, there was a tendency towards buffer being less effective than RISA and RISA being less effective than NaOCl. This trend is confirmed by the outcomes of the dentine tubule study where significantly less viable bacteria were seen in the NaOCl group compared to RISA and buffer. However, a reverse trend is seen in the results after irrigation phase 2. In phase 2 the effect of removal of the post treatment remaining biofilm from phase 1 is evaluated. Here, more biofilm is removed in the ultrasound and buffer group than in the RISA group and in the RISA group more than in the NaOCl group. Perhaps RISA and NaOCl canor toughen the remaining biofilm which already has been described for NaOCl (Busanello *et al.* 2019). This coincides with the fact that the composition of the regrown biofilm is identical as the remaining biofilm for RISA and NaOCl and not for the buffer and ultrasound group. It is known that a chemical reaction can induce a dormant state of the biofilm and that persister cells are present in the biofilm both impeding removal (Koo *et al.* 2017). Furthermore, it is known that biofilms can survive NaOCl (Stewart *et al*. 2001) resulting in post-treatment biofilm persistence (Nair *et al.* 2005, Ricucci & Siqueira 2010). Depending on the environmental conditions, the remaining biofilm can re-grow (Chávez de Paz *et al.* 2008, Shen *et al.* 2010, Ohsumi *et al*. 2015, Shen *et al*. 2016) sustaining periapical disease (Siqueira & Rôças 2008). Therefore, investigating aspects of its structure could aid in the development of effective removal regimes (Peterson *et al*. 2015). However, on the dentine disc model buffer, NaOCl and RISA do not seem to influence the viscoelastic properties of remaining biofilm. Sodium hypochlorite exhibits limited penetration due to the immediate NaOCl consumption related to its reaction with the organic biofilm substrate (Stewart *et al.* 2001, Stewart 2003). This effect is more pronounced at low concentrations of NaOCl, increasing biofilm thickness and density. The biofilms grown on the dentine discs present a bacterial dense structure which is low in water and EPS content (Busanello *et al.* 2019). The dense bacterial aggregation and the low water content impede deeper transport of solutes, thereby limiting NaOCl penetration. This could also account for RISA and buffer irrigants. This biofilm could also be denser in structure than the biofilm of *E. faecalis* used in the dentine tubule model.

Translation to the clinic

For the dentine disc model and the root canal model a bacterial dense biofilm has been used mimicking the ground (basal) layer of the biofilm and a biofilm packed in narrow structures like lateral morphological features of the root canal. It can be concluded that for this type of biofilm more time is needed than 30 second for an effective chemical effect of NaOCl and RISA. However, the chemical reaction on the post-treatment remaining biofilm matrix could hamper its removal because chemical agents can alter the mechanical properties of the biofilm matrix, which may be explained by an influence of these agents on the matrix network formation (Körstgens 2001, Busanello *et al.* 2019). This alteration can directly influence the removal of the biofilm (Brindle *et al.* 2011).

Because of the OCT, multiple scans could be made of the same biofilm and the effect of different treatment steps on the same biofilm could be evaluated. This showed that a final flow with high flow rate using syringe irrigation was very effective. This could be translated to the clinic.

As is already known, the complex root canal anatomy impedes biofilm removal. Remaining biofilm after root canal treatment is the reality, chemical attacks will chemically influence the matrix of the remaining biofilm and this biofilm can eventually also regrow. A goal of root canal treatment could be to chemically disturb the remaining biofilm as much as possible hampering regrowth. A strong chemical attack at the end of the root canal treatment could be advised.

Conclusion

In this research set up, it seems that the mechanical effect of syringe irrigation at low and high flow rate is important for biofilm removal. The mechanical effect of the lateral flow of ultrasound could be effective when the surface contact biofilm-irrigant is small. The mechanical effect of ultrasound in cleaning an isthmus like structure was not more effective than syringe irrigation with a low flow rate.

After irrigation with NaOCl, significantly less viable bacteria were present in the dentinal tubules. After the different irrigation procedures, still biofilm was left in the dentinal tubules which could regrow/survive in a period of five days in the root canals without extra nutrition. Both RISA and NaOCl seemed to stabilize the remaining biofilm.

References

1. Abbott PV, Heijkoop PS, Cardaci SC, Hume WR, Heithersay GS (1991) An SEM study of the effects of different irrigation sequences and ultrasonics. *International Endodontic Journal* **24**, 308–16.
2. de Almeida J, Hoogenkamp M, Felippe WT, Crielaard W, van der Waal SV (2016) Effectiveness of EDTA and Modified Salt Solution to Detach and Kill Cells from Enterococcus faecalis Biofilm. *Journal of Endodontics* **42**, 320-3.
3. Andrade FB, Arias MP, Maliza AG, Duarte MA, Graeff MS, Amoroso-Silva PA, Midena RZ, Moraes IG (2015) A new improved protocol for in vitro intratubular dentinal bacterial contamination for antimicrobial endodontic tests: standardization and validation by confocal laser scanning microscopy. *Journal of Applied Oral Science* **23**, 591-8.
4. Arias-Moliz MT, Ordinola-Zapata R, Baca P, Ruiz-Linares M, Ferrer-Luque CM (2014) Antimicrobial activity of a sodium hypochlorite/etidronic acid irrigant solution. *Journal of Endodontics* **40**, 1999-2002.
5. Arias MP, Maliza AG, Midena RZ, Graeff MS, Duarte MA, Andrade FB (2016) Effect of ultrasonic streaming on intra-dentinal disinfection and penetration of calcium hydroxide paste in endodontic treatment. *Journal of Applied Oral Science* **24**, 575-581.
6. Baron A, Lindsey K, Sidow SJ, Dickinson D, Chuang A, McPherson JC 3RD (2016) Effect of a Benzalkonium Chloride Surfactant-Sodium Hypochlorite Combination on Elimination of Enterococcus faecalis. *Journal of Endodontics* **42**, 145-9.
7. Bowen WH, Koo H (2011) Biology of Streptococcus mutans-derived glucosyl-transferases: role in extracellular matrix formation of cariogenic biofilms. *Carie Research* **45**, 69-86.
8. Brindle ER, Miller DA, Stewart PS (2011) Hydrodynamic deformation and removal of Staphylococcus epidermidis biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. *Biotechnology and Bioengineering* **108**, 2968– 77.
9. Busanello FH, Petridis X, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019) Chemical biofilm removal capacity of endodontic irrigants as a function of biofilm structure: optical coherence tomography, confocal microscopy and viscoelasticity determination as integrated assessment tools. *International Endodontic Journal* **52**, 461-74.
10. Cerca N, Pier GB, Vilanova M, Oliveira R, Azeredo J (2005) Quantitative analysis of adhesion and Staphylococcus epidermidis. *Research Microbiology* **156**, 506-14.

11. Chávez de Paz LE, Dahlen G, Molander A, Moller A, Bergenholtz G (2003) Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. *International Endodontic Journal* **36**, 500–8.
12. Chávez de Paz LE (2007) Redefining the persistent infection in root canals: possible role of biofilm communities. *Journal of Endodontics* **33**, 652-62.
13. Chávez de Paz LE, Hamilton IR, Svensater G (2008) Oral bacteria in biofilms exhibit slow reactivation from nutrient deprivation. *Microbiology* **154**, 1927–38.
14. Duque JA, Duarte MA, Canali LC, Zancan RF, Vivan RR, Bernardes RA, Bramante CM (2017) Comparative Effectiveness of New Mechanical Irrigant Agitating Devices for Debris Removal from the Canal and Isthmus of Mesial Roots of Mandibular Molars. *Journal of Endodontics* **43**, 326-31.
15. Flemming H-C, Neu TR, Wozniak DJ (2007) The EPS matrix: the "house of biofilm cells". *Journal of Bacteriology* **189,** 7945-47.
16. Flemming HC, Wingender J (2010) The biofilm matrix. *Nature Reviews. Microbiology*. **8**, 623-33.
17. Giardino L, Del Fabbro M, Cesario F, Fernandes FS, Andrade FB (2018) Antimicrobial effectiveness of combinations of oxidant and chelating agents in infected dentine: an ex vivo confocal laser scanning microscopy study. *International Endodontic Journal* **51**, 448-456.
18. Giardino L, Del Fabbro M, Morra M, Pereira T, Bombarda de Andrade F, Savadori P, Generali L (2019) Dual Rinse(®) HEDP increases the surface tension of NaOCl but may increase its dentin disinfection efficacy. *Odontology* **107**, 521-9.
19. Gulabivala K, Patel B, Evans G, Ng YL (2005) Effects of mechanical and chemical procedures on root canal surfaces. *Endodontic Topics* **10**, 103–22.
20. Heim S, Lleo MM, Bonato B, Guzman CA, Canepari P (2002) The viable but nonculturable state and starvation are different stress responses of Enterococcus faecalis, as determined by proteome analysis. *Journal of Bacteriology* **184,** 6739–45.
21. He Y, Peterson BW, Jongsma MA, Ren Y, Sharma PK, Busscher HJ, Van der Mei HC (2013) Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms. *PLoS ONE* **8**: e63750.
22. Jiang LM, Verhaagen B, Versluis M, van der Sluis LW (2010) Influence of the oscillation direction of an ultrasonic file on the cleaning efficacy of passive ultrasonic irrigation. *Journal of Endodontics* **36**,1372-6.

23. Kinniment SL, Wimpenny JW, Adams D, Marsh PD (1996) Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* **142**, 631-8.
24. Kolenbrander PE, Palmer RJ, Jr, Periasamy S, Jakubovics NS (2010) Oral multispecies biofilm development and the key role of cell-cell distance. *Nature Reviews. Microbiology* **8,** 471–80.
25. Koo H, Allan RN, Howlind RP, Hall-Stoodleye L, Stoodley P. 2017. Targeting microbial biofilms: current and prospective therapeutic strategie. *Nature Reviews. Microbiology* **15**, 740–55.
26. Korstgens V, Flemming HC, Wingender J, Borchard W (2001) Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. *Journal of Microbiological Methods* **46,**9 –17.
27. Lei L, Shao M, Yang Y, Mao M, Yang Y, Hu T (2016) Exopolysaccharide dispelled by calcium hydroxide with volatile vehicles related to bactericidal effect for root canal medication. *Journal of Applied Oral Sciences* **24,** 487-95.
28. Liu T, Huang Z, Ju Y, Tang X (2019) Bactericidal efficacy of three parameters of Nd:YAP laser irradiation against Enterococcus faecalis compared with NaOCl irrigation. *Lasers in Medical Science* **34**, 359-66.
29. Love RM (2001) Enterococcus faecalis — a mechanism for its role in endodontic failure. *International Endodontic Journal* **34,** 399–405.
30. Ma J, Wang Z, Shen Y, Haapasalo M (2011) A new noninvasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy. *Journal of Endodontics* **37**, 1380-5.
31. Macedo RG, Robinson J, Verhaagen B, Walmsley D, Versluis M, Cooper P, van der Sluis LWM (2014) A novel optical methodology for quantifying the removal of a biofilm-mimicking hydrogel from lateral morphological features of the root canal. *International Endodontic Journal* **47**, 1040-51.
32. Marinho AC, Martinho FC, Goncalves LM, Rabang HR, Gomes BP (2015) Does the Reciproc file remove root canal bacteria and endotoxins as effectively as multifile rotary systems? *International Endodontic Journal* 48, 542-548.
33. Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15,** 187–196.
34. Morago A, Ordinola-Zapata R, Ferrer-Luque CM, Baca P, Ruiz-Linares M, Arias-Moliz MT (2016) Influence of Smear Layer on the Antimicrobial Activity of a Sodium

Hypochlorite/Etidronic Acid Irrigating Solution in Infected Dentin. *Journal of Endodontics* **42**, 1647-50.

35. Morago A, Ruiz-Linares M, Ferrer-Luque CM, Baca P, Rodríguez Archilla A, Arias-Moliz MT (2019) Dentine tubule disinfection by different irrigation protocols. *Microscopy Research and Technique* **82**, 558-63.
36. Naenni N, Thoma K, Zehnder M (2004) Soft tissue dissolution capacity of currently used and potential endodontic irrigants. *Journal of Endodontics* **30**, 785-7.N
37. air PN, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **99**, 231–252.
38. Ohsumi T, Takenaka S, Wakamatsu R, *et al*. (2015) Residual structure of Streptococcus mutans biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. *PLoS ONE* **10**, e0116647.
39. Otsu N (1979) A threshold selection method from gray-level histograms. *IEEE Transactions on Systems, Man and Cybernetic* **9**, 62-6.
40. Pereira TC, Boutsioukis C, Dijkstra RJB, Petridis X, Versluis M, de Andrade FB, van der Meer WJ, Sharma P, van der Sluis LWM, So MVR *in progress. Submitted to International Endodontic Journal* Biofilm removal from an artificial isthmus and lateral canal during syringe irrigation at various flow rates: a combined experimental and Computational Fluid Dynamics approach.
41. Peters O, Schönenberger K, Laib A (2001) Effects of four Ni–Ti preparation techniques on root canal geometry assessed by micro computed tomography. *International Endodontic Journal* **34**, 221-230.
42. Peterson BW, He Y, Ren Y *et al*. (2015) Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiology Reviews* **39**, 234-45.
43. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019a) Chemical efficacy of several NaOCl concentrations on biofilms of different architecture: new insights on NaOCl working mechanisms. *International Endodontic Journal* **52**, 1773-88.
44. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Prashant SK, van der Sluis LWM (2019b) Factors affecting the chemical efficacy of 2% sodium hypochlorite against oral

steady-state dual-species biofilms: Exposure time and volume application. *International Endodontic Journal* **52***,* 1182-95.

