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FACULDADE DE ODONTOLOGIA DE PIRACICABA – FOP

RODRIGO ARRUDA VASCONCELOS

Monitoramento clínico, microbiológico e imunológico de canais radiculares de pacientes submetidos à terapia endodôntica com diagnóstico de pulpite irreversível

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Clínica Odontológica, na Área de Endodontia.

Orientadora: Prof.ª Dr.ª Brenda Paula Figueiredo de Almeida Gomes

Este exemplar corresponde à versão final da tese defendida pelo aluno Rodrigo Arruda Vasconcelos e orientada pela Profa. Dra. Brenda Paula Figueiredo de Almeida Gomes.

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 23 de março de 2021, considerou o candidato RODRIGO ARRUDA VASCONCELOS aprovado.

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PROF. DR. MARLOS BARBOSA RIBEIRO

A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistemade Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

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língua que as articule, retém-na um nó na
garganta e só nos olhos é que se podem ler.”
(José Saramago)

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RESUMO

Esta Tese foi dividida em 3 capítulos que tiveram como **objetivos**: **Capítulo I)** Investigar o perfil microbiano, os níveis de endotoxinas, ácido lipoteicóico, citocinas (TNF-α, IL-1α, IL-1β, IL-10), metaloproteinases de matriz (MMP-2, -3, -8, -9, -13), prostaglandina E2 (PGE2), substância P e percepção de dor nas diferentes etapas do tratamento endodôntico, realizado com clorexidina (CLX) 2% gel ou hipoclorito de sódio (NaOCl), e uso de medicação intracanal (MIC) por 30 dias de dentes com pulpite irreversível sintomática (PIS); **Capítulo II)** Quantificar os níveis de TGF-β1 liberados da matriz dentinária utilizando diferentes protocolos de irrigação e avaliar a atividade antimicrobiana para otimizar estratégias terapêuticas. Além disso, este capítulo avaliou a quantidade de debris extruídos apicalmente após o preparo químico-mecânico (PQM) associado à irrigação ultrassônica passiva utilizando 4 soluções irrigadoras diferentes; e **Capítulo III)** Discutir o crescente número de pacientes com necessidade de tratamento endodôntico devido a sintomas agudos e sugerir possível associação com alterações psicológicas relacionadas a pandemia do SARS-CoV-2. **Métodos: Capítulo I)** Foram selecionados 20 pacientes com PIS. Amostras clínicas dos canais radiculares e tecidos periapicais foram realizadas antes do PQM, após o PQM (coletas microbiológicas), 48 horas após o PQM (coletas imunológicas) e após 30 dias de MIC à base de hidróxido de cálcio. O perfil microbiano foi avaliado através de cultura microbiana, nested PCR (reação em cadeia da polimerase) e *checkerboard DNA-DNA hybridization*. Os níveis de endotoxinas foram quantificados através do teste LAL Pyrogent 5000. Os níveis de LTA, substância P e PGE2 foram quantificados através de ELISA, e os níveis de citocinas e metaloproteinases de matriz através de Imunoensaio multiplex. A percepção da dor foi avaliada através de escala visual analógica; **Capítulo II)** A liberação de TGF-β1 da matriz dentinária foi quantificada através do teste de ELISA após a realização de protocolos simulando sessão única (ácido etidrônico + NaOCl 2% ou ácido etidrônico + água destilada) ou duas sessões (NaOCl 2% + hidróxido de cálcio/ CLX 2% gel + EDTA 17% ou CLX 2% gel + hidróxido de cálcio/ CLX 2% gel + EDTA 17%). A atividade antimicrobiana foi avaliada através de microscopia confocal de varredura a laser após 21 dias de contaminação com saliva. No outro estudo foram selecionados 60 incisivos inferiores. O PQM foi realizado com instrumento Reciproc e irrigação ultrassônica passiva. Os protocolos utilizados envolveram o NaOCl 6%, CLX 2% gel em associação a solução salina, solução de

CLX 2% e solução salina. Os debris extruídos apicalmente foram coletados em tubos Eppendorf e a quantidade de debris foi calculada através da diferença entre o peso final e o peso inicial (em gramas); **Capítulo III)** Foi escrito um comentário geral sobre uma possível relação entre PIS e transtornos psiquiátricos durante a pandemia. Foi realizada análise estatística com nível de significância de 5%. **Resultados: Capítulo I)** O tratamento endodôntico foi eficaz na redução dos níveis de bactérias e seus fatores de virulência, bem como modificou os níveis de citocinas, metaloproteinases de matriz, substância P e PGE2. Bactérias resistentes, tais como, *E. faecalis* e *F. nucleatum* foram encontradas após PQM e MIC; **Capítulo II)** maiores níveis de TGF-β1 foram liberados após utilização de MIC e CLX 2% gel como solução irrigadora. Além disso, este protocolo foi o mais efetivo na redução bacteriana no interior dos túbulos dentinários; A realização do tratamento endodôntico com CLX 2% gel associada a solução salina promoveu menor extrusão de debris; **Capítulo III)** Estudos têm mostrado que durante a pandemia houve aumento dos casos de PIS, apesar da redução dos atendimentos odontológicos. Também foi demonstrado aumento nos casos de alterações psicológicas tendo como consequência o aumento de mediadores inflamatórios. **Concluiu-se**: **Capítulo I)** Dentes com PIS apresentam um perfil microbiano complexo, com presença de bactérias Gram-positivas, Gram-negativas, anaeróbios facultativos e estritos. O tratamento endodôntico se mostrou efetivo na redução dos níveis de bactérias, endotoxinas e ácido lipoteicóico em dentes com PIS. O tratamento endodôntico foi eficaz na redução dos marcadores inflamatórios. CLX e NaOCl apresentaram efeitos similares nos parâmetros analisados. **Capítulo II)** O NaOCl dificulta a liberação de TGF-β1 da matriz dentinária, podendo reduzir as chances da criação de um microambiente propício para a regeneração pulpar. A utilização de substâncias quelantes (EDTA ou HDEP) e CLX permitiu a liberação de TGF-β1 da matriz dentinária, além de apresentar atividade antimicrobiana; houve extrusão apical de debris durante a técnica de irrigação ultrassônica passiva. O PQM realizado com CLX 2% gel associado a solução salina foi o método que permitiu a menor extrusão de debris; Capítulo III) O crescente aumento no número de pacientes que procuram atendimento por sintomatologia aguda pode estar relacionado à mudança de hábitos do indivíduo e aos transtornos psiquiátricos desencadeados pelo medo da SARS-CoV-2 e pelas medidas restritivas de isolamento impostas.

Palavras-chave: Bactérias. Endodontia. Inflamação. Pulpite.

ABSTRACT

This Thesis was divided into 3 chapter with the following objectives: **Chapter I)** To investigate the microbial profile, levels of endotoxins, lipoteichoic acid, cytokines (TNF-α, IL-1α, IL-1β, IL-10), matrix metaloproteinases (MMP-2, -3, -8, -9, -13), prostaglandin E2 (PGE2), substance P and perception of pain in the different stages of endodontic treatment, performed with 2% chlorhexidine (CHX) or 6% sodium hypochlorite (NaOCl), and calcium hydroxide-based intracanal medication (ICM) in teeth with symptomatic irreversible pulpitis (SIP); **Chapter II)** To quantify the levels of TGF-β1 from root dentine matrix using different irrigation protocols and to evaluate the antimicrobial activity in order to optimise treatment strategies. Furthermore, this chapter evaluated the amount of apically extruded debris after chemo-mechanical preparation (CMP) associated with passive ultrasonic irrigation (PUI) using four different root canal irrigants, namely, 6% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX) gel + saline solution (2% CHXg + SS), 2% CHX solution (2% CHXs) and SS alone; and **Chapter III)** To point out the increasing number of patient with a need of endodontic treatment do to acute symptoms and to hypothesise its association with psychological disorders related to the SARS-CoV-2 pandemic. **Methods: Chapter I)** Twenty teeth with SIP were selected. Clinical samples were collected from root canals and periapical tissues before CMP, after CMP (microbiological samples), 48 hours after CMP (immunological samples), and after 30 days of calcium hydroxide-based ICM. Microbial profile was assessed by culture method, nested PCR (polymerase chain reaction), and checkerboard DNA-DNA hybridization. The levels of endotoxins were quantified by sing LAL Pyrogent 5000. The levels of LTA, substance P and prostaglandin E2 (PGE2) were quantified by using ELISA, and the levels of cytokines and matrix metalloproteinases using multiplex immunoassay. Perception of pain was assessed by using a visual analogue scale; **Chapter II)** Liberation of TGF-β1 from dentine matrix was quantified by using ELISA after protocols simulating single (etidronic acid + 2% NaOCl or etidronic + distilled water) or two sessions (2% NaOCl + calcium hydroxide/2% CHX gel + 17% EDTA or 2% CHX gel + calcium hydroxide/2% CHX gel + 17% EDTA). Antimicrobial activity was assessed by using confocal laser scanning microscope after 21 days of contamination with saliva. The second study, a total of 60 lower incisors were selected. CMP was performed by using Reciproc instrument and passive ultrasonic irrigation. Protocols

