UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

THAIS CRISTINA PEREIRA

**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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Tese constituída de artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo em convênio com a Universidade de Groningen (Holanda) para obtenção da dupla titulação de Doutor em Ciências Odontológicas Aplicadas no Programa de Ciências Odontológicas Aplicadas, na área de concentração Endodontia.

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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	<i>Zero refreshment</i>
1R	<i>One refreshment</i>
2R	<i>Two-refreshments</i>
2D	<i>Two-dimensional</i>
<i>A. naeslundii</i>	<i>Actinomyces naeslundii</i>
ANOVA	<i>Analysis of Variance</i>
ATCC	<i>American type culture collection</i>
B	<i>Buffer solution</i>
BHI	<i>Brain Heart Infusion</i>
CaCl ₂	<i>Calcium chloride</i>
CDFF	<i>Constant Depth Film Fermentor</i>
CFD	<i>Computational Fluid Dynamics</i>
CH	<i>Calcium hidroxyde</i>
CI	<i>Confidence Interval</i>
CLSM	<i>Confocal Laser Scanning Microscopy</i>
D	<i>Diameter</i>
EDTA	<i>Ethylenediaminetetraacetic acid</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EPS	<i>Extracellular polymeric substances</i>
E(t)	<i>Total stress exerted by the biofilm</i>
E	<i>Stress exerted by the biofilm</i>
f	<i>Effect size</i>
Fig.	<i>Figure</i>
G	<i>Gauge</i>
g	<i>Grams</i>
g/L	<i>Grams per liter</i>
IT	<i>Initial Treatment</i>
KCl	<i>Potassium chloride</i>
K ₂ HPO ₄	<i>Dipotassium phosphate</i>
KH ₂ PO ₄	<i>Monopotassium phosphate</i>
L	<i>Lenght</i>

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

xg	<i>Centrifugal force</i>
°C	<i>Degree Celsius</i>
τ_i	<i>Decay time</i>
%	<i>Percent</i>
<	<i>Less-than</i>
$\geq$	<i>Greater than or equal to</i>

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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).

Lysakowska *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, Riccucci & 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 isthmuses (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 (Damaschke *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 toxic 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 30° 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.

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.

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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 São 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 analisados por meio do MCVL para avaliar a viabilidade bacteriana e a presença 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 análise

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estatística para verificar qual tratamento será mais 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 radiculares frente a diferentes protocolos de irrigação, tendo assim resultados que vão auxiliar na rotina clínica.

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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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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BAURU, 27 de Março de 2018

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

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