45. Ricucci D, Loghin S, Siqueira Jr JF (2013) Exuberant biofilm infection in a lateral canal as the cause of short-term endodontic treatment failure: report of a case. *Journal of Endodontics* **39**, 712-8.
46. Ricucci, D, Siqueira JF, Bate AL, Pitt Ford, TR (2009) Histologic Investigation of Root Canal–treated Teeth with Apical Periodontitis: A Retrospective Study from Twenty-four Patients. *Journal of Endodontics* **35**, 493–502.
47. Ricucci D, Siqueira JF Jr (2010) Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *Journal of Endodontics* **36**, 1277-88.
48. Robinson JP, Macedo RG, Verhaagen B, Versluis M, Cooper PR, van der Sluis LWM, Walmsley AD (2018) Cleaning lateral morphological features of the root canal: the role of streaming and cavitation. *International Endodontic Journal* **51**, e55-64.
49. Rodrigues CT, de Andrade FB, de Vasconcelos LRSM, Midena RZ, Pereira TC, Kuga MC, Duarte MAH, Bernardineli N (2018) Antibacterial properties of silver nanoparticles as a root canal irrigant against Enterococcus faecalis biofilm and infected dentinal tubules. *International Endodontic Journal* **51**, 901-911.
50. Rozenbaum RT, van der Mei HC, Woudstra W, de Jong ED, Busscher HJ, Sharma PK (2019) Role of Viscoelasticity in Bacterial Killing by Antimicrobials in Differently Grown Pseudomonas aeruginosa Biofilms. *Antimicrobial Agents and Chemotherapy* **63**, e01972-18.
51. Sabins RA, Johnson JD, Hellstein JW (2003) A comparison of the cleaning efficacy of short-term sonic and ultrasonic passive irrigation after hand instrumentation in molar root canals. *Journal of Endodontics* **29**,674-8.
52. Sarbinoff JA, O'Leary TJ, Miller CH (1983) The comparative effectiveness of various agents in detoxifying diseased root surfaces. *Journal of Periodontology* **54**, 77-80.
53. Sen BH, Wesselink PR, Türkün M (1995) The smear layer: a phenomenon in root canal therapy. *International Endodontic Journal* **28**, 141-8.
54. Shen Y, Stojicic S, Haapasalo M (2010) Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture. *Journal of Endodontics* **36**, 1820–3.
55. Shen Y, Zhao J, de la Fuente-Nuñez C *et al*. (2016) Experimental and theoretical investigation of multispecies oral biofilm resistance to chlorhexidine treatment. *Scientific Reports* **6**, 27537.

56. Silva LA, Leonardo MR, Assed S, Tanomaru Filho M (2004) Histological study of the effect of some irrigating solutions on bacterial endotoxin in dogs. *Brazilian Dental Journal* **15**, 109-14.
57. Siqueira JF Jr, Rôças IN (2008) Clinical implications and microbiology of bacterial persistence after treatment procedures. *Journal of Endodontics* **34**, 1291–301.
58. van der Sluis LW, Wu MK, Wesselink PR (2005) The efficacy of ultrasonic irrigation to remove artificially placed dentine debris from human root canals prepared using instruments of varying taper. *International Endodontic Journal* **38**, 764-8.
59. van der Sluis LW, Versluis M, Wu MK, Wesselink PR (2007) Passive ultrasonic irrigation of the root canal: a review of the literature. *International Endodontic Journal* **40**, 415-26.
60. Song F, Ren D (2014) Stiffness of cross-linked poly(dimethylsiloxane) affects bacterial adhesion and antibiotic susceptibility of attached cells. *Langmuir* **30**, 10354-62.
61. Spratt DA, Pratten J, Wilson M, Gulabivala K (2001) An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *International Endodontic Journal* **34**, 300-7.
62. Stewart PS (2003) Diffusion in biofilms. *Journal of Bacteriology* **185**, 1485–91.
63. Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. *Nature Reviews Microbiology* **6**, 199-210.
64. Stewart PS, Rayner J, Roe F, Rees WM (2001) Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *Journal of Applied Microbiology* **91**, 525–32.
65. Swimberghe RCD, Coenye T, De Moor RJG, Meire MA (2019) Biofilm model systems for root canal disinfection: a literature review. *International Endodontic Journal* **52**, 604-628.
66. Tartari T, Bachmann L, Maliza AG, Andrade FB, Duarte MA, Bramante CM (2016) Tissue dissolution and modifications in dentin composition by different sodium hypochlorite concentrations. *Journal of Applied Oral Sciences* **24**, 291-8.
67. Tawakoli PN, Ragnarsson KT, Rechenberg DK, Mohn D, Zehnder M (2017) Effect of endodontic irrigants on biofilm matrix polysaccharides. *International Endodontic Journal* **50**, 153-160.
68. Trevors JT (2011) Viable but non-culturable (VBNC) bacteria: gene expression in planktonic and biofilm cells. *Journal of Microbiology Methods* **86**, 266-73.
69. Verhaagen B, Boutsioukis C, Sleutel CP, Kastrinakis E, van der Sluis L, Versluis M (2014) Irrigant transport into dental microchannels. *Microfluidics and Nanofluidics* **16,** 1165-77.

70. van der Waal SV, van der Sluis LW, Özok AR, Exterkate RA, van Marle J, Wesselink PR, de Soet JJ (2011) The effects of hyperosmosis or high pH on a dual-species biofilm of Enterococcus faecalis and Pseudomonas aeruginosa: an in vitro study. *International Endodontic Journal* **44**, 1110-7.
71. van der Waal, S. V., Scheres, N., de Soet, J. J., Wesselink, P. R., & Crielaard, W. (2014). Cytotoxicity, interaction with dentine and efficacy on multispecies biofilms of a modified salt solution intended for endodontic disinfection in a new in vitro biofilm model. *International Endodontic Journal* **42**, 153–161.
72. van der Waal SV, Oonk CA, Nieman SH, Wesselink PR, de Soet JJ, Crielaard W (2015) Additional disinfection with a modified salt solution in a root canal model. *Journal of Dentistry* **43**, 1280-4.
73. van der Waal SV, Connert T, Crielaard W, de Soet JJ (2016) In mixed biofilms Enterococcus faecalis benefits from a calcium hydroxide challenge and culturing. *International Endodontic Journal* **49**, 865-73.
74. van der Waal SV, de Almeida J, Krom BP, de Soet JJ, Crielaard W (2017) Diffusion of antimicrobials in multispecies biofilms evaluated in a new biofilm model. *International Endodontic Journal* **50**, 367-376.
75. Wagner M, Horn H (2017) Optical Coherence Tomography in biofilm research: a comprehensive review. *Biotechnology and Bioengineering* **114,** 1386-1402.

2.3 ARTICLE 3 - The effect of refreshment, exposure time, flow rate and irrigant on biofilm removal from lateral morphological features of the root canal.

Abstract

Aim To evaluate the effect of irrigant refreshment and exposure time of a 2% sodium hypochlorite solution (NaOCl) on biofilm removal from lateral morphological features of the root canal using two different flow rates.

Methodology A dual-species biofilm was formed by Constant Depth Film Fermentor (CDFF) for 96 hours in isthmus and lateral canal like structures in inserts. These inserts where placed in a root canal model facing the main canal. NaOCl and demineralized water (control group) were used as irrigant solutions. Both substances were applied at a flow rate of 0.05 and 0.1 mL/second. The samples were divided in three groups with zero, one or two refreshments in a total exposure time of 15 minutes. A three-way Analysis of Variance (ANOVA) was performed to investigate the interaction among the independent variables and the effect of consecutive irrigant refreshment on percentage of biofilm removal. A Tukey *post hoc* test was used to evaluate the effect of each independent variable on percentage biofilm removal in the absence of statistically significant interactions.

Results For the lateral canal, NaOCl removed significantly more biofilm irrespective of the number of refreshments and exposure time. There was no significant effect in biofilm removal between the consecutive irrigant refreshments measured in the same biofilm. For the isthmus, NaOCl removed significant more biofilm irrespective of the number of refreshments and exposure time; both NaOCl and a high flow rate significant removed more biofilm when the exposure time was analyzed. Evaluating the effect of consecutive irrigant refreshment on the same biofilm, 2% NaOCl, 0.1 mL/s flow rate and one or two refreshments removed more biofilm.

Conclusions In this model, refreshment did not improve biofilm removal from lateral morphological features in the root canal. NaOCl removed more biofilm from the lateral canal and isthmus-like structure. A higher flow rate removed significantly more biofilm from the isthmus like structure. There was always remaining biofilm left after the irrigation procedures.

Keywords: Biofilms; irrigation; optical coherence tomography; removal; sodium hypochlorite.

Introduction

Sodium hypochlorite (NaOCl) is the irrigant of choice during root canal treatment (Zehnder 2006, Dutner *et al.* 2012). It has gained its popularity mainly due to its action against micro-organisms (McDonnel & Russel 1999) and biofilm (Arias-Moliz *et al*. 2009, Bryce *et al.* 2009) as well as its capacity to dissolve pulp tissue (Sirtes *et al*.2005) and organic components of the smear layer (Baumgartner & Mader 1987). However, NaOCl does not dissolve inorganic tissue (Sen *et al*. 1995) and is not able to fully penetrate the biofilm (van der Waal *et al*. 2014, 2017, Petridis *et al*. 2019a) especially when the biofilm is densely packed with bacteria (Busanello *et al*. 2019, Petridis *et al*. 2019 a,b). Furthermore, biofilms withstand NaOCl treatment (Stewart *et al.* 2001), which is also corroborated in *in situ* investigations of root canal specimens (Nair *et al*. 2005, Ricucci & Siqueira 2010). Taking into account that post-treatment remaining biofilm can re-grow (Chávez de Paz *et al.* 2008, Shen *et al.* 2010, Ohsumi *et al.* 2015, Shen *et al.* 2016), failure of apical periodontitis to resolve is an imminent risk. Therefore, getting a better understanding of the properties of NaOCl is warranted in order to improve its antibiofilm efficacy.

NaOCl reacts by direct contact between free available chlorine molecules and organic matter (Moorer & Wesselink 1982). The flux of molecules will take place through diffusion or convection, with diffusion being slower than convection (Verhaagen *et al*. 2014). The surface contact irrigant - biofilm as well as the volume of irrigant will directly influence the availability of the free chlorine molecules to the biofilm. In the highly complex root canal system, the surface contact irrigant - biofilm is limited especially for lateral morphological features like isthmuses and lateral canals. Moreover, it is not known whether the volume of irrigant present in the root canal contains enough free chlorine to allow for diffusion into the biofilm present in lateral morphological features.

The concentration of NaOCl will determine the amount of free chlorine. Clinically concentrations ranging between 1 and 5% are commonly used (Dutner *et al.* 2012). Refreshment is widely thought to be an effective method of compensation for the loss of chemical efficiency of a lower concentration of NaOCl (Moorer & Wesselink 1982, Zenhder 2006). However, this has never been proven. Using a numerical diffusion model to predict the efficacy of diffusion, it has been advised to constantly apply fresh irrigant alongside the opening of an isthmus or lateral canal like structure to enhance diffusion (Verhaagen *et al*. 2014). Also, it has been demonstrated that lower concentration NaOCl solutions have a significantly less effective reaction rate compared to high concentration solutions, even after multiple refreshments of the former

(Macedo *et al.* 2010). Moreover, a constant flow of a 2% NaOCl using syringe irrigation did not remove more biofilm from lateral morphological features than a non-chemical control solution (Pereira *et al*. in progress b). In addition, within 30 s of exposure time, increasing NaOCl concentration did not significantly improve the removal of biofilm from lateral morphological features (Pereira *et al*. in progress a).

Taking all the above into consideration, it is interesting to explore whether refreshment of a NaOCl solution of clinically relevant concentration, applied at different flow rates and for clinically relevant application times could enhance biofilm removal from lateral morphological features in the root canal. Hence, the aim of this study was to:

- evaluate the effect of 2% NaOCl refreshments, exposure time and flow rate on its capacity to remove biofilm from lateral morphological features of the root canal using an *in vitro* root canal model.

The null hypothesis was that refreshment and exposure time will not make a significant difference in removing biofilm from lateral morphological features from the root canal.

Material and Methods

Bacterial strains and growth conditions

A single colony of *Streptococcus oralis* J22 and *Actinomyces naeslundii* T14V-J1 grown on blood agar plates was used to inoculate 10 mL modified brain heart infusion broth (BHI, Oxoid Ltd., Basingstoke, Hampshire, UK) (37.0 g/L BHI, 1.0 g/L yeast extract, 0.02 g/L NaOH, 0.001 g/L Vitamin K1, 5 mg/L L-cysteine-HCl, pH 7.3) as previously described (Busanello *et al.* 2019, Petridis *et al*. 2019). The pre-cultures were separately stored for 24 h in ambient air for *S. oralis* and in anaerobic chamber for *A. naeslundii*. Subsequently, bacteria were harvested by centrifugal force (6350 ×g) and washed twice in sterile adhesion buffer (0.147 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.136 g/L KH_2PO_4, 3.728 g/L KCl, pH 6.8). The bacterial pellets were suspended in 10 mL sterile adhesion buffer and sonicated intermittently in ice-water for 3 × 10 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break bacterial chains. Following this, bacteria were counted using a Bürker-Türk counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) and the suspensions were diluted in 250 mL of adhesion buffer in a concentration of 6×10^8 bacteria/mL for *S. oralis* and 2×10^8 bacteria/mL for *A. naeslundii* in order to obtain the dual-species bacterial suspension.

Specimen preparation

Transparent PolyDiMethylSiloxane (PDMS) (PolyDiMethylSiloxane; Sylgard 184, Dow-Corning, Midland, MI) root canal models, with a small pocket (R= 2.5 mm) in the apical area perpendicular to the root canal were created using a D-size finger spreader (Dentsply Maillefer, Ballaigues, Switzerland) as described in Macedo *et al.* (2014). PDMS pocket inserts with anatomical features resembling an isthmus or lateral canal were created using molds containing a thin metal strip (width 3 mm, thickness 0.15 mm, length 3 mm, total volume 1.35 mm^3) or a small cylinder (length 3.0 mm and thickness 0.25 mm, total volume 0.29 mm^3), respectively.