used were 6% NaOCl, 2% CHX gel associated with saline solution, 2% CHX solution and saline solution alone. Extruded debris were collected in Eppendorf tubes and the amount of debris was calculated by the difference between the final and initial weight of the tubes (in grams); Chapter III) It was written a general comment with regard to possible relationship between SIP and psychiatric disturbs during the pandemic. Significance level was set at 5% in all tests. **Results: Chapter I)** Endodontic treatment was effective in reducing the levels of bacteria e their virulence factors, as well as the levels of cytokines, matrix metalloproteinases, substance P and PGE2. Resistant microbial species including *E. faecalis* and *F. nucleatum* were detected after CMP and ICM; Chapter II) Higher levels of TGF-β1 were released after ICM and 2% CHX gel as root canal irrigant; Endodontic treatment performed with 2% CHX gel + saline solution provided lower levels of extruded debris; **Chapter III)** Although the number of dental appointments is reduced, increase number of patients presenting with pulpitis has been observed. Furthermore, it has been reported increased number of patients with psychological disorders, and consequently, higher levels of inflammatory mediators. **In conclusion: Chapter I)** Teeth with SIP present a complex polymicrobial profile with the presence of Gram-positive, Gram-negative, facultative, and strict anaerobes. Endodontic treatment was effective in reducing the levels of bacteria, endotoxins and lipoteichoic acid in teeth with SIP. Endodontic treatment was effective in reducing the levels of inflammatory biomarkers. CHX and NaOCl presented similar effects in the analysed parameters. **Chapter II)** NaOCl hampers the liberation of TGF-β1 from dentine matrix, which may reduce the chances of a conductive environment for revitalisation. Chelating substances (EDTA or HEDP) and CHX allowed the liberation of TGF-β1 from dentine matrix, beside presenting antimicrobial activity; Apically extruded debris were observed using passive ultrasonic irrigation. CMP performed with 2% CHX gel associated with saline solution minimised debris extrusion; **Chapter III)** The recent increase in the number of patients seeking endodontic treatment due to acute symptomatology could be related to the change of the individual´s habits and to the psychiatric disorders triggered by the fear of SARS-CoV-2 and the restrictive/isolation measures imposed during the outbreak.

Keywords: Bacteria. Endodontics. Inflammation. Pulpitis.

SUMÁRIO

ANEXOS

1 INTRODUÇÃO

A cavidade oral é um dos ambientes do corpo humano com maior densidade microbiana (Jiang et al., 2014), composto por mais de 600 espécies procarióticas documentadas pelo *Human Oral Microbiome Database* (HOMD) (Dewhirst et al., 2010). Para que ocorra a manutenção da homeostase, ou seja, condição de equilíbrio do microambiente bucal, é fundamental a presença destes micro-organismos (Wade, 2013). Entretanto, em um ambiente disbiótico, as bactérias comensais organizadas em forma de biofilmes, associado a alterações do meio ambientes, tais como redução do pH e presença de açúcar, são capazes de produzir ácidos (e.g., lático, propiônico e acético) a partir do processo de fermentação, promovendo a dissolução da hidroxiapatita do esmalte e dentina, levando a formação da cárie dental (Marsh, 1994, 2006; Sheiham & James, 2015). A literatura aponta diferenças entre a microbiota das lesões superficiais de cárie, contidas em região de esmalte dental, comparada a cáries profundas em dentina (Edwardsson, 1974; Hoshino 1985; Hahn et al., 1991; Aas et al., 2008). Alterações ecológicas existentes entre ambos os sítios podem ser responsáveis pelas alterações microbiológicas existentes (Rôças et al., 2015).

Ao longo dos anos, diversos estudos evidenciaram a crucial importância dos micro-organismos no desenvolvimento e perpetuação das alterações de origem pulpar e perirradicular (Kakehashi et al., 1965; Gomes et al., 1996ab; Siqueira & Rôças, 2007; Rahimi et al., 2014; Barbosa-Ribeiro et al., 2016). Desta maneira, diversas espécies bacterianas identificadas em cáries profundas também são detectadas em infecções endodônticas (Munson et al., 2002; Sakamoto et al., 2006; Aas et al., 2008; Siqueira & Rôças, 2009; Santos et al., 2011; Gross et al., 2012; Hong et al., 2013; Schulze-Schweifing et al., 2014; Rôças et al., 2016), sugerindo que, além de estarem envolvidas em danos ao tecido pulpar, estas lesões dentinárias podem ser a fonte primária de micro-organismos que iniciam as infecções endodônticas (Rôças et al. 2016). Estudos prévios sugerem significativas diferenças entre as espécies microbianas encontradas na cárie dental e canais radiculares. Desta maneira, autores associam bactérias dos gêneros *Actinomyces*, *Lactobacillus*, *Neisseria*, *Streptococcus* e *Veillonella* à cárie dental (Gross et al., 2012) e representantes dos gêneros *Dialister*, *Fusobacterium*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas* e *Prevotella* aos canais radiculares (Sakamoto et al, 2006; Keskin et al., 2017). Entretanto, as amostras analisadas nos estudos supracitados não correspondem ao mesmo elemento dental.

Por outro lado, estudo realizado mostrou similaridade microbiana entre cárie dental e canais radiculares de dentes com pulpite irreversível, uma vez que foram coletadas amostras clínicas de dentes com exposição da lesão de cárie à câmara pulpar (Rôças et al., 2016). Recentemente, coletas microbiológicas de cárie dental profunda e canais radiculares de dentes com pulpite irreversível sintomática, revelaram similaridade entre ambos os sítios, como presença de bactérias dos gêneros *Dialister*, *Enterococcus*, *Fusobacterium*, *Parvimonas*, dentre outros (Arruda-Vasconcelos et al., 2021).

O método de cultura microbiana associado a técnicas e meios de cultura que favorecem o crescimento *in vitro* de bactérias anaeróbias estritas foi considerado por muitos anos como padrão ouro em pesquisas envolvendo identificação de microrganismos em endodontia. Entretanto, com o desenvolvimento de técnicas aprimoradas cultura-independentes, tais como, a reação em cadeia da polimerase (PCR) e suas variações, *checkerboard DNA-DNA hybridization*, clonagem e sequenciamento genético e, ultimamente, o sequenciamento de alto rendimento [*i.e.*, *Next Generation Sequencing* (NGS)] tem permitido a detecção de uma vasta diversidade microbiana em canais radiculares em diferentes condições clínicas (Gomes et al., 2015; Zahran et al., 2021). O NGS permite o sequenciamento e a análise de dados em um nível mais profundo comparado às demais técnicas moleculares. Devido ao grande número de leituras de sequências, a técnica NGS pode identificar táxons de alta e baixa abundância em uma comunidade microbiana. Infelizmente, o custo elevado é um grande limitador para utilização rotineira em laboratórios (Gomes et al., 2021).

De acordo com a *American Association of Endodontists* (AAE, 2013), a pulpite pode ser classificada em reversível ou irreversível, podendo esta última ser de natureza sintomática ou assintomática. O diagnóstico da pulpite irreversível sintomática é baseada em achados subjetivos e objetivos que sugerem um grau de inflamação da polpa vital, no qual é incapaz de se regenerar. Portanto, nestas condições, o tratamento endodôntico é indicado (Levin et al., 2009). Características clínicas desta condição pulpar podem incluir dor aguda ao estímulo térmico, dor persistente (geralmente 30 segundos ou mais após a remoção do estímulo), espontaneidade (dor não provocada) e dor referida. Às vezes, a dor pode ser acentuada por alterações posturais, como deitar-se ou inclinar-se e a administração

de analgésicos de venda livre são, geralmente, ineficazes. A pulpite é caracterizada por inflamação do tecido pulpar em resposta a agentes de origem microbiana, química e física (Ricucci et al., 2014). O principal fator associado a esta inflamação é a presença do biofilme bacteriano da cárie dental (Rôças et al., 2016).

Classicamente, as bactérias são divididas em dois grupos de acordo com as características das paredes celulares de seus representantes: Gram-positivas e Gram-negativas. Tal classificação é de grande relevância no contexto das infecções endodônticas, uma vez que estas bactérias apresentam em sua estrutura o ácido lipoteicóico (LTA), nas Gram-positivas, e os lipopolissacarídeos (endotoxinas, LPS), nas Gram-negativas. Tanto o LPS quanto o LTA são importante fatores de virulência liberados durante a multiplicação ou morte bacteriana e apresentam propriedades patogênicas similares, ocasionando resposta inflamatória no hospedeiro, e subsequente dano aos tecidos pulpar e perirradicular (Ginsburg, 2002; Barbosa-Ribeiro et al., 2016; Martinho et al., 2017).

O ácido lipoteicóico é o principal constituinte da parede celular de bactérias Gram-positivas (Tian et al. 2020). Desempenha função importante na adesão bacteriana às paredes dentinárias, formação de biofilme e liberação de mediadores inflamatórios (Bhakdi et al., 1991; Yoo et al., 2019). O LTA pode persistir no interior dos canais radiculares por longos períodos, resultando em inflamação crônica (Ahn et al., 2019; Zou et al., 2020). A eliminação do LTA do interior dos canais radiculares tem se mostrado desafiadora (Barbosa-Ribeiro et al., 2016; Aveiro et al., 2020; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021).

O LPS é o maior componente da membrana externa das bactérias Gram-negativas, aproximadamente 75%, o qual é essencial para atividades celulares, tais como crescimento, redução da permeabilidade da membrana, estabilidade e integridade estrutural, e proteção contra agressões externas (Rietschel & Brade, 1992; Caroff & Karibian, 2003; Marinho et al., 2018). O LPS tem sido relacionado a sinais e sintomas clínicos, tais como dor espontânea, reabsorção óssea, sensibilidade a percussão e dor à palpação (Jacinto et al., 2005; Vianna et al., 2007; Gomes et al., 2012; Martinho et al., 2014, 2017; Machado et al., 2020).