Constant depth film fermenter biofilm

Steady-state biofilms were grown using a constant depth film fermenter (CDFF) in which a constant dropwise supply of nutrients combined with a repeated cycle of compression/scraping leads to a dental plaque-like bacterial dense biofilm (Kinniment *et al.* 1996, Rozenbaum *et al.* 2017). Lateral canal and isthmus inserts were coated with reconstituted human saliva. Briefly, whole human saliva was pooled from at least 20 volunteers of both genders (saliva was collected in agreement with the guidelines set out by the Medical Ethical Committee of the University Medical Centre Groningen, Groningen, The Netherlands, approval letter 06-02-2009) and freeze-dried. Next, it was dissolved in 20 ml adhesion buffer (1.5 g/L), stirred for 2 hours and centrifuged at 10,000 ×g, at 10ºC, for 5 minutes. Both inserts were conditioned with the reconstituted saliva by dropwise inoculation in the CDFF at a constant low platform rotation that was stopped after 1 h. Samples were incubated with the saliva under static conditions for 14 h. After this, 250ml dual-species bacterial suspension was introduced in the CDFF over 1 hour at a constant slow rotation of the CDFF table. Rotation was stopped and bacteria were allowed to adhere for 1 h to the saliva coated inserts. Subsequently, rotation was resumed and continuous supply of modified BHI (45 mL/h) was started so biofilms could growth over the next 96 h at 37ºC.

Irrigation protocols and OCT analysis

After 96 h, the inserts with lateral canal and isthmus-like structures filled with biofilm were removed from the CDFF, carefully placed in the root canal model and analyzed using optical coherence tomography (Thorlabs Ganymade II, Newton, NJ, USA). The purpose of these OCT scans was to determine the initial biofilm volume present (pre-treatment scan). The experimental groups were established based on an irrigation procedure of 15 min with an irrigant solution (NaOCl or sterile demineralized water-control), applied at 2 different flow rates (0.05 or 0.1

mL/s) and different number of refreshments (0, 1 or 2). The 120 samples of lateral canal and 120 samples of isthmus-like inserts were randomly divided into 12 experimental groups (n=10). Six groups were irrigated with demineralized water and the other six with 2% NaOCl. For each irrigant, three groups were irrigated at 0.05 mL/s and the other three at 0.1 mL/s. For each irrigant and flow rate, three different protocols were used: no refreshment (0R), 1 refreshment at 7.5 minutes (1R) and 2 refreshments at 5 and 10 minutes (2R). In all the groups, OCT scans were made after the irrigation procedure and before each refreshment (post-treatment scan) (Fig. 1). In the group with 2 refreshments, the percentage of biofilm removal after each step was evaluated (consecutive irrigant refreshment).

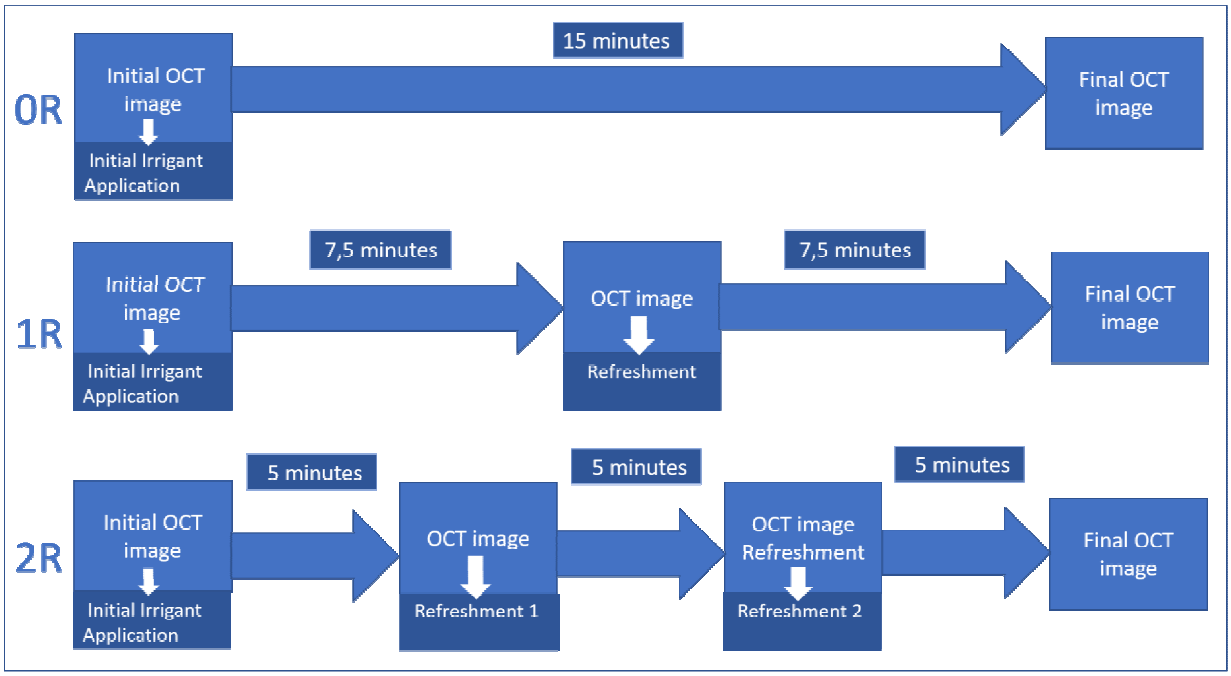


Figure 1- Schematic representation of the experimental groups.

The 2% NaOCl concentration was obtained from a standard solution of NaOCl 12-15% (Sigma-Aldrich, St Louis, Missouri, USA) by iodometric titration before every experiment. The irrigation process was performed with a 5mL irrigation syringe (Ultradent Products Inc., South Jordan, UT, USA) and 30G irrigation needle (Endo-Eze - Ultradent Products Inc., South Jordan, UT, USA). During irrigation, the needle was placed at 2mm coronal from the apical endpoint of the root canal. In the 0.05 mL/s flow rate groups, 1.5 mL of the irrigating solution was released during 30s by in and outward movements of 5mm amplitude. In the 0.1 mL/s flow rate groups, the 1.5 mL of the irrigants were released in 15 s.

OCT image analysis

The software program Image J FIJI (National Institutes of Health, Bethesda, MD, USA) was used for the quantitative image analyses. By acquiring 3D scans, containing 750 slices of 750 x 373px (field of view 5.0 mm, refractive index 1.33), the volume of biofilm residing inside the isthmus or lateral canal like structures could be determined. All 750 slices per sample were analyzed based on their greyscale composition, and by thresholding to only select the biofilm and filtering the background noise. Percentage biofilm removal was calculated by determining the difference in biofilm volume between pre-treatment scans and post-treatment scans after the refreshments and after the 15 minutes of irrigant exposure.

Statistical analysis

Statistical analysis was performed using SPSS software (Version 24.0, IBM Corp., Armonk, New York, USA). A three-way Analysis of Variance (ANOVA) was performed to investigate the interaction among the three independent variables, namely, *irrigant* (demineralized water, 2% NaOCl), *flow rate* (0.05, 0.1 mL/s) and *exposure time* (5, 7.5, 15 min) or *irrigant* (demineralized water, 2% NaOCl), *flow rate* (0.05, 0.1 mL/s) and *irrigant refreshment* (0, 1, 2) on *percentage biofilm removal* (dependent variable). In the absence of statistically significant interactions, a main effect analysis was carried out in order to investigate the effect of each independent variable on percentage biofilm removal (Tukey *post hoc* test). A three-way mixed ANOVA was performed in order to investigate the effect of consecutive irrigant refreshment on percentage biofilm removal, whereby *irrigant* (demineralized water, 2% NaOCl) and *flow rate* (0.05, 0.1 mL/s) were the between-subjects independent variables and the *consecutive irrigant refreshments of the same biofilm* in the group with 2 refreshments the within-subjects independent variable. In the absence of statistically significant interactions, a main effect analysis was carried out in order to investigate the effect of each independent variable on percentage biofilm removal (Tukey *post hoc* test).

RESULTS

Lateral canal

Chemical effect of the irrigant on biofilm removal

Three-way ANOVA yielded no significant interaction among the independent variables on percentage biofilm removal. The main effect analysis revealed no significant effect of exposure time and flow rate on biofilm removal. The main effect of irrigant on biofilm removal was significant (P = 0.005) with 2% NaOCl removing significantly more biofilm than demineralized water irrespective of the exposure time and flow rate (Fig. 2).

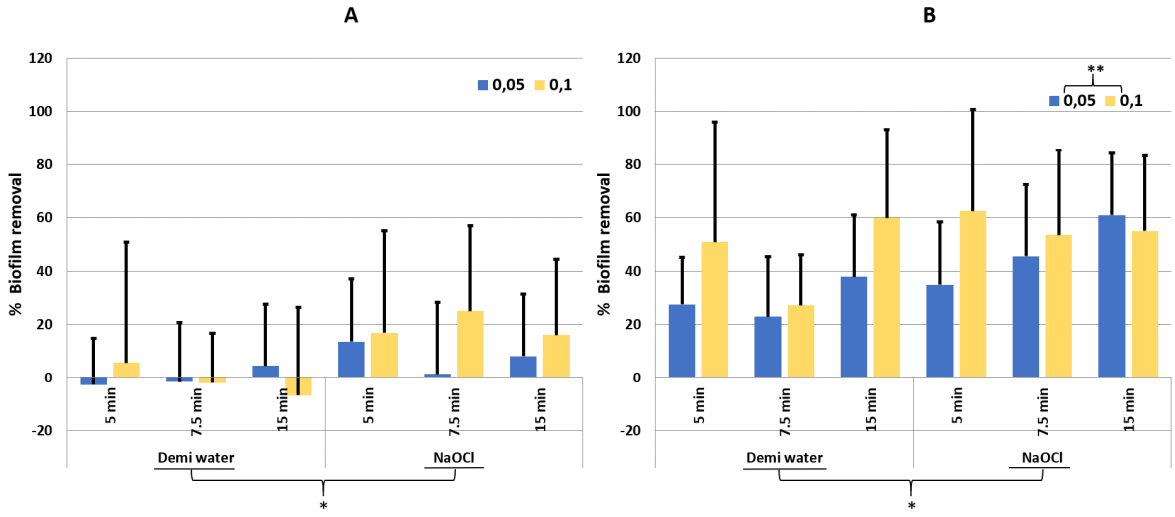


Figure 2 - Chemical effect of an irrigant (demineralized water, 2% NaOCl) after 3 different exposure times (5, 7.5 or 15 min.) applied with either flow rate 0.05 ml/s (dark grey) or 0.1 ml/s (light grey) on biofilm removal (%) from a lateral canal (A) or isthmus (B) like structure. * indicates significant difference between the irrigants, whereas ** indicates a significant difference for the flowrate (ml/s).

Effect of irrigant refreshment on biofilm removal measured after 15 min

Three-way ANOVA yielded no significant interaction among the independent variables on percentage biofilm removal. The main effect analysis revealed no significant effect of refreshment and flow rate on biofilm removal. The main effect of irrigant on biofilm removal was significant (P < 0.001), with 2% NaOCl removing significantly more biofilm than demineralized water irrespective of refreshment and flow rate (Fig. 3).

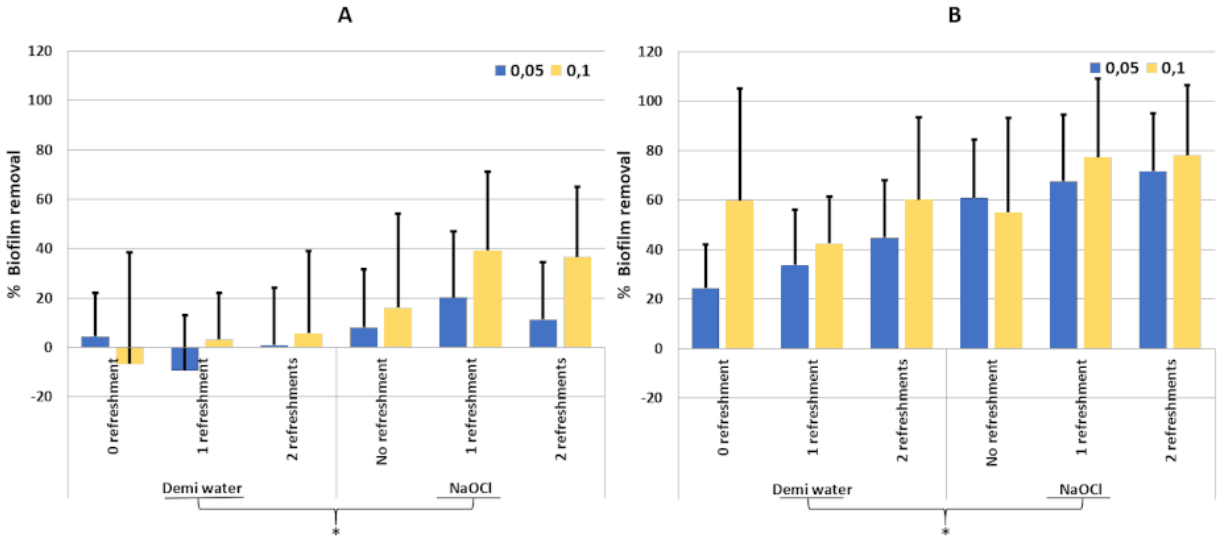


Figure 3 - The influence of the number of refreshments of demineralized water or 2% NaOCl applied with either flow rate 0.05 ml/s (dark grey) or 0.1 ml/s (light grey) on biofilm removal (%) after a total exposure time of 15 minutes from a lateral canal (A) or isthmus (B) like structure. Significant difference between the irrigants is indicated by an *.

Effect of consecutive irrigant refreshment of the same biofilm on biofilm removal

Three-way mixed ANOVA yielded no significant interaction among the independent variables on percentage biofilm removal. The main effect analysis revealed no significant effect of consecutive irrigant refreshment and flow rate on biofilm removal. The main effect of irrigant on biofilm removal was significant ($P = 0.018$), with 2% NaOCl removing significantly more biofilm than demineralized water irrespective of consecutive irrigant refreshment and flow rate (Fig. 4).