Em casos de pulpite, a invasão microbiana estimula o recrutamento de células

para o local da injúria e, consequentemente, acúmulo de mediadores inflamatórios (citocinas e quimiocinas) que contribuem para os processos destrutivos e reparadores nos tecidos pulpares (Cooper et al., 2010; Khorasani et al., 2020). Citocinas são polipeptídios liberados por macrófagos, neutrófilos, e outras células, sendo estas propostas como biomarcadores do processo inflamatório (Elsalhy et al., 2013). As citocinas desempenham papel importante na modulação dos processos imune e inflamatório, sendo classificadas em pro-inflamatórias (mediação da intensificação da inflamação) e anti-inflamatórias (supressão da inflamação) (Elsalhy et al., 2013).

O fator de necrose tumoral alfa (TNF-α) é um biomarcador proeminente e absolutamente central no início da cascata de inflamação do sistema imunológico. TNF-α está relacionada a dilatação e aumento da permeabilidade vascular, causando o extravasamento de leucócitos para o local da infecção (Stashenko et al., 1987). Além disso, induz a quimiotaxia e ativação de neutrófilos (Stashenko et al., 1987; Hirsch et al., 2017), indução da produção de citocinas, ativação e expressão de moléculas de adesão, e estimulação de proliferação celular (Elsalhy et al., 2013). TNF-α coordena a resposta inicial do hospedeiro, representando um importante ponto nas alterações inflamatórias (Hehlgans & Pfeffer, 2005). Pezelj-Ribaric et al., (2002) mostraram que dentes com pulpite irreversível sintomática apresentaram maiores níveis de TNF-α que dentes com pulpite irreversível assintomática e polpa normal.

As interleucinas IL-1α e IL-1b são citocinas com estruturas moleculares similares (Zehnder et al., 2003). IL-1α desempenha importante função na proteção do organismo contra invasores externos (bactérias e vírus) (Champagne et al., 2003). IL-1β tem sido associada a sinais e sintomas clínicos e reabsorção óssea (Lim et al. 1994; Zehnder et al., 2003; Gonçalves et al., 2013; Rechenberg et al., 2016).

IL-10 é uma importante citocina anti-inflamatória (Elsalhy et al., 2013). IL-10 atua como inibidor da atividade de macrófagos e da síntese de outras citocinas, tais como TNF-α e IL-1β em diversos tipos celulares (Elsalhy et al., 2013). Estudos prévios mostram maiores níveis de IL-10 durante o processo inflamatório, demonstrando um efeito imunomodulatório (Markert, 2003), ou seja, atua na tentativa de modular a resposta do hospedeiro contra determinados patógenos, tais como bactérias e vírus. De tal modo, foi observado maior expressão de IL-10 em dentes com cárie profunda que em dentes com lesões superficiais (Hahn et al., 2000).

As metaloproteinases de matriz (MMPs) são um grupo de enzimas proteolíticas com capacidade de degradar os componentes da matriz extracelular, tais como colágeno, elastinas, fibronectinas, lamininas e proteoglicanas (Eba et al., 2012; Mente et al., 2016; Barbosa-Ribeiro et al., 2019). As MMPs são produzidas por macrófagos, monócitos, fibroblastos, e outras células (Navarro et al., 2006). Desta forma, acredita-se que as metaloproteinases de matriz desempenham papel central na degradação tecidual (Eba et al., 2012). Em relação ao ambiente oral, as MMPs estão envolvidas em diversos eventos fisiológicos e patológicos, tais como, erupção dental, mineralização dentária, processos de cárie, doença periodontal, reabsorção óssea, metástases de tumores, e outros (Takata et al., 2000; Beertsen et al., 2002; Cotrim et al., 2002; Souza & Line, 2002; Souza et al., 2003; Fanchon et al., 2004; Vaananen et al., 2004; Navarro et al., 2006).

Diferentes tipos de células e tecidos do corpo humano sintetizam prostaglandinas E2 (PGE2) que é considerado a principal prostaglandina na inflamação aguda (Park et al., 2006). Durante o processo inflamatório, células como macrófagos, fibroblastos e células dendríticas sintetizam PGE2 que está envolvida na percepção da dor pela redução do limiar dos nociceptores e pela compressão de fibras nervosas devido a vasodilatação (Karataş et al., 2020). Além disso, PGE2 tem sido associada a degradação de colágeno e aumento da permeabilidade vascular (Martinho et al., 2011).

A substância P é um neuropeptídeo liberado pelas terminações nervosas em consequência de estímulos térmico, químico e/ou mecânico (Caviedes-Bucheli et al., 2008, 2009; Bıçakcı et al., 2016). Substância P desempenha papel importante nas alterações inflamatórias incluindo vasodilatação, extravasamento plasmático e liberação de citocinas e prostaglandinas (Awawdeh et al., 2002; Bamini et al., 2019; Arslan et al., 2020).

O principal objetivo do tratamento endodôntico consiste na prevenção ou eliminação das lesões perirradiculares. O emprego de técnicas cuja desinfecção é realizada do sentido coroa-ápice são preconizadas com o objetivo de promover menor extrusão de debris contaminados para os tecidos perirradiculares, e consequentemente minimizar dor e desconforto ao paciente (Tanalp & Güngör, 2014). Para tanto, se faz necessária a limpeza, modelagem, desinfecção e obturação dos

canais radiculares (Carvalho et al., 2019). Desta maneira, a realização do tratamento endodôntico de forma criteriosa através da instrumentação e irrigação é fundamental para atingir altos índices de sucesso (Vianna et al., 2007; Gomes et al., 2009b; Gomes et al., 2012; Marinho et al., 2015; Gomes et al., 2015; Barbosa-Ribeiro et al., 2016; Duque et al., 2019; Louzada 2019). A utilização de uma substância química auxiliar se mostra essencial na redução da carga microbiana e, consequentemente, de seus fatores de virulência (Gomes et al., 2015; Barbosa-Ribeiro et al., 2016, 2019, 2020; Louzada, 2019; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021), favorecendo o reparo tecidual.

O hipoclorito de sódio é a substância química auxiliar mais utilizada na endodontia, uma vez que apresenta excelente propriedade antimicrobiana e capacidade de dissolução de tecidos necróticos e constituintes orgânicos da *smear layer* (Zhender, 2006). Como alternativa a uma eficaz desinfeção do sistema de canais radiculares, a utilização da clorexidina tem sido preconizada (Barbosa-Ribeiro et al., 2016, 2019, 2020; Louzada, 2019; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021). Dentre as vantagens da clorexidina, pode-se destacar o amplo espectro de ação, adsorção aos tecidos mineralizados, permitindo liberação lenta e prolongada – substantividade, baixa toxicidade, ação reológica e tixotropia (Gomes et al., 2013). Clinicamente, tem se mostrado eficaz na remoção do LPS quando utilizada como substância química auxiliar durante o preparo químico-mecânico (Gomes et al. 2013; Marinho et al., 2015; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021).

Embora o preparo químico-mecânico seja altamente eficaz na redução dos patógenos endodônticos e seus fatores de virulência (Endo et al., 2013; Barbosa-Ribeiro et al., 2016, 2019, 2020; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021), a utilização de uma medicação intracanal pode contribuir com o controle da infecção no interior do sistema de canais radiculares (Barbosa-Ribeiro et a., 2019; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021). O hidróxido de cálcio é amplamente utilizado como medicação intracanal por apresentar propriedades antimicrobianas, inativação de LPS, indução de tecido mineralizado e biocompatibilidade (Leonardo et al., 2000, 2006; Lu et al., 2006; Manzur et al., 2007; Guerreiro-Tanomaru et al., 2012). Apesar de suas excelentes propriedades, a associação de diferentes substâncias ao hidróxido de cálcio tem sido proposta, em especial a clorexidina (Souza-Filho et al., 2008; Gomes et al., 2013; Barbosa-Ribeiro

et al., 2016; Martinho et al., 2018; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021).

De acordo com a Organização Mundial da Saúde (OMS, 2017), aproximadamente 3,5 bilhões de indivíduos apresentam alguma doença bucal, sendo que 2,3 bilhões apresentam cáries em dentes permanentes e que mais de 530 milhões de crianças apresentam cárie em dentes decíduos. Procedimentos endodônticos regeneradores têm sido amplamente indicados para pacientes jovens com dentes portadores de ápices incompletamente formados (He et al., 2017; de-Jesus-Soares et al., 2020; Ivica et al., 2020). Embora o tratamento endodôntico convencional proporcione altas taxas de sucesso, este tem o objetivo de preencher do espaço previamente ocupado pelo tecido pulpar com um material sintético, sem que a vitalidade do elemento dental seja restabelecida. Atualmente, os procedimentos endodônticos regeneradores têm sido mais difundidos para dentes de pacientes adultos com ápice completamente formado, nestes casos, os principais objetivos são a remissão de sinais e sintomas clínicos, e o redesenvolvimento do processo de neurogênese (Lim et al., 2020).

Observando a literatura, notamos a falta de estudos em dentes com pulpite irreversível, que é o último estágio da vitalidade pulpar, antes que ocorra a necrose. Enquanto na pulpite reversível, a inflamação da polpa consegue ser controlada através da remoção do estímulo causal (i.e., cárie); na pulpite irreversível, esta inflamação afetou gravemente a polpa e o tratamento endodôntico é indicado. O conhecimento dos fatores microbiológicos e imunológicos envolvidos nesta infecção poderão ser uteis para a endodontia regenerativa, para a identificação de biomarcadores importantes na determinação do estado pulpar a partir das amostras coletadas de sua porção coronária. Todos estes fatores foram motivadores para o desenvolvimento desta pesquisa.

Esta Tese foi dividida em 3 capítulos, e os principais objetivos foram:

Capítulo I

1) Investigar o perfil microbiano, e os níveis de endotoxinas e ácido lipoteicóico na dentina infectada e em canais radiculares em dentes com pulpite irreversível sintomática (artigo 1);

2) Monitorar os efeitos do preparo químico-mecânico realizado com hipoclorito de sódio e medicação intracanal à base de hidróxido de cálcio nos níveis e diversidade bacteriana, endotoxinas e ácido lipoteicóico em dentes com pulpite irreversível sintomática (artigo 2);

3) Investigar os níveis de citocinas e metaloproteinases de matriz nos tecidos periapicais de dentes com pulpite irreversível sintomática (artigo 3);

4) Monitorar os efeitos do tratamento endodôntico no perfil microbiano e quantificar os níveis de PGE2 e substância P em dentes com pulpite irreversível sintomática (artigo 4).

Capítulo II

1) Avaliar a quantidade de debris extruídos apicalmente após o preparo químico-mecânico associado à irrigação ultrassônica passiva utilizando 4 soluções irrigadoras diferentes (artigo 5);

2) Quantificar os níveis de TGF-β1 liberados da matriz dentinária utilizando diferentes protocolos de irrigação e avaliar a atividade antimicrobiana para otimizar estratégias terapêuticas (artigo 6).

Capítulo III

1) Discutir o crescente número de pacientes com necessidade de tratamento endodôntico devido a sintomas agudos e sugerir possível associação com alterações psicológicas relacionadas a pandemia do SARS-CoV-2 (artigo 7).

Capítulo I

2 ARTIGOS

2.1 INVESTIGATION OF MICROBIAL PROFILE, AND LEVELS OF ENDOTOXIN AND LIPOTEICHOIC ACID IN TEETH WITH SYMPTOMATIC IRREVERSIBLE PULPITIS: A CLINICAL STUDY.

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Abstract

Aim To investigate the microbial profile, and levels of endotoxin (LPS) and lipoteichoic acid (LTA), in infected dentine (ID) and root canals (RC) at different phases of root canal treatment in teeth with symptomatic irreversible pulpitis.

Methodology Ten volunteers were included, and samples were collected from ID and root canal lumen using sterile spoon excavators and paper points, respectively. RC samples were performed before (S1) and after (S2) chemo-mechanical canal preparation (CMP), and after intracanal medication (ICM) (S3). Checkerboard DNA-DNA hybridization was used for microbial analysis. The levels of LPS and LTA were evaluated using the limulus amebocyte lysate assay and ELISA, respectively. Shapiro-Wilk's test was used to verify data normality. Friedman's test was used to evaluate statistical differences using checkerboard DNA-DNA hybridization in the ID and RC at the different phases of the root canal treatment. Post hoc Dunn's multiple comparison test was used to verify significant differences recorded at the different time-points. The levels of LPS and LTA were analysed statistically by using repeated measures ANOVA and Tukey's post hoc test to evaluate differences in both sites. The significance level was set at 5% ($P < 0.05$).

Results A total of 40 DNA probes were used for microbial investigation in ID and RC using checkerboard DNA-DNA hybridization. The levels and complexity of bacteria were similar in the ID and initial RC samples. The levels of LPS and LTA in ID were significantly higher than the initial RC samples (S1) ($P < 0.05$). CMP was effective in significantly decreasing the levels of bacteria, LPS and LTA ($P < 0.05$). ICM did not provide additional reduction in the levels of bacteria and LPS ($P > 0.05$). However, a significant reduction in the levels of LTA were observed after ICM ($P < 0.05$).

Conclusion The microbial profile of infected dentine and root canals of teeth with irreversible pulpitis was complex, harbouring different species including Gram-positive and Gram-negative, cocci and bacilli, and facultative and strict anaerobes. Root canal treatment was effective in reducing the levels of bacteria, LPS and LTA from root canals of teeth with pulpitis.

Keywords: bacteria, checkerboard DNA-DNA hybridization, endodontic treatment, endotoxin, intracanal medication, lipoteichoic acid.

Introduction

Clinically, pulpitis can be classified as reversible, in which the elimination of the causative effect allows the pulp tissue to return to health, and irreversible, which is characterised by pulp exposure to the caries biofilm resulting in root canal treatment being required (Levin *et al*. 2009, Ricucci *et al*. 2014, Rôças *et al*. 2015, 2016). Inflammation of pulp tissue, and consequently symptomatology, is understood to be related to the biofilm and the depth of the carious infection (Rôças *et al*. 2015, Zheng *et al*. 2019). Indeed, initially it is the dentinal tubules which enable the communication of bacteria, their virulence factors and antigens with the dental pulp if appropriate treatment is not undertaken (Rôças *et al*. 2015, Zheng *et al*. 2019).

Lipopolysaccharides (endotoxin, LPS) and lipoteichoic acid (LTA) are virulence factors derived from Gram-negative and Gram-positive bacteria, respectively. LPS and LTA are released during bacterial multiplication and present similar pathogenic properties, which drive the hosts' inflammatory response and cause damage to pulp and periradicular tissues (Ginsburg 2002, Barbosa-Ribeiro *et al*. 2016, Martinho *et al*. 2017). Over the years, the levels of virulence factors have been associated with clinical signs and/or symptoms including bone resorption, spontaneous pain, tenderness to percussion and pain on palpation (Jacinto *et al*. 2005, Vianna *et al*. 2007, Gomes *et al*. 2012, Martinho *et al*. 2014, 2017).

Chemo-mechanical canal preparation (CMP) and intracanal medicament (ICM) are important steps for microbial control within the root canal (RC) system (Gomes *et al*. 2015, Barbosa-Ribeiro *et al*. 2016, 2019). Chlorhexidine (CHX) has been proposed as an alternative to sodium hypochlorite use during canal preparation (Gomes *et al*. 2013) due to its properties including broad spectrum, substantivity, lubrication and decreased cytotoxicity compared with sodium hypochlorite, allowing effective clinical performance (Gomes *et al*. 2013). Calcium hydroxide [$Ca(OH)_2$] is an intracanal medicament (ICM) widely used in Endodontics (Barbosa-Ribeiro *et al*. 2016, 2019, Martinho *et al*. 2018, Louzada *et al*. 2020). In order to increase the antimicrobial activity of $Ca(OH)_2$ against resistant bacteria, its combined use with other substances (e.g. CHX) has been suggested (Martinho *et al*. 2018, Barbosa-Ribeiro *et al*. 2019, Duque *et al*. 2019, Louzada *et al*. 2020).

Previous studies have evaluated the microbiota of dentinal caries (Aas *et al*. 2008, Rôças *et al*. 2015, 2016, Zheng *et al*. 2019). Others have investigated the infectious content from root canals in various clinical situations (Martinho *et al*. 2014, Barbosa-Ribeiro *et al*. 2016, 2019, Duque *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020, Machado *et al*. 2020). However, few studies have evaluated the effects of root canals procedures on the infectious content in teeth with irreversible pulpitis. Thus, the aim of this clinical study was to investigate the microbial profile, and levels of LPS and LTA in infected dentine and root canals at different phases of root canal treatment in teeth with symptomatic irreversible pulpitis. The null hypotheses tested were as follows: a) root canal procedures have no effects on the levels and types of microorganisms, and b) there would be no effects on the levels of LPS and LTA in teeth with irreversible pulpitis.

Materials and methods

Previous studies have described the methods for clinical and sampling procedures, microbiological assessment using checkerboard DNA-DNA hybridization and quantification of the levels of LPS and LTA (Socransky *et al*. 1994, Vianna *et al*. 2008, Barbosa-Ribeiro *et al*. 2019, Duque *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020).

This study was approved by the Human Research Ethics Committee of the Piracicaba Dental School, State University of Campinas – UNICAMP, Piracicaba, SP, Brazil (protocol CAAE 86140218.0.0000.5418). All patients signed an informed consent prior to their participation.

Patient selection

The present observational (cross-sectional) study was performed in compliance with STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines. A total of ten volunteers needing root canal treatment diagnosed with symptomatic irreversible pulpitis who attended the Dental Emergencies Service at the Piracicaba Dental School, State University of Campinas – UNICAMP, from September 2018 to August 2019 were included. Irreversible pulpitis was determined based on the

criteria reported by the American Association of Endodontists Consensus Conference on diagnostic terminology (Levin *et al*. 2009). Patients presented with sharp, spontaneous, and lingering pain (often 30 seconds or longer after stimulus removal). They reported that over-the-counter analgesics were ineffective.

Inclusion and exclusion criteria

Inclusion criteria comprised teeth with mature root apexes on radiographs with absence of periapical lesion, presence of coronal carious lesions with vital pulp, prolonged response to cold testing with Endo-Ice (1,1,1,2 tetrafluoroethane; Hygenic Corp., Akron, OH, USA), positive response to electric testing (Odous De Deus, Belo Horizonte, MG, Brazil), the presence of bleeding on access cavity and those that could be appropriately isolated.

The exclusion criteria were: teeth with no response to cold testing, no bleeding on access cavity, teeth with cracks, presence of caries in the root surface, teeth that could not be isolated with a rubber dam, root filled teeth, teeth with periapical pathosis, teeth with periodontal pockets deeper than 3 mm. In addition, volunteers who had received antibiotic treatment within the previous 3 months and those who were taking medications known to influence the immune response, and individuals reporting systemic disease starting with ASA 3 (American Society of Anaesthesiology) were not included.

Endodontic procedures and sample collection

One experienced operator performed all the clinical procedures and sample collection.

Clinical procedures and sample collection were performed as is described in previous studies (Barbosa-Ribeiro *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020). Briefly, patient's facial skin was decontaminated with 2% CHX solution (Drogal, Piracicaba, SP, Brazil) prior to intervention. Local anaesthesia (2% lidocaine with 1:100.000 epinephrine) was applied and the teeth were isolated with a rubber dam to prevent infiltration of saliva.