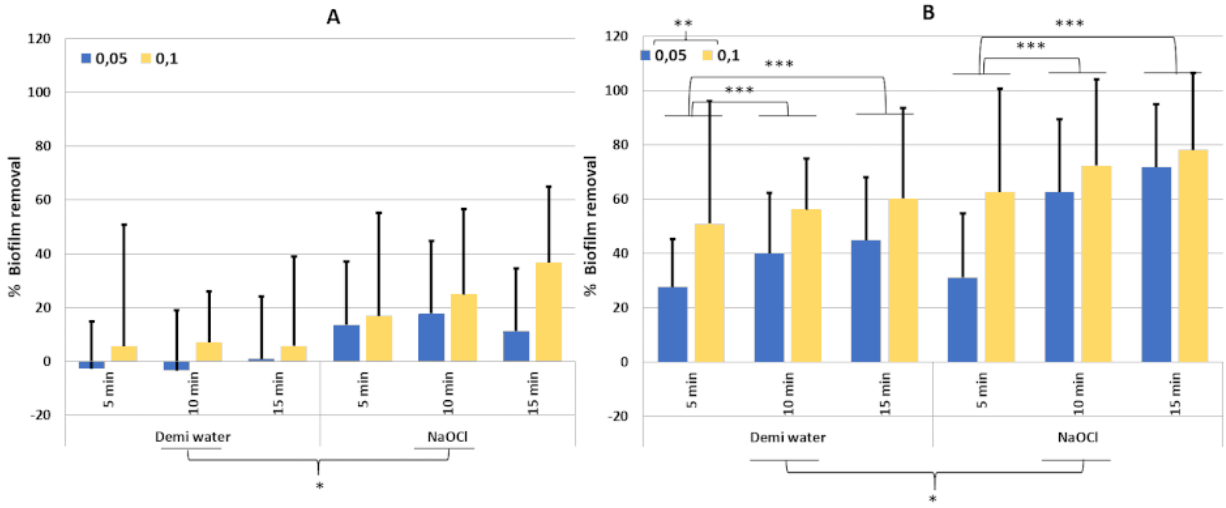


Figure 4 - The influence of consecutive irrigation steps (5, 10 and 15 min) using demineralized water or 2% NaOCl, applied with either flow rate 0,05 ml/s (dark grey) or 0.1 ml/s (light grey) on biofilm removal (%) from a lateral canal (A) or isthmus (B) like structure. Significant difference between the irrigants is indicated by an * whereas ** indicates the significant difference for the flowrates (ml/s). *** shows significant influence between the different irrigation steps.

Isthmus

Chemical effect of the irrigant

Three-way ANOVA yielded no significant interaction among the independent variables on percentage of biofilm removal. The main effect analysis revealed no significant effect of exposure time on biofilm removal. The main effects of irrigant and flow rate on biofilm removal were significant (P = 0.018 and P = 0.029, respectively), with 2% NaOCl and 0.1 mL/s removing significantly more biofilm irrespective of the exposure time (Fig. 2).

Effect of irrigant refreshment on biofilm removal measured after 15 min

Three-way ANOVA yielded no significant interaction among the independent variables on percentage biofilm removal. The main effect analysis revealed no significant effect of refreshment and flow rate on biofilm removal. The main effects of irrigant on percentage biofilm removal was significant (P < 0.001), with 2% NaOCl removing significantly more biofilm irrespective of refreshment and flow rate (Fig. 3).

Effect of consecutive irrigant refreshment on biofilm removal

Three-way ANOVA yielded no significant interaction among the independent variables on percentage biofilm removal. The main effect analysis revealed no significant effect of flow rate on biofilm removal. The main effects of irrigant, flow rate and consecutive irrigant refreshment on percentage biofilm removal were significant ($P = 0.04$, $P = 0.034$ and $P = 0.003$, $P < 0.001$) with respectively 2% NaOCl, 0.1 mL/s and one or two refreshments removing more biofilm (Fig. 4).

Discussion

In this study, a root canal model with lateral morphological features filled with biofilm was used and the outcome measure was percentage biofilm removal evaluated by optical coherence tomography (OCT). OCT is unique in the sense that it enables a longitudinal evaluation of biofilm without the need for prior staining. This allows for measurements before and after each irrigation step. This implies that each biofilm is its own control. This is important in biofilm research given that biofilm growth is difficult to standardize notwithstanding standardized laboratory procedures (Swimberghe *et al.* 2019). A bacterial dense dual species *in vitro* biofilm (*S. oralis* and *A. naeslundii*) was used due to its strong collateral bonds improving biofilm cohesion and adhesion (He *et al.* 2013, Busanello *et al.* 2018). In an earlier study using this typical model, biofilm removal highly correlated with the irrigant flow pattern and streaming velocities (Pereira *et al.* in progress). Furthermore, the bacteria comprising this biofilm are often found in infected root canals (Chávez de Paz *et al.* 2003), the biofilm adheres to PDMS (Song *et al.* 2015) and its viscoelastic properties resemble close to those of an *in vivo* oral biofilm (He *et al.* 2013). Importantly, the root canal model used in the present study is a closed system. Biofilm removal from a closed system is hampered compared to the removal from an artificial open isthmus between two canals. More importantly, this mimics the clinical circumstances.

The negative values in Fig. 2, 3 and 4, related to the lateral canal like structure, indicate biofilm expansion. The fluid flow produced during syringe irrigation can induce wall shear stress, possibly leading to a mechanical disruption of biofilm causing absorption of energy into the biofilm resulting in volumetric expansion (Busscher *et al*. 2003). Deformation surpassing the yield point could disrupt top layers of biofilm, or its EPS matrix (cohesive failure), or could completely remove the biofilm (adhesive failure). If deformation is still in the plastic range but below the yield point, biofilm is expanded but not removed (Busscher *et al.* 2003). Disruption of the top layers or EPS matrix or expansion of the biofilm eases irrigant penetration in the biofilm

and could therefore enhance the chemical effect of irrigants (He *et al*. 2013). Moreover, a disruption of the biofilm matrix could leave 'footprints' in the post treatment remaining biofilm, which may facilitate or impede adhesion of microorganisms, thereby influencing reorganization of the biofilm (Busscher *et al*. 2003).

Microorganisms in the root canal live in a biofilm state, and are consequently protected by a matrix structure (EPS). Antimicrobials need to diffuse into the EPS matrix to enhance an effect. Therefore, the potential of EPS penetration, disruption and killing of the micro-organisms are all inherently related. Disruption of the top layers of the biofilm or EPS matrix or expansion of the biofilm induced by shear stress facilitate irrigant penetration in the biofilm and could enhance the chemical effect of irrigants (He *et al*. 2013). Furthermore, antimicrobials can alter the mechanical properties of EPS, which may be explained by an effect on the EPS network formation (Körstgens 2001). This alteration can directly influence the removal of the biofilm (Brindle *et al*. 2011) and influence the data.

NaOCl removed significantly more biofilm from the lateral canal and isthmus-like structure than the non-chemical active control. This is in contrast to an earlier study using the same model (Pereira *et al*. in progress). However, in that study, irrigant exposure time was 30 s and the flow was constant. In the present study, the irrigants were applied during 30 or 15 s (flow rate 0.05 or 0.1 mL/s) after which the irrigants were left in the root canal for 5 – 15 min without irrigant flow depending on the refreshment schedule. This indicates that more time than 30 s was needed for a significant biofilm removal to occur through chemical-driven diffusion when the biofilm is dense and the contact surface small, both mimicking the clinical situation. However, no difference in biofilm removal was seen between 5, 7.5 and 15 min, indicating that after 5 min no significant removal occurred.

Refreshment of irrigant had no influence on biofilm removal from both the isthmus and the lateral canal like structures. Apparently, application of fresh NaOCl close to the biofilm did not improve biofilm removal. Possibly because the refreshment was performed after 5 minutes and the results show that 5 minutes were enough for diffusion. This is in line with the conclusion of Macedo *et al*. (2010, 2014) that refreshment will not compensate for the use of a lower concentration of NaOCl. Possibly in our model the amount of free chlorine present in the root canal was enough to sustain diffusion. Furthermore, without refreshment already about 60% of the biofilm had been removed. Most likely the removal of the last part of the biofilm is difficult and requires other mechanisms. De Beer *et al*. (1994), studied the penetration of chlorine through a *Pseudomonas aeruginosa/Klesiella pneumoniae* biofilm and concluded that diffusion into the biofilm is a slow process, chlorine is reduced in the matrix, diffusion rate depends on the

concentration, there is a diffusion-reaction mechanism and large variation due to local differences (highly resistant spots). These highly resistant spots show a higher cell density with subpopulations with a higher reducing capacity per cell. Furthermore, these spots have a higher density of EPS with a higher reducing potential. Combined with a fast regrowth after an antimicrobial treatment, these highly resistant spots are serious threats. In the biofilm used in this actual root canal model these highly resistant spots have been identified and impede removal (Busanello *et al*. 2019).

Mass transfer inside a biofilm occurs by convection and (mainly) diffusion (De Beer *et al*. 1994). The surface-averaged relative effective diffusion coefficient (D_{rs}) decreases from the top of the biofilm toward the bottom. The D_{rs} profiles differ for biofilms of different ages and generally decrease over time. In addition, different biofilms showed similar D_{rs} profiles near the top of the biofilm but different D_{rs} profiles near the bottom of the biofilm (Renslow *et al*. 2010). This bottom layer also determines the attachment to the surface, in our case PDMS, and is normally the most difficult to remove (Derlon *et al*. 2008).

Higher flow rate removed significantly more biofilm only from the isthmus like structure. Earlier studies using the same root canal model also demonstrated that flow rate had a higher impact on biofilm removal from an isthmus like structure than a lateral canal like structure (Macedo *et al*. 2014, Pereira *et al*. in progress a,b). The distribution of irrigant will depend on the anatomy of the root canal (Gulabivala *et al*. 2005). Because the opening of the isthmus is larger than the lateral canal, the former allows a steady jet of irrigant solution, favoring a slow and steady viscous biofilm removal after an initial rapid and unsteady removal (Jiang *et al*. 2010, Verhaagen *et al*. 2014) whereas the latter favors removal by pieces (Macedo *et al.* 2014).

In the group with 2 refreshments, biofilm removal was analyzed after each 5-min time interval. After every step significantly more biofilm was removed. Since no significant difference was seen between exposure time of 5 and 15 min, this difference has to be caused by the flow rate of the irrigant. However, the total amount of biofilm removed after 15 min was not significantly more than the group without irrigant refreshment.

Biofilm research has its difficulties and every methodology has its own drawbacks (Swimberghe *et al.* 2019). Notwithstanding the fact that the biofilms were grown under standardized conditions all isthmus and lateral canal-like structures contained different volumes of biofilm. OCT enables a longitudinal evaluation of biofilm allowing measurements at different time points using the biofilm as its own control. Nonetheless, this variation in volume can cause a relatively big standard deviation.

Only few studies have used a cell rich biofilm with viscoelastic properties of an oral biofilm and a more realistic surface contact irrigant-biofilm. It seems that biofilm removal from the last part of the biofilm is somewhat more difficult than expected from earlier work. This could be related to the resistant spots or the typical location and accessibility. However, it correlates better with clinical outcome of root canal treatment where it seems to be impossible to remove biofilm completely using NaOCl as irrigant. The recalcitrance of the biofilm and the complex root canal anatomy seems to be crucial. Furthermore, in the clinical situation, lateral morphological features in the root canal are probably covered with dentine debris produced during instrumentation of the root canal (Paque *et al*. 2012) further hampering biofilm removal (Arias-Moliz *et al.* 2016).

Conclusions

In this typical root canal model with lateral morphological features, refreshment did not improve biofilm removal suggesting the root canal itself contained a sufficient amount of reactive molecules for biofilm removal. NaOCl removed more biofilm from the lateral canal and isthmus structure and a higher flow rate removed more biofilm from the isthmus like structure. There was always remaining biofilm left after the irrigation procedures.

References

1. Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P (2009) Enterococcus faecalis biofilms eradication by root canal irrigants. *Journal of Endodontics* **35**, 711-4.
2. Arias-Moliz MT, Morago A, Ordinola-Zapata R, Ferrer-Luque CM, Ruiz-Linares M, Baca P (2016) Effects of Dentin Debris on the Antimicrobial Properties of Sodium Hypochlorite and Etidronic Acid. *Journal of Endodontics* **42**, 771-5.
3. Baumgartner JC, Mader CL (1987) A scanning electron microscopic evaluation of four root canal irrigation regimens. *Journal of Endodontics* **13**, 147-57.
4. de Beer D, Srinivasan R, Stewart PS (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology* **60**, 4339–44.
5. Brindle ER, Miller DA, Stewart PS (2011) Hydrodynamic deformation and removal of Staphylococcus epidermidis biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. *Biotechnology and Bioengineering* **108**, 2968– 77.