The operatory field, crown and surrounding structures was disinfected using the following protocol: 30% H_2O_2 (v/v) for 30 seconds, 2.5% NaOCl (30 seconds) and

subsequently neutralised with 5% sodium thiosulfate. The disinfection of these sites was monitored by taking a swab sample from both external and internal surfaces of the crown and from the surrounding structural area. The swab sample was streaked on a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA – LAB M, Heywood, UK) before being incubated anaerobically and aerobically, respectively, for up to 14 days (Gomes *et al*. 2015, Duque *et al*. 2019, Louzada *et al*. 2020). Subsequently DNA extraction from the swab and PCR analysis using universal bacterial primers was performed. If any positive cultures or the presence of DNA amplification products were obtained, then the patient was excluded from the study.

Initially, samples collected from the ID were obtained using sterile spoon excavators. The ID sample was collected from the tooth and placed into a sterile tube containing 1 mL of pre-reduced transport medium (VMGA III, viability medium, Göteborg, anaerobically prepared and sterilised) for microbiological assessment, and into a cryogenic tube for LPS and LTA analysis. Samples were frozen at -80 °C prior to further analyses.

A 2-stage access cavity was prepared using a sterile high-speed diamond round bur. The water supply was disconnected from the equipment and refrigeration/irrigation was performed manually using a sterile saline solution and syringe. The first stage aimed to remove major contaminants. In the second stage of access cavity preparation, the disinfection protocol was repeated as described above prior to accessing the pulp chamber. All procedures were performed under strict aseptic conditions.

Root canal treatment was performed under magnification using a dental operating microscope (DF Vasconcellos S/A, São Paulo, SP, Brazil). After pulp tissue extirpation, the working length (WL) was estimated by pre-operative radiography and confirmed using an electronic apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel) and a size 20 K-file (Dentsply Sirona, Ballaigues, Switzerland).

For initial endodontic sampling (S1) one sterile absorbent paper point (size 20, Cell Pack, Dentsply Sirona) was inserted into the full length of the canal and immobilised in position for 1 minute. The paper point was transferred to a sterile cryotube for LPS and LTA analysis. Then, three sterile absorbent paper points (size

20, Dentsply Sirona) were positioned consecutively as described. The paper points were removed and placed in a sterile tube containing VMGA III. When the root canals were dry, they were moistened with sterile saline solution to guarantee microbial sampling. The tubes were frozen at -80 °C prior to further analysis.

Reciproc R25 and R40 files (VDW, Munich, Germany) were used during the canal preparation according to the manufacturer's protocol in a reciprocating motion generated by an engine-driven electric motor (VDW Silver). Reciproc files were applied in an in-and-out pecking motion (approximately 3 mm amplitude) with slight apical pressure. After three pecking motions, the file was removed from the root canal and cleaned using a sterile gauze. Next, a size 20 K-file was placed at WL for patency confirmation. This protocol was repeated until the Reciproc file reached the WL (zero point displayed on the apex locator). The auxiliary chemical substance used during canal preparation was a 2% CHX gel (Endogel, Essencial Pharma Ltd. Itapetininga, SP, Brazil), which consisted of a gel base (1% natrosol) with CHX gluconate (pH 7). Natrosol gel (hydroxymethyl cellulose) is highly inert, non-ionic and water-soluble agent (Vianna *et al*. 2008, Gomes *et al*. 2015). Prior to the insertion of each instrument, the canals were flooded with the 2% CHX gel using a 27-G needle attached to a syringe. In order to remove debris originated from the mechanical instrumentation, 5 mL of saline solution was used immediately after the use of each endodontic file. Inactivation of CHX was performed with a solution of 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin for 1 min and then removed from the canal using 5 mL of saline solution. A second sample (S2) was obtained as described above and the canals were dried with sterile paper points before the use of an intracanal medicament.

A freshly prepared $Ca(OH)_2$-based ICM was used in all cases by mixing $Ca(OH)_2$ powder with 2% CHX gel (ratio of 1:1), which was placed in the full WL of the canal using Lentulo spiral fillers (Dentsply Sirona) (Louzada *et al*. 2020). The ICM was applied for 30 days. A sterilised cotton pellet was used to condense the $Ca(OH)_2$ paste at the level of the canal orifice. The access cavity sealing was performed with a 2-mm thickness of temporary cement (Cimpat; Septodont, Saint-Maur-des-Fossés, France), followed by a light-cured composite resin (Filtek Z350 XT; 3M Dental Products, St Paul, MN, USA) in combination with a single bond adhesive (3M ESPE).

After 30 days, the canals were accessed aseptically as described above and the ICM was removed using 5 mL of sterile saline solution and a master apical file (size 40) plus 2 further files. In addition, 17% ethylenediaminetetraacetic acid (EDTA) was ultrasonically activated for 3 cycles of 20 seconds by using ultrasonic device (Satelec/Acteon, Mount Laurel, NJ, USA) with Irrisonic E1 tip (Helse Ultrasonic, Santa Rosa de Viterbo, SP, Brazil) for complete removal of the ICM. This procedure was performed under a dental operating microscope to ensure complete elimination of the ICM. Then, the canals were rinsed with sterile saline and $Ca(OH)_2$ was neutralised with 5 mL of 0.5% citric acid for 1 min and removed with 5 mL of sterile saline. Immediately after this procedure, a third sample (S3) was obtained as described for the S1 and S2 procedures.

A final rinse with 3 mL of 17% EDTA ultrasonically activated was performed as described above. Irrigation with 5 mL of sterile saline was used for EDTA removal. The canals were dried with sterile paper points and filled with a single Reciproc gutta-percha cone and endodontic sealer Endométhasone N (Septodont, Saint-Maur-des-Fossés, France). A 2-mm layer of temporary cement (Cimpat, Septodont), followed by a light cured composite (Filtek Z350; 3M ESPE) combined with single-bond adhesive (3M ESPE) were used for the restoration of access cavities.

It is important to highlight that root canal treatment was performed even if the patients were not included in this study.

Checkerboard DNA-DNA hybridization

Microbiological assessment using checkerboard DNA-DNA hybridization (CB) was performed in accordance with previous studies (Socransky *et al*. 1994, Vianna *et al*. 2008, Aveiro *et al*. 2020, Louzada *et al*. 2020).

Briefly, the presence, levels, and proportions of 40 bacterial species were determined using the CB method (Table 1). Microbial DNA from American Type Culture Collection (ATCC) bacterial strains and endodontic samples were obtained according to the manufacturer's instructions. The probes were extracted and purified by using the QIAamp DNA Mini Kit; Qiagen, Hilden, Germany. The concentration of DNA was determined with a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE) with absorbance set at 260 nm.

The samples were boiled for 10 minutes and neutralised with 0.8 mL 5 mol/L ammonium acetate. The released DNA was then placed into extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated onto a 15 − 15 positively charged nylon membrane (Boehringer, Mannheim, Germany), and fixed to the membrane by incubation at 120 °C for 20 minutes. A Miniblotter 45 (Immunetics) device was used to hybridize the 40 digoxigenin-labeled whole-genomic DNA probes at right angles to the lanes of the collected clinical samples. Bound probes were detected by using phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent; Amersham Biosciences, Chicago, IL, USA). Signals were visually evaluated by comparison with 2 standards. These standards consisted of a mixture of 10^5 and 10^6 cells from each bacterium tested placed in the outer 2 lanes of each membrane. The signals were coded into 6 different scores according to the following count levels:

0 = undetected

1 = less than 10^5 cells

2 = ~10^5 cells

3 = between 10^5 and 10^6 cells

4 = ~10^6 cells

5 = >10^6 cells.

The sensitivity of this assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Quantification of LPS levels

Quantification of LPS levels was performed according to previous studies (Duque *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020). For quantification of LPS concentrations in the ID and at different phases of the root canal procedures (before and after canal preparation, and after ICM) the turbidimetric test (Bio Whitaker, Inc., Walkersville, MD, USA) was used with LAL assay detection limit of 0.01 – 100 EU/mL.

A standard curve was determined by using the LPS provided in the kit at a known concentration (*i.e.,* 100 EU/mL). The standard curve was used to calculate the concentration of LPS from each collected sample. The concentrations of LPS in the standard curve were 0.01, 0.10, 1, and 10 EU/mL, according to the manufacturer's recommendations. Subsequently the software calculates the LPS amounts correcting for the dilution factor.

The reactions were carried out in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA, USA) was incubated in a heating block set at 37 °C throughout the experiment. Initially, the samples were suspended in 1 mL of LAL Reagent Water supplied with the kit and agitated in a vortex mixer for 1 minute and diluted up to 10^{-1}. One hundred µL of the blank plus standard LPS solutions at the concentrations described above along with 100 µL of each sample were added in duplicate to wells of the 96-well microplate.

The test procedure was performed according to the manufacturer's instruction. The LPS levels were measured individually by using an ELISA plate reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) with absorbance at 340 nm. The mean absorbance value of the standards was directly proportional to the concentration of LPS, with the concentrations of the unknown LPS being determined from the standard curve.

Quantification of LTA levels

Quantification of LTA levels was based on assays described in previous studies (Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020, Louzada *et al*. 2020). The concentrations of LTA in the ID and at different phases of the endodontic treatment were quantified by using the human LTA ELISA Kit (My BioSource, San Diego, CA, USA).

The standard, control and sample solutions were added to an ELISA multiwell plate, which had been precoated with the specific monoclonal antibody for LTA supplied by the manufacturer. Anti-LTA antibodies labelled with biotin were added to bind to streptavidin conjugated with horseradish peroxidase (streptavidin-HRP), thus forming an immune complex. The plate was incubated for 1 hour at 37 °C and then washed for removal of unbound enzymes. A substrate solution containing hydrogen

peroxide and chromogen was added to the reaction.

The LTA levels were evaluated by an ELISA plate reader with an absorbance set at 450 nm and the assay was normalized for negative control values. Each densitometric value, expressed as mean and standard deviation, was obtained from two independent experiments.

Statistical analysis

Data collected at different time-points were tabulated in an Excel spreadsheet (Microsoft, WA, USA) and statistically analysed by using SPSS software for Windows (SPSS Inc., Chicago, IL, USA). The number of individuals was based on previous studies evaluating similar parameters (Aveiro *et al*. 2020, Louzada *et al*. 2020). For the quantitative variables, the Post Hoc statistical power was calculated using G*Power 3.1.9.7 for Windows. The achieved power was calculated giving $\alpha = 0.05$, the sample size ($n = 10$) and the effect size according to Cohen (1988). The statistical power observed was $(1-\beta) = 0.80$. Shapiro-Wilk's test was used to verify data normality. Parametric tests were used in the presence of normal distribution and non-parametric tests in cases of absence of normal distribution of the data. Friedman's test was used to evaluate statistical differences using checkerboard DNA-DNA hybridization in the ID and RC at the different phases of the endodontic treatment. Post hoc Dunn's multiple comparison test was used to verify statistical differences recorded at the different time-points. The levels of LPS and LTA were analysed statistically using repeated measures ANOVA and Tukey's post hoc test to evaluate differences in the ID and RC at different phases of the endodontic treatment in teeth with irreversible pulpitis. The significance level was set at 5% ($P < 0.05$).

Results

Clinical features

Figure 1 shows a flow diagram of patients seeking root canal treatment. A total of 47 individuals was assessed for eligibility. Thirty-seven were excluded given the following reasons: Presented periodontal pockets > 3mm (n = 7), were under antibiotic therapy (n = 5), declined to participate (n = 12), did not return in the appropriate day

for post-intracanal medicament sampling (n = 9) and received intervention before being referred to endodontic treatment (n = 4). Therefore, 10 patients were allocated to intervention.

Pulp vitality of the 10 teeth with symptomatic irreversible pulpitis was confirmed by prolonged and exacerbated lingering pain to cold sensitivity testing. In addition, the electric test was also performed with positive readings. Confirmation of pulp vitality was also verified by the presence of bleeding in the coronal access. All patients had spontaneous pain and tenderness to percussion when they attended the clinic. Pain on palpation was not reported. Two males and eight females, with age ranging from 23 to 58 years old (mean age 39.2±9.22 years), comprised the study population.

Sample collection were performed in 7 molars (4 maxillary and 3 mandibular), 2 premolars (1 maxillary and 1 mandibular) and 1 maxillary canine. Five of the teeth had leakage in the coronal restoration with the presence of secondary caries lesion. Five teeth had dentinal caries with no restoration. In all cases pulp exposure was observed. Periodontal pockets were ≤ 3mm with no pathological dental mobility. No symptomatology was reported before RC filling.

Microbial assessment

The disinfection protocol of the operatory field and teeth's surrounding structures was proven to be effective as no microbial growth on the control plates after 14 days and no DNA amplification products were evident on the agarose gels.

Checkerboard DNA-DNA hybridization

A total of 40 DNA probes were used for the investigation of the microbiota in ID and RC in teeth with irreversible pulpitis using CB. Figure 2 shows the prevalence of the different bacteria in ID and RC samples at the different stages of the root canal treatment. Bacteria were recovered from all ID and RC. Samples collected from the ID and from S1 (initial samples from the canals) presented increased numbers of bacterial species, without statistically significant differences between them.

After canal preparation (S2), there was a significant microbial reduction in the root canal ($P < 0.05$), which was not observed after ICM (S3, $P > 0.05$).

The most prevalent species in the ID were *E. hirae*, *F. nucleatum* sp. v*incentii*, *E. faecalis*, *F. periodonticum*, *T. forsythia*, *S. epidermidis*, *L. buccalis* and *S. mutans* (all 10/10)*, E. faecium, T. socransckii, D. pneumosintes, T. deniticola* and *C. sputigena* (all 8/10). Notably, *P. gingivalis*, *E. saburreum* and *S. gordonii* were not detected. *S. mutans* was associated with higher DNA load (10^6 cells) (Figure 3).

Regarding the RC samples, at baseline (S1), *E. hirae*, *F. nucleatum* sp. v*incentii*, *P. micra*, *C. ochracea*, *C. showae*, *F. periodonticum*, *F. nucleatum, T. forsythia* and *L. buccalis* (all 10/10) predominated. From a total of 40 investigated species, 2 were not detected (*P. gingivalis* and *E. saburreum*). *V. parvula* presented with the greatest DNA load (> 10^6 cells) (Figure 4).

After CMP (S2), a total of 7 species were not detected. In addition, there was a reduction in 34 microbial species when compared with S1. After ICM (S3), a total of 10 species were not detected. Canal medication resulted in a reduction of 18 species (Figure 5).

Levels of LPS

Table 2 provides an overview of the levels of LPS in ID and RC samples at the different stages of the root canal treatment. The levels of LPS in the ID [42.52 (±8.11) EU/mL] were significantly higher ($P < 0.05$) compared with those from the RC [0.44 (±0.03) EU/mL] in the initial samples. After CMP (S2), there was a significant reduction in the levels of LPS in the root canals ($P < 0.05$). ICM did not provide additional reduction in the levels of LPS compared with S2.

Levels of LTA

The levels of LTA in the ID and in the RC samples at different phases of the root canal treatment are shown in detail in figure 6. LTA was detected in all samples from ID and RC. The levels of LTA in the ID [507.60 (±33.25) pg/mL] were significantly higher ($P < 0.05$) compared with levels in the initial samples (S1) from the RC [440.60 (±29.73) pg/mL]. After CMP (S2), there was a significant reduction in the levels of LTA ($P < 0.05$). Furthermore, after ICM (S3), a significant reduction in the levels of LTA was observed ($P < 0.05$).

Discussion

The lack of literature reporting the effects of root canal treatment on the levels of bacteria and their virulence factors in teeth with symptomatic irreversible pulpitis were the motivating factors for this clinical investigation. The subjects included were those presenting with irreversible pulpitis due to the presence of carious lesions. Notably, the primary source of bacteria was dental caries as the teeth had healthy periodontal conditions and no history of trauma.

During the root canal treatment, the auxiliary chemical substance of choice was 2% CHX gel, not only because of its broad spectrum and lower cytotoxicity compared with NaOCl, but also because of its rheological action, which localises the debris originating from the mechanical instrumentation in suspension, facilitating its removal from the root canals (Gomes *et al*. 2013, 2015, Arruda-Vasconcelos *et al*. 2019). Notably studies observed no difference regarding antimicrobial activity between 2% CHX and 5.25% NaOCl (Gomes *et al*. 2013, Barbosa-Ribeiro *et al*. 2016, 2019). Moreover, both auxiliary chemical substances have been associated with reduction in the levels of LPS and LTA (Endo *et al*. 2012, Barbosa-Ribeiro *et al*. 2016, Neelakantan *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020).

The checkerboard DNA-DNA hybridization method was used for the evaluation of the microbial profile in ID and RC. This molecular technique has previously been successfully used to assess bacterial content at both sites (Rôças *et al*. 2015, Aveiro *et al*. 2020, Louzada *et al*. 2020, Machado *et al*. 2020) and it allows the assessment of up to 40 bacterial species concomitantly (Socransky *et al*. 1994, Vianna *et al*. 2008, Aveiro *et al*. 2020, Louzada *et al*. 2020) instead of using a single target-bacteria comparison. Nevertheless, a limitation of this method is the possibility of cross-reactions between species as a result of common regions of DNA shared by closely related species. However, the prevalence of cross-reactions associated with this diagnostic test was shown to be low between different species of the same genera, with virtually no cross-reactions being observed (Socransky *et al*. 2004). A total of 37 out of the 40 investigated species were detected in the ID samples which demonstrated the polymicrobial community in ID and RC. Previously the importance of key-species in the development of dental caries such as bacteria from the genus *Streptococcus* has been reported and the present study detected *S. mutans* in all ID samples in

agreement with these previous findings (Kianoush *et al*. 2014). A high prevalence of species from the genera *Prevotella*, *Treponema* and *Tannerella* were also observed, also corroborating with a previous study (Rôças *et al*. 2016). *S. mitis* was detected in 10% of the ID samples, agreeing with the findings of Gross *et al*. (2010) who also found lower levels of *S. mitis* in deep caries. Interestingly, the prevalence of *E. faecalis*, a facultative Gram-positive species strongly associated with root canal treatment failure due to its resistance to disinfection agents (Gomes *et al*. 2006, 2008, Barbosa-Ribeiro *et al*. 2016) was detected in all ID samples. These data were different from those of a previous publication using reverse-capture CB assay (Rôças *et al*. 2015).

With regard to the RC samples, 38 out of the 40 species analysed were observed. The increased number of species in teeth with irreversible pulpitis emphasizes the complexity of the microbiota within the RC. In the initial samples (S1), *E. faecalis* was detected in 70% of the cases. Although *E. faecalis* has been associated with persistent/secondary endodontic infection (Gomes *et al*. 2008, Barbosa-Ribeiro *et al*. 2016), it has also been detected in primary endodontic infections (Aveiro *et al*. 2020, Machado *et al*. 2020) and teeth with vital pulps associated with chronic periodontal disease (Louzada *et al*. 2020) using the same technique for microbiological assessment.