6. Bryce G, O'Donnell D, Ready D, Ng YL, Pratten J, Gulabivala K (2009) Contemporary root canal irrigants are able to disrupt and eradicate single- and dual-species biofilms. *Journal of Endodontics* **35**, 1243-8.
7. Busanello FH, Petridis X, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019) Chemical biofilm removal capacity of endodontic irrigants as a function of biofilm structure: optical coherence tomography, confocal microscopy and viscoelasticity determination as integrated assessment tools. *International Endodontic Journal* **52**, 461-74.
8. Busscher HJ, Rustema-Abbing M, Bruinsma GM, de Jager M, Gottenbos B, van der Mei HC (2003) Non-contact removal of coadhering and non-coadhering bacterial pairs from pellicle surfaces by sonic brushing and de novo adhesion. *European Journal of Oral Sciences* **111**, 459-64.
9. Chávez de Paz LE, Dahlen G, Molander A, Moller A, Bergenholtz G (2003) Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. *International Endodontic Journal* **36**, 500–8.
10. Chávez de Paz LE, Hamilton IR, Svensater G (2008) Oral bacteria in biofilms exhibit slow reactivation from nutrient deprivation. *Microbiology* **154**, 1927–38.
11. Derlon N, Masse A, Escaudie R, Bernet N, Paul E (2008) Stratification in the cohesion of biofilms grown under various environmental conditions. *Water Research* **42**, 2102-10.
12. Dutner J, Mines P, Anderson, A (2012). Irrigation Trends among American Association of Endodontists Members: A Web-based Survey. *Journal of Endodontics* **38**, 37–40.
13. Gulabivala K, Patel B, Evans G, Ng YL (2005) Effects of mechanical and chemical procedures on root canal surfaces. *Endodontic Topics* **10**, 103–22.
14. He Y, Peterson BW, Jongsma MA, *et al.* (2013) Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms. *PLoS ONE* **8**, e63750.
15. Jiang LM, Verhaagen B, Versluis M, van der Sluis LW (2010) Influence of the oscillation direction of an ultrasonic file on the cleaning efficacy of passive ultrasonic irrigation. *Journal of Endodontics* **36**, 1372-6.
16. Kinniment SL, Wimpenny JW, Adams D, Marsh PD (1996) Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* **142**, 631-8.
17. Korstgens V, Flemming HC, Wingender J, Borchard W (2001) Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. *Journal of Microbiological Methods* **46,**9 –17.

18. Korstgens V, Flemming HC, Wingender J, Borchard W (2001) Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. *Journal of Microbiological Methods* **46**, 9 –17.
19. Macedo RG, Wesselink PR, Zaccheo F, Fanali D, van Der Sluis LWM (2010) Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. *International Endodontic Journal* **43**, 1108-15.
20. Macedo RG, Robinson J, Verhaagen B, Walmsley D, Versluis M, Cooper P, van der Sluis LWM (2014) A novel optical methodology for quantifying the removal of a biofilm-mimicking hydrogel from lateral morphological features of the root canal. *International Endodontic Journal* **47**, 1040-51.
21. McDonnell G, Russell AD (1999) Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Review* **12**, 147-79.
22. Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15** 187–196.
23. Nair PN, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **99,** 231–52.
24. Ohsumi T, Takenaka S, Wakamatsu R *et al*. (2015) Residual structure of Streptococcus mutans biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. *PLoS ONE* **10**, e0116647.
25. Paqué F, Rechenberg DK, Zehnder M (2012) Reduction of hard-tissue debris accumulation during rotary root canal instrumentation by etidronic acid in a sodium hypochlorite irrigant. *Journal of Endodontics* **38**, 692-5.
26. Pereira TC, Boutsioukis C, Dijkstra R, Petridis X, Versluis M, Andrade FB, van der Meer J, Sharma P, der Sluis LWM, So M *in progress Submitted to International Endodontic Journal* Biofilm removal from an artificial isthmus and lateral canal during syringe irrigation at various flow rates: a combined experimental and Computational Fluid Dynamics approach.
27. Pereira TC, Dijkstra R, van der Waal S, Petridis X, van der Meer J, Sharma, van der Sluis LWM, de Andrade FB *in progress Submitted to International Endodontic Journal* Chemical and mechanical influence of root canal irrigation on biofilm removal from lateral morphological features and dentinal tubules.

28. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019a) Chemical efficacy of several NaOCl concentrations on biofilms of different architecture: new insights on NaOCl working mechanisms. *International Endodontic Journal* **52**, 1773-88.
29. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Prashant SK, van der Sluis LWM (2019b) Factors affecting the chemical efficacy of 2% sodium hypochlorite against oral steady-state dual-species biofilms: Exposure time and volume application. *International Endodontic Journal* **52***,* 1182-95.
30. Renslow RS, Majors PD, McLean JS, Fredrickson JK, Ahmed B, Beyenal H (2010) In situ effective diffusion coefficient profiles in live biofilms using pulsed-field gradient nuclear magnetic resonance. *Biotechnology Bioengineering* **106**,928-37.
31. Ricucci D, Siqueira Jr, JF (2010) Fate of the Tissue in Lateral Canals and Apical Ramifications in Response to Pathologic Conditions and Treatment Procedures. *Journal of Endodontics* **36**, 1–15.
32. Rozenbaum RT, van der Mei HC, Woudstra W, de Jong ED, Busscher HJ, Sharma PK. (2019) Role of viscoelasticity in bacterial killing by antimicrobials in differently grown P. aeruginosa biofilms. *Antimicrobial Agents and Chemotherapy* **63**, e01972–18.
33. Sen BH, Safavi KE, Spångberg LS (1999) Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *Journal of Endodontics* **25**, 235-8.
34. Shen Y, Stojicic S, Haapasalo M (2010) Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture. *Journal of Endodontics* **36**, 1820–3.
35. Shen Y, Zhao J, de la Fuente-Núñez C, *et al*. (2016) Experimental and theoretical investigation of multispecies oral biofilm resistance to chlorhexidine treatment. *Scientific Reports* **6**, 27537.
36. Sirtes G, Waltimo T, Schaetzle M, Zehnder M (2005) The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. *Journal of Endodontics* **31**, 669-71.
37. Song F, Koo H, Ren D (2015) Effects of material properties on bacterial adhesion and biofilm formation. *Journal of Dental Research* **94**, 1027-34.
38. Stewart PS, Rayner J, Roe F, Rees WM (2001) Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *Journal of Applied Microbiology* **91**, 525–32.

39. Swimberghe RCD, Coenye T, De Moor RJG, Meire MA (2019) Biofilm model systems for root canal disinfection: a literature review. *International Endodontic Journal* **52**, 604-28.
40. Verhaagen, B., Boutsioukis, C., Sleutel, C. P., Kastrinakis, E., van der Sluis, L. W. M. & Versluis, M. (2014) Irrigant transport into dental microchannels. In : *Microfluidics and Nanofluidics*. **16**, p. 1165-77.
41. van der Waal SV, Scheres N, de Soet JJ, Wesselink PR, Crielaard W (2014) Cytotoxicity, interaction with dentine and efficacy on multispecies biofilms of a modified salt solution intended for endodontic disinfection in a new in vitro biofilm model. *International Endodontic Journal* **42**, 153–161.
42. van der Waal SV, de Almeida J, Krom BP, de Soet JJ, Crielaard W (2017) Diffusion of antimicrobials in multispecies biofilms evaluated in a new biofilm model. *International Endodontic Journal* **50**, 367-76.
43. Zehnder M (2006) Root canal irrigants. *Journal of Endodontics* **32**, 389-98.

3 DISCUSSION

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The complex root canal anatomy combined with a microbial invasion resulting in biofilm formation in the root canal system hinder disinfection during endodontic treatment (Peters *et al*. 2001; Ricucci *et al*. 2013). Disinfection of the root canal system is being studied as a surrogate for healing of apical periodontitis (Caputa *et al*. 2019). It is known that bacteria play a key role in the initiation and maintenance of apical periodontitis (Chávez de Paz 2007), and until now, no disinfection procedure is able to completely eliminate biofilm and bacteria from the root canal system leading to treatment failure (Siqueira 2001, Nair *et al*. 2005, Gordon *et al*. 2007, Ricucci *et al*. 2013). This calls for studies related to optimization of the root canal disinfection procedures (Zehnder 2006). To do these studies it is important to have *in vitro* models mimicking as closely as possible the clinical situation. However, it is important to notice that we do not know the structure, cohesion and adhesion of the biofilm in the root canal. As is mentioned in the introduction, through studies using molecular techniques we have insight in the type of microorganisms invading the root canal but not on the structure of the biofilm they form. Within this thesis we have worked on both aspects, testing new disinfection procedures and improving *in vitro* research model used for irrigation research.

Disinfection procedures

The new irrigation solution RISA was compared to the golden standard NaOCl

A new antibiofilm irrigation solution, RISA, was tested. This irrigant is developed to diffuse better in areas of the root canal system that are difficult to reach like lateral canals, isthmuses, oval extensions and dentinal tubules. **RISA was not more effective than the gold standard NaOCl in removing biofilm from lateral canal and isthmus like structures**. Furthermore, NaOCl was more effective in killing the bacteria in the dentinal tubules. However, in this study the irrigant was applied for 30 seconds, perhaps RISA needs a longer exposure time to the biofilm for a greater effectiveness.

Introduction of an extra final irrigation with high flow rate to the irrigation protocol

During root canal irrigation, the irrigant used is applied in the root canal with a syringe and a needle. Little information is available on the mechanical effect of the flow rate on irrigant penetration and biofilm removal in areas beyond the main root canal, such as isthmuses and lateral canals. An extra final irrigation of the root canal at a high flow rate with a non-chemical active buffer was introduced. In our research set up this final irrigation (phase 2 in the articles) is always performed after the irrigation procedure in the experimental groups. **Interestingly, the mechanical effect of this final flow with high flow rate always had an additional effect on biofilm removal, indicating the importance of the mechanical effect of high flow rate syringe irrigation.**

Effect of flow rate

The influence of flow rate on the removal of biofilm from lateral morphological features in the root canal was studied. **Increased flow rate was always associated with more biofilm removal from the isthmus.**

Mechanical effect of ultrasonic activation of a non-chemically active solution

When the mechanical effect of ultrasonic activation of a buffer was tested, for biofilm removal from the lateral canal like structure it was the most effective procedure. This correlated with the results for the tubule model where more polysaccharides from the biofilm matrix and non-viable bacteria were removed from the dentinal tubules than the other groups. Furthermore, in the regrown biofilm less viable bacteria were present than in the other groups suggesting a long-term effect on the biofilm.

Effect of refreshment and exposure time on biofilm removal

The influence of exposure time and refreshments of the irrigant solution on biofilm removal from lateral morphological features was described. Moreover, the effect of consecutive refreshments using the same biofilm as reference was analysed. NaOCl and demineralised water (control group) were used as irrigant solutions applying two different flow rates (0.05 and 0.1 mL/s). In all comparisons, for both isthmus and lateral canal like

structures, NaOCl removed significant more biofilm. The flow rate had an influence on biofilm removal on isthmus-like structures when the refreshment and sequential refreshments were analysed. The flow rates used in this study are considered low, which explains its influence only in the structure with greater surface contact area that allows the continuous flow of the irrigant, removing more biofilm (Jiang *et al*. 2010, Verhaagen *et al*. 2014). The effectiveness of NaOCl on biofilm removal from lateral morphological features was described showing different results. When NaOCl was maintained for 5, 7.5, 10 and 15 minutes it removed significantly more biofilm, whereas in 30 seconds it did not show a statistical difference with the control. Thus, it can be concluded that a longer exposure time was necessary to affect the biofilm structure and lead to its removal. After 5 minutes of exposure to NaOCl no significant biofilm removal occurred, also when the irrigant was refreshed close to the biofilm. This finding suggests enough free chlorine is available in the root canal to sustain diffusion into the biofilm for 15 minutes. However, most of the biofilm removal takes place in the first 5 minutes. Probably, refreshment of the irrigant and renewing the concentration of free chlorine, is not enough to remove the remaining biofilm. This could be related to the efficacy of diffusion or the recalcitrance of the biofilm. However, when the same biofilm was evaluated after consecutive refreshments, every 5 minutes significant more biofilm was removed however altogether not significantly more than compared to 15 minutes exposure without refreshment.

Models for *'in vitro'* research

Root canal model with lateral morphological features

Because OCT is a non-invasive scanning technique that exempts the staining procedure, it allows longitudinal evaluation of the biofilm before and after distinct disinfection procedures, which makes it possible to use each specimen as its own control. OCT is introduced as biofilm evaluation technique in the root canal model with lateral morphological features. With this technique, it is possible to evaluate the influence of each disinfection procedure and also whether a combination of procedures can improve biofilm removal. When evaluating root canal irrigation, it is possible to separately evaluate the chemical and mechanical effect of this procedure by the use of chemical agents at low flow

rates and high flow rates with inert solutions, respectively. Thus, a better understanding of irrigation mechanisms and relation with the biofilm is possible.

The CDFF dual-species biofilm model was based on the model of Macedo *et al*. (2014), which evaluated the removal of biofilm-mimicking hydrogel from PolyDiMethylSiloxane (PDMS) lateral canal and isthmus-like structures. However, in the studies presented here, for the first time a real dual-species biofilm was formed inside the structures in order to evaluate biofilm removal. Biofilms are not as homogeneous in their structure as the hydrogel and also have preferential growth in some locations and corners in the lateral canal and the isthmus. The Constant Depth Film Fermentor (CDFF) was used to promote biofilm growth (Kinniment *et al.* 1996, Rozenbaum *et al.* 2017), which allows the formation of a strong biofilm with a dense basal/ground bacterial mass that hampers penetration of antimicrobials in these dense layers. The basal layer of the biofilm is its foundation that will support biofilm growing, and directly influences its structure (Peterson *et al*. 2012). The bacteria used in this biofilm are early colonizers, *Streptococcus oralis* J22, a biofilm initial colonizer and *Actinomyces naeslundii* T14V-J1, an important species for biofilm maturation, adhesion and coadhesion of the biofilm to a substratum (Al-Ahmad *et al*. 2007, Riihinen *et al*. 2010, He *et al*. 2013, Busanello *et al*. 2018). Therefore, adhesion to the PDMS root canal model with lateral morphological features was improved (Song et al. 2015), and a biofilm with a resistant structure was formed. Moreover, since the root canal space presents a very limited space for irrigant contact with the biofilm, the PDMS model used presents great similarity with the real clinical situation. This results in very limited space for irrigation, partly the reason for the problems removing biofilm from the root canal system. Besides, it is a distal-closed model that makes biofilm irrigation more difficult, by preventing the continuous flow of the irrigating solution that would increase debridement.