Microbial genera commonly observed in endodontic periodontal lesions, including *Tannerella* and *Treponema* (Gomes *et al*. 2015, Duque *et al*. 2019, Louzada *et al*. 2020) were also detected in canals from teeth with irreversible pulpitis. On the other hand, *P. gingivalis* was not detected in the RC. This is not consistent with the previous literature that associates this species with endo-perio lesions (Gomes *et al*. 2015, Louzada *et al*. 2020) or long-standing infections (Aveiro *et al*. 2020). Notably the number of detected species in the RC were higher compared with those from teeth with vital pulps associated with chronic periodontal disease (Louzada *et al*. 2020) or with necrotic pulps (Aveiro *et al*. 2020).

Canal preparation and intracanal medicament reduced the levels of bacteria within the RC samples, which corroborates data from previous studies (Barbosa-Ribeiro *et al*. 2016, 2019, Duque *et al*. 2019, Aveiro *et al*. 2020, Louzada *et al*. 2020). Therefore, and importantly, root canal treatment was shown to reduce the microbial population of the canals of teeth with irreversible pulpitis, resulting in the first null

hypothesis being rejected.

Studies have extensively investigated the levels of LPS, especially in necrotic pulps (Jacinto *et al*. 2005, Vianna *et al*. 2007, Martinho *et al*. 2017, Aveiro *et al*. 2020, Machado *et al*. 2020). Notably, higher levels of LPS are associated with symptomatology (Jacinto *et al*. 2005, Vianna *et al*. 2007), bone resorption and presence of inflammation (Jacinto *et al*. 2005, Vianna *et al*. 2007, Gomes *et al*. 2012, Martinho *et al*. 2017, Aveiro *et al*. 2020). The present results revealed higher levels of LPS in ID samples compared with the levels obtained initially from the RC samples. Samples collected from ID were similar to those reported in the samples collected initially from the canals of teeth with asymptomatic apical periodontitis (Aveiro *et al*. 2020), higher than in teeth with persistent/secondary endodontic involvement (Endo *et al*. 2012, Machado *et al*. 2020) or in teeth with vital pulps associated with chronic periodontal disease (Duque *et al*. 2019, Louzada *et al*. 2020).

Regarding the initial samples collected from the RC of teeth with irreversible pulpitis, the levels of LPS were equivalent to those from teeth with pulp vitality and associated periodontal disease (Duque *et al*. 2019, Louzada *et al*. 2020), agreeing with the findings of Schein & Schilder (2006), who investigated the LPS levels in normal and in irreversibly inflamed pulps. Canal preparation was shown to be effective in the reduction of LPS from canals (88.63% reduction), agreeing with previous studies (Duque *et al*. 2019, Aveiro *et al*. 2020). Levels of LPS were reduced after the use of a $Ca(OH)_2$-based ICM; however, no significant difference was observed, corroborating previous findings (Louzada *et al*. 2020).

The number of clinical studies monitoring the levels of LTA at different phases of the root canal treatment is limited (Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020, Louzada *et al*. 2020). LPS and LTA exhibit similar pathogenic properties (Ginsburg 2002, Hahn & Liewehr 2007, Barbosa-Ribeiro *et al*. 2016). In addition, complete removal of LTA from root canals is challenging (Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020, Louzada *et al*. 2020), due to its binding to dentine surfaces (Ciardi *et al*. 1977, Hahn & Liewehr 2007). This is the first clinical investigation undertaken to assess the levels of LTA in ID and RC at different stages of root canal treatment in teeth with irreversible pulpitis. The concentration of LTA in ID samples was comparable with those teeth with primary endodontic infection (Aveiro *et al*. 2020), lower compared with

the canals of persistent/secondary infection (Barbosa-Ribeiro *et al*. 2016) and higher than in canals of teeth with vital pulps associated with chronic periodontal disease (Louzada *et al*. 2020).

The concentration of LTA in the canals at S1 was lower compared with those from the canals in persistent/secondary endodontic infection with apical periodontitis (Barbosa-Ribeiro *et al*. 2016), which may be explained by the long-standing infection with an increased presence of Gram-positive species. Similar levels of LTA were observed in a previous study in canals from teeth with primary infection (Aveiro *et al*. 2020), in which the prevalence of Gram-negative species is to be expected.

Chemo-mechanical canal preparation was effective in significantly reducing LTA levels within the canals, agreeing with previous studies (Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020). CHX gel application has been reported to be effective in the reduction of LTA from canals (Barbosa-Ribeiro *et al*. 2016), supporting the present data. Conversely, different outcomes were obtained from another investigation (Louzada *et al*. 2020) where a significant reduction in the levels of LTA was found after intracanal medicament and not after chemo-mechanical canal preparation. The use of a $Ca(OH)_2$-based intracanal medicament provided additional reduction in the levels of LTA, agreeing with this previous study (Barbosa-Ribeiro *et al*. 2016). The endodontic procedures were effective in the reduction of the levels of LPS and LTA; thus, the second null hypothesis was rejected.

Monitoring the effectiveness of each phase of the root canal treatment is an important tool to evaluate different parameters (*i.e.* bacteria and their virulence factors), and to observe the levels achieved immediately before canal filling. Although root canal treatment in teeth with irreversible pulpitis can be performed in a single visit, the use of an intracanal medicament can provide additional reduction in the levels of bacteria and their virulence factors. Therefore, in specific situations where the canal filling is not achieved (persistent bleeding; patients with systemic conditions or with special needs; and lack of time to complete the treatment), the use of an intracanal medicament can improve treatment outcomes.

Conclusions

The microbial profile of infected dentine and root canals of teeth with irreversible pulpitis is complex, harbouring different species including Gram-positive and Gram-negative, cocci and bacilli, and facultative and strict anaerobes. Root canal treatment was effective in reducing the levels of bacteria, and endotoxin and lipoteichoic acid from root canals of teeth with pulpitis.

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Figures and tables

Table 1. DNA probes used for the characterization of the microbiota using checkerboard DNA-DNA hybridization in infected dentine and root canals at different stages of root canal treatment in teeth with irreversible pulpitis.

Microorganism	ATCC
Actinomyces israelii	*12102*
Actinomyces odontolyticus	17929
Actinomyces oris	43146
Aggregatibacter actinomycetemcomitans (a+b)	43718 + 29523
Campylobacter rectus	33238
Campylobacter showae	51146
Capnocytophaga gingivalis	33624
Capnocytophaga ochracea	33596
Capnocytophaga sputigena	33612
Dialister pneumosintes	G8427
Eikenella corrodens	23834
Enterococcus faecalis	29212
Enterococcus faecium	6569
Enterococcus hirae	10541
Eubacterium nodatum	33099
Eubacterium saburreum	33271
Fusobacterium nucleatum sp. *nucleatum*	25586
Fusobacterium nucleatum sp. *polymorphum*	10953
Fusobacterium nucleatum sp. *vincentii*	49256
Fusobacterium periodonticum	33693
Gemella morbillorum	27824
Leptotrichia buccalis	14201
Neisseria mucosa	19696
Parvimonas micra	33270
Porphyromonas endodontalis	35406
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Prevotella melaninogenica	25845

Prevotella nigrescens	33563
Propionibacterium acnes (I+II)	11827 + 11828
Selenomonas noxia	43541
Streptococcus gordonii	10558
Streptococcus intermedius	27335
Streptococcus mitis	49456
Streptococcus mutans	25175
Streptococcus oralis	35037
Tannerella forsythia	43037
Treponema denticola B1	Forsyth
Treponema socranskii S1	Forsyth
Veillonella parvula	10790

Table 2. Mean (±standard deviation) of the concentrations of LPS levels (EU/mL) in infected dentine root canals and at different phases of root canal treatment (before and after chemo-mechanical canal preparation, and after intracanal medication) in teeth with irreversible pulpitis.

	Clinical phase			
	ID	**Before CMP**	**After CMP**	**After ICM**
LPS	42.52 (±8.11)[a]	0.44 (±0.03)[b]	0.05 (±0.01)[c]	0.04 (±0.01)[c]

Figure 1. Flow diagram of patients seeking dental emergencies service.