Biofilm removal was correlated with the fluid flow of the irrigant. For this, a Computational Fluid Dynamics (CFD) model was used to visualise and determine flow velocity and related shear stress in the isthmus and lateral canal-like structures. Originally, CFD was created for industrial and engineering purposes as a method to observe flow patterns and physical and chemical phenomena by mathematical modelling and computer simulation (Tilton 1999, Arvand *et al*. 2005, Boutsioukis *et al*. 2010). Boutsioukis *et al*. (2010) adapted this model to evaluate the velocity of the irrigant inside the root canals. When three different flow rates were used, irrigant velocity was measured in all internal areas of the structures by CFD and then, compared with the average of the removed biofilm showing substantial

correlation between these two parameters. Besides, the areas closer to the needle tip presented higher streaming velocity and also greater biofilm removal. **Thus, we concluded that higher irrigant velocities promote more biofilm removal.** Moreover, it was found that irrigant velocity increases with the increase of flow rate during irrigation. NaOCl was used as root canal irrigant at three concentrations, 2, 5 and 10%. No significant differences were seen between the different concentrations but flow rate of the irrigant significantly influenced biofilm removal. The model seemed to be suitable to detect differences between irrigation protocols.

Dentinal tubule model

The intratubular contamination model showed *E. faecalis* (ATCC 29212) ability to deeply penetrate the dentinal tubules. With this model, different endodontic disinfection procedures have been analysed, evaluating the antimicrobial effectiveness and Extracellular Polymeric Substances (EPS) removal, consequently showing how far the antimicrobial agents can demonstrate an effect on bacteria in areas not touched by instrumentation. What we see is the concentration that diffused in the dentinal tubules which is high enough to result in an antibacterial effect.

We described the dentinal tubule model with some modifications, such as a standardized flow rate during root canal irrigation, the EPS removal analysis and the human teeth as specimens instead of bovine teeth. A recent systematic review compared ultrasonic activated irrigation (US) with conventional syringe irrigation and showed the importance of standardizing irrigation protocols, such as flow rate, during research on root canal irrigation. The authors concluded that the absence of standardization in the research protocols could lead to not realistic outcomesfor the conventional syringe irrigation when compared to US (Caputa *et al*. 2019). The second modification is important because the EPS is responsible to provide the biofilm its structure, mechanical strength and protection against antimicrobial agents (De Beer *et al*. 1994; Flemming & Wingender 2010). Thus, the evaluation of EPS is a valuable tool when studying disinfection. The use of human teeth is a really important change because it approaches the clinical situation. Human teeth have smaller root canals and smaller dentinal tubules than bovine teeth. Thus, bacteria penetration is more difficult in smaller dentinal tubules, but in the same way it also hampers penetration of the antimicrobial agents. By the results obtained NaOCl showed significant less viable bacteria than the other tested irrigation

solutions, even with different exposure times. These results show the consistence of both models, but in order to make a more clinical realistic *in vitro* study, human teeth are preferred.

Bacteria viability and EPS removal was assessed in this model by CLSM which is a widely used technique when studying disinfection in endodontics. Despite of the valuable information provided by CLSM analysis, this technique has some disadvantages such as the fact that only a very small part of the biofilm is scanned. The penetration of the staining is around 60μm, which limits the area to be analysed. Besides, interpretation of the LIVE/DEAD staining is difficult because it is performed with the use of SYTO 9 (green staining) which stains all bacteria and Iodide Propidium (PI - red staining) which stains bacteria with damaged membrane. Then, the interpretation between the red and green channels could be biased and incomplete replacement of SYTO9 by PI in non-viable cells could lead to higher numbers of supposedly present viable cells. Moreover, staining of the biofilm matrix is difficult because no universal stain colours all the matrix components and, a 'black' area could be a non-stained area of the biofilm or the absence of a biofilm (Flemming & Wingerder 2010, Azeredo *et al*. 2016, Stiefel *et al*. 2016).

Combination of the two models

Because of CLSM limitations and its non-conservative character of analysis, the root canal and the dentine tubule models were correlated using the same experimental groups and protocols. In the root canal model with lateral morphological features, OCT was used. Therefore, the direct effect of an irrigation procedure on the biofilm structure could be measured. However, no information on biofilm viability was possible. Thus, a combination of OCT and CLSM can provide additional information about the effect of tested irrigation protocols on bacteria. Moreover, it is known that oral bacteria have the ability to enter in a non-growing state as a mechanism to survive to starved micro-environments (Chávez de Paz, 2007). This fact establishes the importance in evaluating not only the bacteria viability inside the biofilm, but mainly its removal, since remaining bacterial components can also induce or maintain the apical periodontitis (Jacinto *et al*. 2005). Thus, this work shows the importance in using complementary methods of evaluation in research. For root canal disinfection, the use of these two methods allowed to see not only the bacterial killing but also the influence of the tested irrigating protocols on biofilm removal in the total area.

Post-Treatment Remaining Biofilm

As stated earlier, the complex root canal anatomy in combination with the recalcitrance of the biofilm makes disinfection of the root canal system difficult. *In situ* investigations of root canal specimens (Nair *et al*. 2005, Ricucci & Siqueira 2010) have clearly confirmed the presence of post- treatment remaining biofilm. Taking into account that post-treatment remaining biofilm can re-grow (Chávez de Paz *et al.* 2008, Shen *et al.* 2010, Ohsumi *et al.* 2015, Shen *et al.* 2016), failure of apical periodontitis is a challenge to resolve. Thus, post-treatment remaining biofilm will always remain in the root canal and we demonstrated that remaining biofilm can reorganise itself without nutrition. However, disruption of the top layers or EPS matrix or expansion of the biofilm induced by shear stress on the biofilm during irrigation procedures facilitates irrigant penetration in the biofilm and could therefore enhance the chemical effect of irrigants (He *et al*. 2013, Busanello *et al*. 2019, Petridis *et al*. 2019). NaOCl has a different effect on the 'fluffy' organised top layer of the biofilm compared to the cell rich basal or ground layer of the biofilm which is more cell rich (Busanello et al. 2019). The former is relatively easy removed by NaOCl, the latter is more difficult to remove and can stabilise after contact with NaOCl (Busanello et al. 2019). Chemical agents can alter the mechanical properties of the EPS, which may be explained by an influence of these agents on the EPS network formation or structure of the post treatment remaining biofilm (Körstgens 2003, Busanello *et al*. 2019). This alteration can directly influence the removal of the biofilm (Brindle *et al*. 2011, Busanello *et al*. 2019).

Therefore, it is important to study the structure and viability of the post-treatment remaining biofilm. Based on the outcomes of the dentine model described here, it seems that the chemical effect of NaOCl and RISA has a stabilizing effect on the post-treatment remaining biofilm in contrast to the buffer. Thus, using chemically active solutions during root canal treatment could hamper the disinfection procedure if we do not exactly know what happens with the post-treatment remaining biofilm.

Future perspectives

NaOCl is still the best irrigant to use although we do not exactly know its effect on the post treatment remaining biofilm. In our studies, no significant difference between the concentrations in the range of 2 till 10% was seen and refreshment of the irrigant did not

result in more biofilm removal. Probably, the small dimensions of the lateral morphological features in the root canal influenced the results. Probably, the amount of free chlorine in the main canal as reservoir was enough for an effective diffusion. Our *'in vitro'* research model was made from PDMS which does not react with NaOCl in contrast to dentine. However, when used in a dentine model still the initial concentration of the NaOCl solution is more important for the reaction rate than refreshment of the irrigant solution (Macedo *et al*. 2010, 2013).

Syringe irrigation with a high flow rate, also with a not chemically active irrigant, seems to be effective in biofilm removal when the surface contact is somewhat bigger like for isthmus like structures. In contrast for smaller contact surfaces biofilm-irrigant, ultrasonic activation of an irrigant seems to have additional effect. Furthermore, NaOCl is still the irrigant of first choice however we do not know enough of its effect on the post-treatment remaining biofilm. Perhaps our treatment strategies should focus more on the effect on the post-treatment remaining biofilm. Knowing that the first chemical contact of a chemical solution with biofilm already alters the biofilm could indicate that we should postpone the chemical attack during root canal treatment.

We started the general discussion with the remark that research on disinfection of the root canal is a surrogate outcome for healing of apical periodontitis. The research projects presented in this thesis confirm that biofilm removal is difficult. Furthermore, our knowledge of the effect of our treatment protocols on biofilm is still limited. In addition, research is difficult because we do not know the structure of the biofilm residing in the root canals and clinical research is complicated and expensive. Therefore, step-by-step, we learn more and we can try to improve the healing of apical periodontitis.

4 CONCLUSIONS

4 CONCLUSIONS

The irrigant flow rate had more influence on biofilm removal than the irrigant concentration.

The irrigant velocity influenced biofilm removal since in areas with higher velocities more biofilm was removed. A high flow rate was also related with higher irrigant velocity inside the root canal model with lateral morphological features.

The mechanical effect of syringe irrigation showed to be a relevant factor to be observed when studying biofilm removal. Ultrasonic activation of the irrigant showed to be effective in the lateral canal structures and dentine tubules where the contact surface biofilm-irrigant was smaller. Biofilm was able to regrow inside the dentine tubules in a five days period without any extra nutrition.

Sodium hypochlorite and the higher flow rate presented greater biofilm removal from the isthmus like structures, whereas for the lateral canal the flow rate had no influence.

REFERENCES

REFERENCES

1. 1. AAE (2012) Glossary of endodontics terms, 8th edn. American Association of Endodontists, Chicago.

2. Adcock JM, Sidow SJ, Looney SW et al (2011) Histologic evaluation of canal and isthmus debridement efficacies of two different irrigant delivery techniques in a closed system. *Journal of Endodontics* **37**, 544–8.

3. Afkhami F, Akbari S, Chiniforush N. (2017). Entrococcus faecalis Elimination in Root Canals Using Silver Nanoparticles, Photodynamic Therapy, Diode Laser, or Laser-activated Nanoparticles: An In Vitro Study. *Journal of Endodontics* **43**, 279–282.

4. Afkhami F, Pourhashemi SJ, Sadegh M, Salehi Y, Fard MJ (2015) Antibiofilm efficacy of silver nanoparticles as a vehicle for calcium hydroxide medicament against Enterococcus faecalis. *Journal of Dentistry* **43**, 1573–9.

6. Al-Ahmad A, Wunder A, Auschill TM, Follo M, Braun G, Hellwig E, Arweiler NB. (2007) The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization. *Journal of Medical Microbiology* **56**, 681–7.

7. Al-Jadaa A, Paque F, Attin T, Zehnder M (2009) Necrotic pulp tissue dissolution by passive ultrasonic irrigation in simulated accessory canals: impact of canal location and angulation. *International Endodontic Journal* **42**, 59–65.

8. Baker RW (1947) Studies on the reaction between sodium hypochlorite and proteins: 1. Physicochemical study of the course of the reaction. *The Biochemical Journal* **41**, 337–42.

9. Arvand A, Hormes M, Reul H (2005) A validated computational fluid dynamics model to estimate hemolysis in a rotary blood pump. *Artificial Organs* **29**, 531–40.

10. Baumgartner JC, Cuenin PR (1992) Efficacy of several concentrations of sodium hypochlorite for root canal irrigation. *Journal of Endodontics* **18**,605-12.

11. Baumgartner JC, Siqueira J Jr, Sedgley C, Kishen A (2008) Microbiology of endodontic disease. In: Ingle JI, Bakland LK, Baumgartner JC (eds) Ingle's endodontics, BC Decker, Ontario.

11. Bhardwaj SB, Mehta M, Gauba K (2009) Nanotechnology: role in dental biofilms. *Indian Journal of Dentistry Research* **20**, 511–3.

12. Boutsioukis C, Lambrianidis T, Kastrinakis E, Bekiaroglou P (2007) Measurement of pressure and flow rates during irrigation of a root canal ex vivo with three endodontic needles. *International Endodontic Journal* **40**, 504-13.

13. Boutsioukis C, Lambrianidis T, Kastrinakis E (2009) Irrigant flow within a prepared root canal using different flow rates: a Computational Fluid Dynamics study. *International Endodontic Journal* **42**, 144–55.

14. Boutsioukis C, Verhaagen B, Versluis M, Kastrinakis E, van der Sluis LW (2010a) Irrigant flow in the root canal: experimental validation of an unsteady Computational Fluid Dynamics model using high-speed imaging. *International Endodontic Journal* **43**, 393-403.

15. Boutsioukis C, Verhaagen B, Versluis M, Kastrinakis E, Wesselink PR, van der Sluis LWM (2010b) Evaluation of irrigant flow in the root canal using different needle types by an unsteady computational fluid dynamics model. *Journal of Endodontics* **36,** 87–9.

16. Boutsioukis C, Verhaagen B, Kastrinakis E, Versluis M, van der Sluis LWM (2010c) The effect of needle penetration depth on the irrigant flow in the root canal: evaluation by an unsteady Computational Fluid Dynamics model. *Internationl Endodontic Journal* **36,** 1664–8.

17. Brennen CE (1995) Cavitation and bubble dynamics. Oxford University Press, New York (NY), USA. 1st ed.

18. Brindle ER, Miller DA, Stewart PS (2011) Hydrodynamic deformation and removal of Staphylococcus epidermidis biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. *Biotechnology and Bioengineering* **108**, 2968– 77.

19. Bryce G, O'Donnell D, Ready D, Ng YL, Pratten J, Gulabivala K (2009) Contemporary root canal irrigants are able to disrupt and eradicate single- and dual-species biofilms. *Journal of Endodontics* **35**, 1243–8.

20. Burleson A, Nusstein J, Reader A, Beck M (2007) The in vivo evaluation of hand/rotary/ ultrasound instrumentation in necrotic, human mandibular molars. *Journal of Endodontics* **33**, 782–7.

21. Busanello FH, Petridis X, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019) Chemical biofilm removal capacity of endodontic irrigants as a function of biofilm structure: optical coherence tomography, confocal microscopy and

viscoelasticity determination as integrated assessment tools. *International Endodontic Journal* **52**, 461-474.

22. Bystrom A, Claesson R, Sundqvist G. (1985) The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Dental Traumatology* **1**, 170–5.

23. Bystrom A, Happonen RP, Sjogren U, Sundqvist G (1987) Healing of periapical lesions of pulpless teeth after endodontic treatment with controlled asepsis. *Endodontics & Dental Traumatology* **3**, 58-63.

24. Byström A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scandinavian Journal of Dental Research* **89**, 321– 8.

25. Căpută PE, Retsas A, Kuijk L, Chávez de Paz LE, Boutsioukis C. Ultrasonic Irrigant Activation during Root Canal Treatment: A Systematic Review. J Endod.2019 Jan;45(1):31-44.e13.

26. Castelo-Baz P, Martin-Biedma B, Cantatore G, Varela-Patino P (2012) In vitro comparison of passive and continuous ultrasonic irrigation in simulated lateral canals of extracted teeth. *Journal of Endodontics* **38**, 688–91.

27. Chavez de Paz LE (2007) Redefining the persistent infection in root canals: possible role of biofilm communities. *Journal of Endodontics* **33**, 1289.

28. Chávez de Paz LE, Hamilton IR, Svensater G (2008) Oral bacteria in biofilms exhibit slow reactivation from nutrient deprivation. *Microbiology* **154**, 1927–38.

29. Chávez de Paz LE & Marsh PD (2015) Ecology and Physiology of Root Canal Microbial Biofilm Communities. In: Chávez de Paz LE, Sedgley CM, Kishen A, The Root Canal Biofilm, 9 edn: pp 3-22. Berlin, German: Springer.

30. Dammaschke T, Witt M, Ott K, Schafer E (2004) Scanning electron microscopic investigation of incidence, location, and size of accessory foramina in primary and permanent molars. *Quintessence International* **35**, 699–705.

31. De Beer D, Srinivasan R, Stewart PS (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Applied Environmental Microbiology* **60**, 4339–44.

32. Degerness RA, Bowles WR (2010) Dimension, Anatomy and Morphology of the Mesiobuccal Root Canal System in Maxillary Molars. *Journal of Endodontics* **36,** 985–9.

33. Donnermeyer D, Wyrsch H, Bürklein S, Schäfer E (2019) Removal of Calcium Hydroxide from Artificial Grooves in Straight Root Canals: Sonic Activation Using EDDY Versus Passive Ultrasonic Irrigation and XPendo Finisher. *Journal of Endodontics* **45**, 322-326.

34. Druttman ACS, Stock CJR (1989) An in vitro comparison of ultrasonic and conventional methods of irrigant replacement. *International Endodontic Journal* **22**, 174–8.

35. Dutner J, Mines P, Anderson A (2012) Irrigation Trends among American Association of Endodontists Members: A Web-based Survey. *Journal of Endodontics* **38**, 37–40.

36. European Society of Endodontology (ESE) developed by: Duncan HF, Galler KM,Tomson PL, Simon S, El-Karim I, Kundzina R, Krastl G, Dammaschke T, Fransson H, Markvart M, Zehnder M, Bjørndal L (2019) European Society of Endodontology position statement: Management of deep caries and the exposed pulp. *International Endodontic Journal* **52**, 923-34.

37. Ferrari PHP & Cai S (2010) O que o endodontista precisa saber sobre microbiologia. In: Ferrari PHP & Bombana AC, ed. A infecção endodôntica e sua resolução. 1 edn; pp 1-33. São Paulo, Brazil: *Santos*.

38. Ferreira FB, Torres SA, Rosa OP, Ferreira CM, Garcia RB, Marcucci MC, Gomes BP (2007) Antimicrobial effect of propolis and other substances against selected endodontic pathogens. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics.* **104**, 709-16.

39. Figdor D, Sundqvist G (2007) A big role for the very small--understanding the endodontic microbial flora. *Australian Dental Journal* **52**, S38-51.

40. Filho, P. N., Silva, L. A. B., Leonardo, M. R., Utrilla, L. S., & Figueiredo, F. 1999). Connective tissue responses to calcium hydroxide-based root canal medicaments. *International Endodontic Journal* **32**, 303–311.

41. Flemming HC, Wingender J. 2010. The biofilm matrix. *Nature Reviews Microbiology* **8,** 623–33.

42. Gahyva SM, Siqueira JF Jr (2005) Direct genotoxicity and mutagenicity of endodontic substances and materials as evaluated by two prokaryotic test systems. *Journal of Applied Oral Sciences* **13,** 387–92.

43. Gordon W, Atabakhsh VA, Meza F, Doms A, Nissan R, Rizoiu I, Stevens RH (2007) The antimicrobial efficacy of the erbium, chromium: yttrium-scandium-gallium-garnet laser with radial emitting tips on root canal dentin walls infected with Enterococcus faecalis. *The Journal of the American Dental Association* **138**, 992–1002.

44. de Gregorio C, Estevez R, Cisneros R, Heilborn C, Cohenca N (2009) Effect of EDTA, sonic, and ultrasonic activation on the penetration of sodium hypochlorite into simulated lateral canals: an in vitro study. *Journal of Endodontics* **35**, 891–5.

45. Guelon T, Mathias J-D, Stoodley P (2011) 'Advances in Biofilm Mechanics', In: Biofilm highlights, Flemming, H-C, Wingender, J and Szewzyk, U, eds. Springer. 1st ed.

46. Gulabivala K, Aung TH, Alavi A, Ng YL (2001) Root and canal morphology of Burmese mandibular molars. *Internationl Endodontics Journal* **34**, 359-70.

47. Gulabivala K, Patel B, Evans G, Ng YL (2005) Effects of mechanical and chemical procedures on root canal surfaces. Endodontic Topics **10**, 103–22.

48. Gulabivala K, Ng YL, Gilbertson M, Eames I (2010) The fluid mechanics of root canal irrigation. *Physiological Measurement* **31**, R49-84.

49. Gulabivala K & Ng YL (2015) Biofilm-Associated Infections in Root Canals: Treatment and Outcomes. In: Chávez de Paz LE, Sedgley CM, Kishen A, The Root Canal Biofilm, 9 edn: pp 108-258. Berlin, German: Springer.

50. Haapasalo M, Shen Y, Qian W, Gao Y (2010) Irrigation in endodontics. *Dental Clinics of North America* **54**, 291-312.

51. Hargreaves KM, Cohen S (2011) Cohen's pathways of the pulp, 10th edn. Mosby, St. Louis.

52. He Y, Peterson BW, Jongsma MA, Ren Y, Sharma PK, Busscher HJ, van der Mei HC (2013) Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms. *PLoS One 27* **8**, e63750.

53. Hong BY, Lee TK, Lim SM et al. (2013) Microbial analysis in primary and persistent endodontic infections by using pyrosequencing. *Journal of Endodontics* **39**, 1136-40.

54. Hülsmann M, Hahn W (2000) Complications during root canal irrigation—literature review and case reports. *International Endodontic Journal* **33**, 186-93.

55. Hsu YY, Kim S (1997) The resected root surface. The issue of canal isthmuses. *Dental Clinics of North America* **41**, 529–40.

56. Huse SM, Ye Y, Zhou Y, Fodor AA (2012) A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS ONE 7, e34242.

57. Jacinto RC, Gomes BP, Shah HN, Ferraz CC, Zaia AA, Souza-Filho FJ (2005) Quantification of endotoxins in necrotic root canals from symptomatic and asymptomatic teeth. *Journal of Medical Microbiology* **54**, 777-83.

58. Javidi M, Afkhami F, Zarei M, Ghazvini K, Rajabi O (2014) Efficacy of a combined nanoparticulate/calcium hydroxide root canal medication on elimination of Enterococcus faecalis. *Australian Endodontic Journal* **40**, 61–5.

59. Jiang LM, Verhaagen B, Versluis M, van der Sluis LW (2010) Influence of the oscillation direction of an ultrasonic file on the cleaning efficacy of passive ultrasonic irrigation. *Journal of Endodontics* **36**, 1372-6.

60. Kaiser HJ (1964) Management of wide open canals with calcium hydroxide. Read before the American Association of Endodontists, Washington, DC, April 17.

61. Kakehashi S, Stanley HR, Fitzgerald W (1965) The effects of surgical exposures of dental pulps in germ free and conventional laboratory rats. *Oral Surgery Oral Medicine Oral Pathology* **20**, 340-8.

62. Keijser BJ, Zaura E, Huse SM, van der Vossen JM, Schuren FH, Montijn RC, ten Cate JM, Crielaard W (2008) Pyrosequencing analysis of the oral microflora of healthy adults. *Journal of Dental Research* **87**,1016–20.

63. Keles A, Ahmetoglu F, Uzun I (2014) Quality of different gutta-percha techniques when filling experimental internal resorptive cavities: a micro-computed tomography study. *Australian Endodontic Journal* **40**, 131–5.

64. Kinniment SL, Wimpenny JW, Adams D, Marsh PD (1996) Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* **142**, 631-8.

65. Kishen A & Haapasalo M (2015) Laboratory Models of Biofilms: Development and Assessment. In: Chávez de Paz LE, Sedgley CM, Kishen A, The Root Canal Biofilm, 9 edn: pp 3-22. Berlin, German: Springer.

66. Korstgens V, Flemming HC, Wingender J, Borchard W (2001) Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. *Journal of Microbiological Methods* **46,** 9 –17.

67. Langeland K (1987) Tissue response to dental caries. *Endodontic Dental Traumatology* **3**, 149-71.

68. Leonardo MR, Bonetti Filho I, Silva RS, Silva LAB (1993) Penetrabilidade do `curativo de demora' no sistema de canal radicular. Avaliação de diferentes produtos. *Revista Gaúcha de Odontologia* **41**, 199-203.

69. Love RM (2001) Enterococcus faecalis--a mechanism for its role in endodontic failure. *International Endodontic Journal* **34**, 399-405.

70. *Łysako*wska ME, Ciebiada-Adamiec A, Sienkiewicz M, Sokołowski J, Banaszek K. (2016) The cultivable microbiota of primary and secondary infected root canals, their susceptibility to antibiotics and association with the signs and symptoms of infection. *International Endodontic Journal* **49**, 422–30.

71. Macedo RG, Robinson JP, Verhaagen B, Walmsley AD, Versluis M, Cooper PR, van der Sluis LW (2014) A novel methodology providing insights into removal of biofilm-mimicking hydrogel from lateral morphological features of the root canal during irrigation procedures. *International Endodontic Journal* **47,** 1040-51.

72. Macedo RG, Wesselink PR, Zaccheo F, Fanali D, Van Der Sluis LW (2010) Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. *International Endodontic Journal* **43**, 1108-15.

73. Martinho FC, Gomes CC, Nascimento GG, Gomes APM, Leite FRM (2017) Clinical comparison of the effectiveness of 7- and 14-day intracanal medications in root canal disinfection and inflammatory cytokines. *Clinical Oral Investigations* **22**, 523–30.

74. Marques-da-Silva, B., Alberton, C. S., Tomazinho, F. S. F., Gabardo, M. C. L., Duarte, M. A. H., Vivan, R. R., & Baratto-Filho, F. (2019). Effectiveness of five instruments in removing calcium hydroxide paste from simulated internal root resorption cavities in extracted maxillary central incisors. *International Endodontic Journal*.

75. Mohammadi, Z., & Dummer, P. M. H. (2011). Properties and applications of calcium hydroxide in endodontics and dental traumatology. *International Endodontic Journal*, **44**, 697–730.

76. Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 17-52.

77. Molven O, Olsen I, Kerekes K (1991) Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. *Endodontic Dental Traumatology* **7**, 226-29.

78. Monteiro DR, Gorup LF, Takamiya AS, Ruvollo-Filho AC, de Camargo ER, Barbosa DB (2009) The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. *International Journal of Antimicrobials Agents* **34**, 103–10.

79. Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15**,187-96.

80. Mott RL (1999) Applied fluid mechanics, 5th edn. New Jersey, USA: Prentice Hall 1–188:221–38.

81. Naenni N, Thoma K, Zehnder M (2004) Soft tissue dissolution capacity of currently used and potential endodontic irrigants. *Journal of Endodontics* **30**, 785-7.

82. Nair PNR (1987) Light and electron microscopic studies of root canal flora and periapical lesions. *Journal of Endodontics* **13,** 29-39.

83. Nair PN, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **99**, 231–52.

84. Nyvad B, Crielaard W, Mira A, Takahashi N, Beighton D (2013) Dental caries from a molecular microbiological perspective. *Caries Research* **47**, 89– 102.

85. Ohsumi T, Takenaka S, Wakamatsu R, et al. (2015) Residual structure of Streptococcus mutans biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. *PLoS ONE* **10**, e0116647.

86. Ohl C-D, Wolfum B (2003) Detachment and sonoporation of adherent HeLa-cells by shock waveinduced cavitation. *Biochimica et Biophysica Acta* **1624**,131–8.

87. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MS, Gomes de Moraes I, Marciano M, Duarte MA (2012) Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. *International Endodontic Journal* **45**, 162–8.

88. Pak JG, Fayazi S, White SN (2012) Prevalence of periapical radiolucency and root canal treatment: a systematic review of cross-sectional studies. *Journal of Endodontics* **38**, 1170-6.

89. Pereira TC, da Silva Munhoz Vasconcelos LR, Graeff MSZ, Ribeiro MCM, Duarte MAH, de Andrade FB (2019) Intratubular decontamination ability and physicochemical properties of calcium hydroxide pastes. *Clinical Oral Investigations* **23**, 1253-62.

90. Peters O, Schönenberger K, Laib A (2001) Effects of four Ni–Ti preparation techniques on root canal geometry assessed by micro computed tomography. *International Endodontic Journal* **34**, 221-230.

91. Peterson BW, Busscher HJ, Sharma PK, van der Mei HC (2012) Environmental and centrifugal factors influencing the visco-elastic properties of oral biofilms in vitro. *Biofouling* **28**, 913-20.

92. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019a) Factors affecting the chemical efficacy of 2% sodium hypochlorite against oral steady-state dual-species biofilms: Exposure time and volume application. Int Endod J. 52(8):1182-1195.

93. Petridis X, Busanello FH, So MVR, Dijkstra RJB, Sharma PK, van der Sluis LWM (2019b) Chemical efficacy of several NaOCl concentrations on biofilms of different architecture: new insights on NaOCl working mechanisms. Int Endod J 52(12):1773-1788.

94. Pitts NB, Zero DT, Marsh PD *et al*. (2017) Dental caries. *Nature Reviews Disease Primers* **25**, 17030.

95. Pratten J, Barnett P, Wilson M (1998) Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. *Applied and Environmental Microbiology* **64**, 3515– 9.

96. Rasmussen K, Reilly C, Li Y, Jones RS (2016) Real-time imaging of anti-biofilm effects using CP-OCT. *Biotechnology and Bioengineering* **113**, 198–205.

97. Ricucci D, Siqueira JF Jr (2010a) Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *Journal of Endodontics* **36**, 1277-88.

98. Ricucci D, Loghin S, Siqueira Jr JF (2013) Exuberant biofilm infection in a lateral canal as the cause of short-term endodontic treatment failure: report of a case. *Journal of Endodontics* **39**, 712-8.

99. Ricucci D, Langeland K (1997) Incomplete calcium hydroxide removal from the root canal: a case report. *International Endodontic Journal* **30**, 418–21.

100. Ricucci D, Loghin S, Siqueira JF (2014) Correlation between Clinical and Histologic Pulp Diagnoses. *Journal of Endodontics* **40**, 1932–39.

101. Ricucci D, Siqueira JF Jr (2008) Apical actinomycosis as a continuum of intraradicular and extraradicular infection: case report and critical review on its involvement with treatment failure. J Endod 34:1124–9.

102. Ricucci, D., & Siqueira Jr., J. F. (2010b) Fate of the Tissue in Lateral Canals and Apical Ramifications in Response to Pathologic Conditions and Treatment Procedures. Journal of Endodontics, 36(1), 1–15.

103. Riihinen K, Ryynänen A, Toivanen M, Könönen E, Törrönen R, Tikkanen-Kaukanen C (2010) Antiaggregation potential of berry fractions against pairs of Streptococcus mutans with Fusobacterium nucleatum or Actinomyces naeslundii. *Phytotherapy Research* **25,** 8–87.

104. Robinson JP, Macedo RG, Verhaagen B, Versluis M, Cooper PR, van der Sluis LWM,Walmsley AD (2018) Cleaning lateral morphological features of the root canal: the role of streaming and cavitation. International Endodontic Journal *51 Suppl 1*:e55-e64.

105. Rozenbaum RT, van der Mei HC, Woudstra W, de Jong ED, Busscher HJ, Sharma PK (2019) Role of Viscoelasticity in Bacterial Killing by Antimicrobials in Differently Grown Pseudomonas aeruginosa Biofilms. *Antimicrobial Agents and Chemotherapy* **63(4)**, e01972-18.

106. Sánchez-Sanhueza G, Bello-Toledo H, González-Rocha G, Gonçalves AT, Valenzuela V, Gallardo-Escárate C (2018) Metagenomic study of bacterial microbiota in persistent endodontic infections using Next-generation sequencing. *International Endodontic Journal* **51,** 1336-48.

107. Shen Y, Gao Y, James L, Wang Z, Haapasalo M (2012) Methods and models to study irrigation. *Endodontic Topics* **27**, 3–34.

108. Shen Y, Stojicic S, Haapasalo M (2010) Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture. *Journal of Endodontics* **36**, 1820–3.

109. Shen Y, Zhao J, de la Fuente-N u~nez C, et al. (2016) Experimental and theoretical investigation of multispecies oral biofilm resistance to chlorhexidine treatment. *Scientific Reports* 6, 27537.

110. Simcock RM, Hicks ML (2006) Delivery of Calcium Hydroxide: Comparison of Four Filling Techniques. *Journal of Endodontics* **32**, 680–2.

111. Siqueira, JF (2001) Aetiology of root canal treatment failure: why well-treated teeth can fail. International Endodontic Journal, 34(1), 1–10.

112. Siqueira JrJ, Roças I, Favieri A, Lima K (2000) Chemomechanical Reduction of The Bacterial Population in The Root Canal After Instrumentation And Irrigation With 1%, 2.5%, And 5.25% Sodium Hypochlorite. *Journal of Endodontics* **26**, 331–4.

113. Siqueira JF, Lopes HP (1999) Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. *International Endodontic Journal* **32,** 361–9.

114. Siqueira JF, Jr, Rôças IN, Lopes HP (2002) Patterns of microbial colonization in primary root canal infections. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **93**, 174- 8.

115. Siqueira JF Jr, Rocas IN, Ricucci D (2010) Biofilms in endodontic infections. Endod Topics 22:33–49.

116. Siqueira JF Jr (2011) Treatment of Endodontic Infections. London: Quintessence Publishing.

117. Sjogren U, Hagglund B, Sundqvist G, Wing K (1990) Factors affecting the long-term results of endodontic treatment. *Journal of Endodontics* **10**, 498–503.

118. van der Sluis L, Boutsioukis C, Jiang LM, Macedo R, Verhaagen B, Versluis M (2015) Ecology and Physiology of Root Canal Microbial Biofilm Communities. In: Chávez de Paz LE, Sedgley CM, Kishen A, The Root Canal Biofilm, 9 edn: pp 3-22. Berlin, German: Springer.

119. van der Sluis LW, Versluis M, Wu MK, Wesselink PR (2007) Passive ultrasonic irrigation of the root canal: a review of the literature. *International Endodontic Journal* **40,** 415-26.

120. Song F, Koo H, Ren D (2015) Effects of material properties on bacterial adhesion and biofilm formation. Journal of Dental Research **94**, 1027-34.

121. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB (2006) *Enterococcus faecalis*: Its role in root canal treatment failure and current concepts in retreatment. *Journal of Endodontics* **32**, 93-8.

122. Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **85**, 86-93.

123. Svensater G, Bergenholtz G (2004) Biofilms in endodontic infections. *Endodontic Topics* **9**, 27–36.

124. Tilton JN (1999) Fluid and particle dynamics. In: Perry RH, Green DW, Maloney JO (eds) Perry's chemical engineer's handbook, 7th edn. McGraw-Hill, New York, USA, pp 6.1–6.50.

125. Verhaagen, B., Boutsioukis, C., Sleutel, C. P., Kastrinakis, E., van der Sluis, L. W. M. & Versluis, M. (2014) Irrigant transport into dental microchannels. In : Microfluidics and Nanofluidics. **16**, p. 1165-77.

126. Versiani MA & Ordinola-Zapata R (2015) Root Canal Anatomy: Implications in Biofilm Disinfection. In: Chávez de Paz LE, Sedgley CM, Kishen A, The Root Canal Biofilm, 9 edn: pp 3-22. Berlin, German: Springer.

127. Vertucci FJ (2005) Root canal morphology and its relationship to endodontic procedures. *Endodontic Topics* **10**, 3–29.

128. Xavier ACC, Martinho FC, Chung A, Oliveira LD, Jorge AOC, Valera MC, Carvalho CAT (2013) One-Visit Versus Two-Visit Root Canal Treatment: Effectiveness in the Removal of Endotoxins and Cultivable Bacteria. *Journal of Endodontics* **39**, 959–64.

129. van der Waal SV, Connert T, Crielaard W, de Soet JJ (2016) In mixed biofilms Enterococcus faecalis benefits from a calcium hydroxide challenge and culturing. *International Endodontic Journal* **49**, 865-873.

130. Weller RN, Niemczyk SP, Kim S (1995) Incidence and position of the canal isthmus. Part 1. Mesiobuccal root of the maxillary first molar. *Journal of Endodontics* **21**, 380–3.

131. White FM (1999) Fluid Mechanics, 4th edn. Boston, USA: McGraw-Hill: 1–56, 129–214, 541.

132. Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB, Duarte MA (2016) Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. *Journal of Endodontics* **42**, 1822-28.

133. Zehnder M. Root canal irrigants. Journal of Endodontics. 2006 May;32(5):389-98.

APPENDIX

APPENDIX

APÊNCIDE A - DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (Biofilm removal from an artificial isthmus and lateral canal during syringe irrigation at various flow rates: a combined experimental and Computational Fluid Dynamics approach) will be included in (Thesis) of the student (Thais Cristina Pereira) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, January 28th 2020.

Thais Cristina Pereira — Author

Thais Cristina Pereira — Signature

Flaviana Bombarda de Andrade — Author

_____ — Signature

APÊNCIDE B - DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (Chemical and mechanical influence of root canal irrigation on biofilm removal from lateral morphological features of the root canal and dentinal tubules) will be included in (Thesis) of the student (Thais Cristina Pereira) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, January 28th 2020.

Thais Cristina Pereira — Author

Thais Cristina Pereira — Signature

Flaviana Bombarda de Andrade — Author

_____ — Signature

APÊNCIDE C - DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (The effect of refreshment, exposure time, flow rate and irrigant on biofilm removal from lateral morphological features of the root canal) will be included in (Thesis) of the student (Thais Cristina Pereira) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, January 28th 2020.

Thais Cristina Pereira — Author

Thais Cristina Pereira — Signature

Flaviana Bombarda de Andrade — Author

_____ — Signature

ANNEX

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Descontaminação Intratubular por diferentes técnicas de irrigação: avaliação por Microscopia Confocal de Varredura a Laser (MCVL).

Pesquisador: THAIS CRISTINA PEREIRA

Área Temática:

Versão: 1

CAAE: 84327318.2.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.566.816

Apresentação do Projeto:

O projeto de pesquisa "Descontaminação Intratubular por diferentes técnicas de irrigação: avaliação por Microscopia Confocal de Varredura a Laser (MCVL)" apresenta como pesquisador responsável Thais Cristina Pereira e como integrante da equipe de pesquisa a orientadora Profa. Dra. Flaviana Bombarda de Andrade. Como desenho, o presente estudo tem por objetivo avaliar e comparar a habilidade de descontaminação intratubular da irrigação convencional com uma solução tampão, um novo medicamento que possui efeito quelante (RISA), Hipoclorito de Sódio a 2% e Irrigação ativada por ultrassom com uma solução tampão, por meio de Microscopia Confocal de Varredura a Laser (MCVL) em dentes contaminados com Enterococcus faecalis. Além disso, verificar qual protocolo de limpeza é mais eficiente em evitar a recolonização desses micro-organismos no sistema de canais radiculares. Para isso, 88 espécimes obtidos de incisivos inferiores humanos recém extraídos serão contaminados com E. faecalis em um protocolo de contaminação de 5 dias. Após isso, os protocolos de irrigação serão realizados, e então os espécimes serão analizados por meio do MCVL para avaliar a viabilidade bacteriana e a presenção de matriz extracelular dentro dos túbulos dentinários. A avaliação da capacidade de recolonização dos micro-organismos será realizada a partir de espécimes que serão armazenados por 5 dias, selados na abertura coronária e no ápice, em microtubos com BHI após a realização de cada procedimento de irrigação. Após os 5 dias, uma nova análise por MCVL será realizada. Todas as imagens obtidas com o MCVL, serão processadas, os dados serão coletados e submetidos a analise

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estatistica para verificar qual tratamento será mas eficiente na redução de micro-organismos e matriz extracelular e em impedir a recolonização.

Objetivo da Pesquisa:

Avaliar e comparar diferentes protocolos de irrigação utilizados no tratamento endodôntico na descontaminação intratubular por Enterococcus faecalis assim como a presença de matriz extracelular, por meio da quantificação de bactérias vivas e mortas através de Microscopia Confocal de Varredura a Laser.

Avaliação dos Riscos e Benefícios:

O pesquisador informa que:

Riscos: Não oferece riscos a paciente.

Benefícios:

O presente estudo pretende explorar os mecanismos de viabilidade bacteriana dentro do sistema de canais raiduclares frente a diferentes protocolos de irrigação,tendo assim resultados que vão auxiliar na rotina clinica.

Comentários e Considerações sobre a Pesquisa:

A pesquisa não apresenta comprometimento ético e faz parte da linha de pesquisa da orientadora.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados os termos: projeto detalhado, encaminhamento e aquiescência do departamento, declaração de resultados, folha de rosto, questionário técnico, termo de cessão dos dentes.

Recomendações:

Realizar uma emenda com:

1) No projeto de pesquisa se lê: "Para isso, 88 espécimes obtidos de incisivos inferiores humanos recém extraídos serão contaminados com E. faecalis" mas na folha de rosto, assim como no termo de cessão, a amostra será de 96 dentes. Por favor faça a correção.

2) Nas informações básicas do projeto (Plataforma Brasil) está descrito que "não propõe dispensa de TCLE" quando na realidade deve-se propor a dispensa do TCLE e justificá-la (já existe o termo de cessão).

Conclusões ou Pendências e Lista de Inadequações:

Sugiro aprovação mediante emenda de acordo com as recomendações.

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Continuação do Parecer: 2.566.816

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 14/03/2018, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1070766.pdf	22/02/2018 15:23:15		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TermoCessaoDentesCirurgiaoDentista.pdf	22/02/2018 15:22:31	THAIS CRISTINA PEREIRA	Aceito
Folha de Rosto	Folha_rosto.pdf	22/02/2018 15:21:44	THAIS CRISTINA PEREIRA	Aceito
Outros	questionario.pdf	20/02/2018 11:53:02	THAIS CRISTINA PEREIRA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	20/02/2018 11:42:55	THAIS CRISTINA PEREIRA	Aceito
Declaração de Pesquisadores	DeclaracaoCompromissoPesquisadorResultados.pdf	20/02/2018 11:27:46	THAIS CRISTINA PEREIRA	Aceito
Declaração de Pesquisadores	Doc.pdf	20/02/2018 10:45:48	THAIS CRISTINA PEREIRA	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Continuação do Parecer: 2.566.816

BAURU, 27 de Março de 2018

Assinado por:
Ana Lúcia Pompéia Fraga de Almeida
(Coordenador)

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