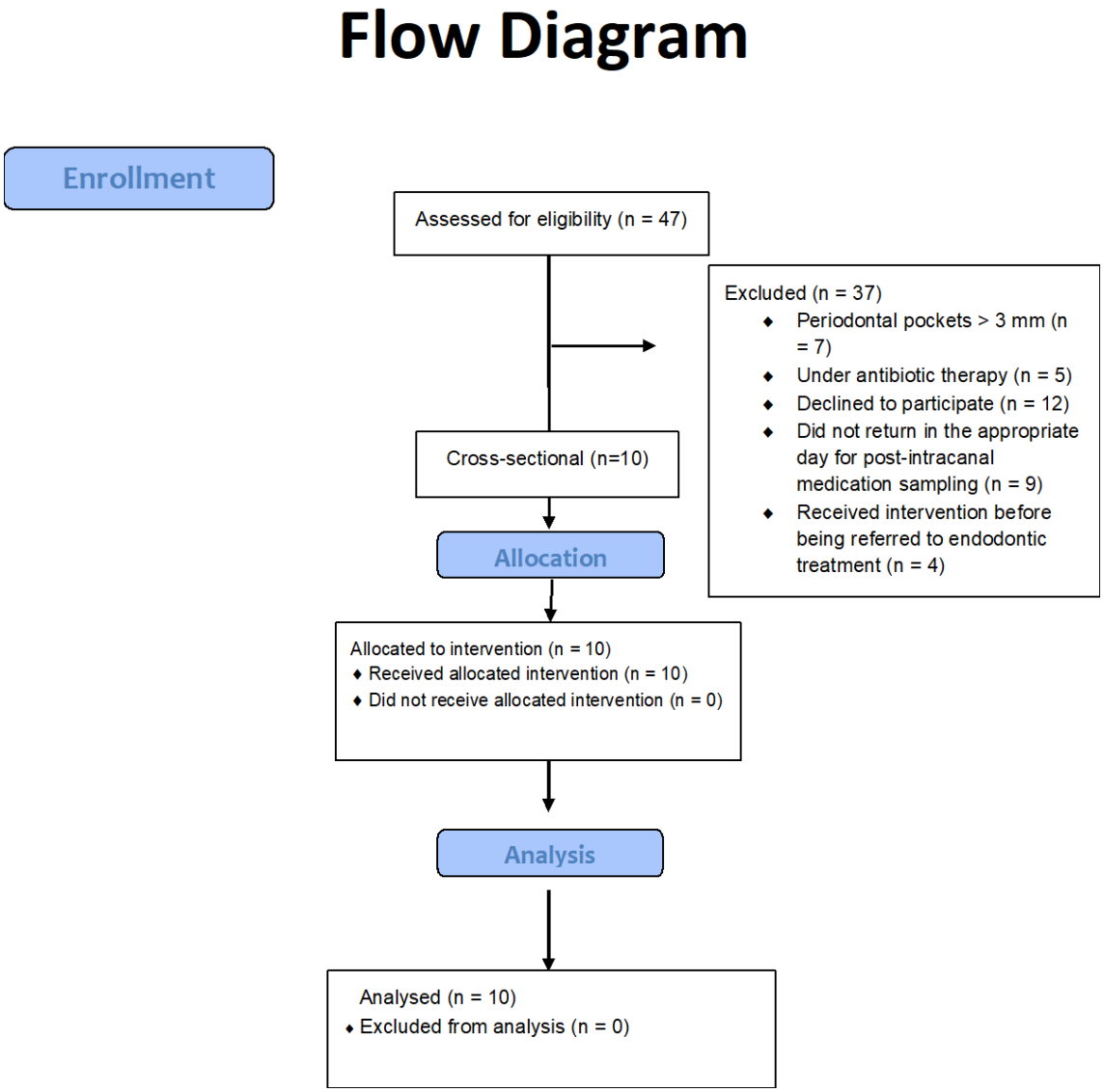


Figure 2. Prevalence of different microbial species in infected dentine and in root canals at different phases of root canal treatment by using checkerboard DNA-DNA hybridization in teeth with irreversible pulpitis.

Figure 3. Microbial load of Gram-positive (●) and Gram-negative (○) species investigated in infected dentine by using checkerboard DNA-DNA hybridization in teeth with irreversible pulpitis.

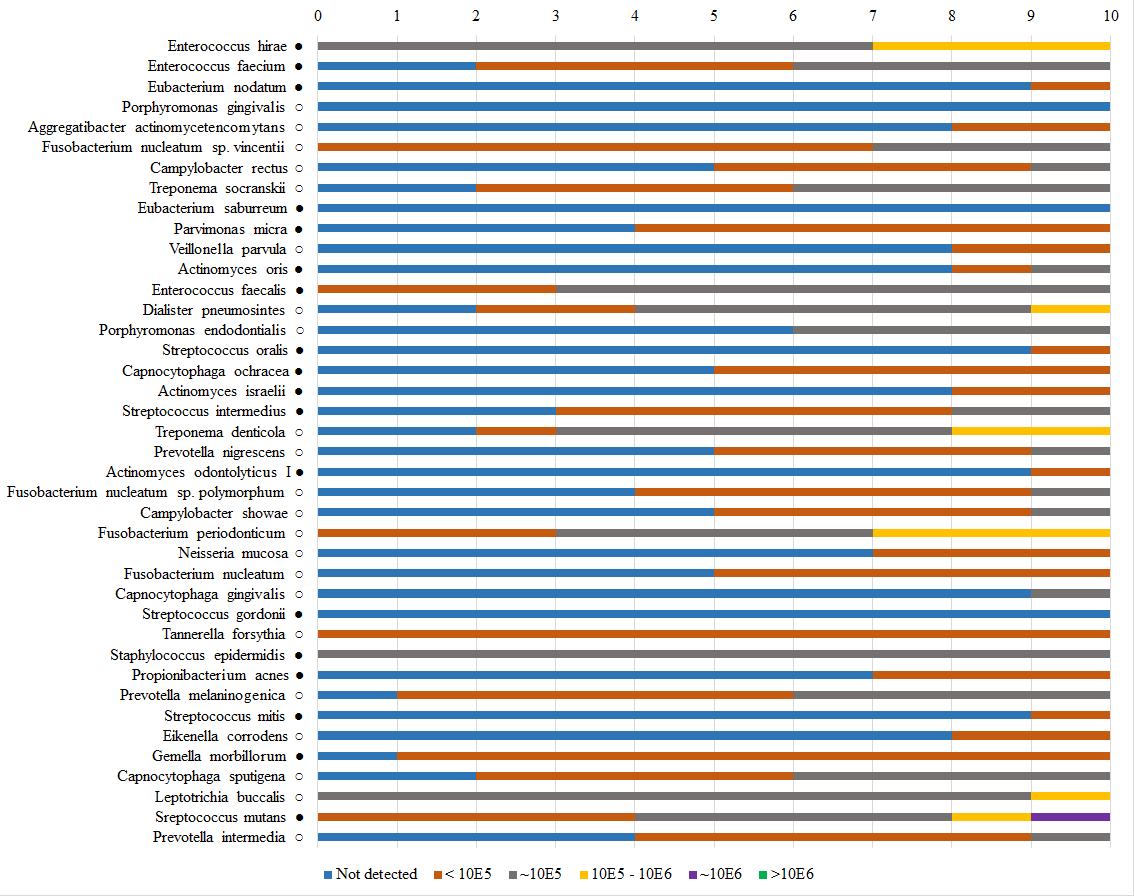


Figure 4. Microbial load of Gram-positive (●) and Gram-negative (○) species investigated in root canals before chemo-mechanical canal preparation by using checkerboard DNA-DNA hybridization in teeth with irreversible pulpitis.

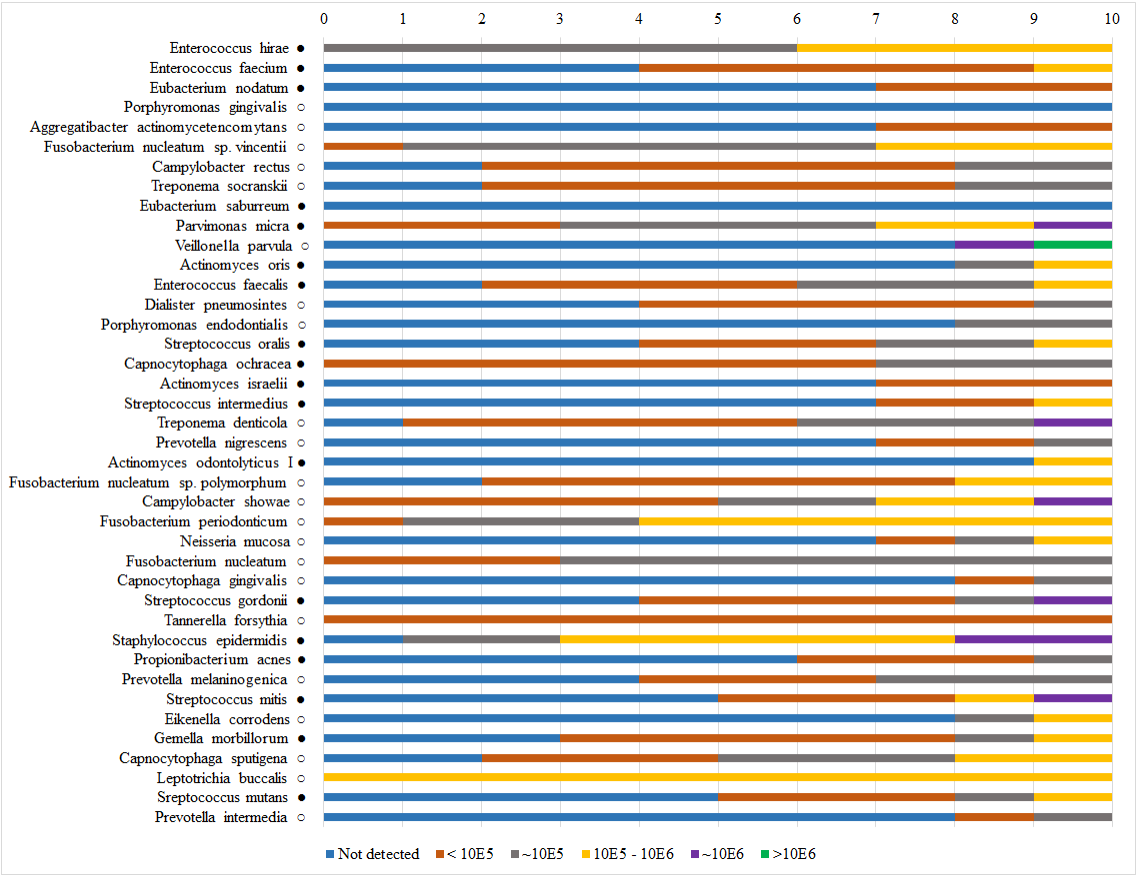


Figure 5. Microbial load of Gram-positive (●) and Gram-negative (○) species investigated in root canals after chemo-mechanical canal preparation by using checkerboard DNA-DNA hybridization in teeth with irreversible pulpitis.

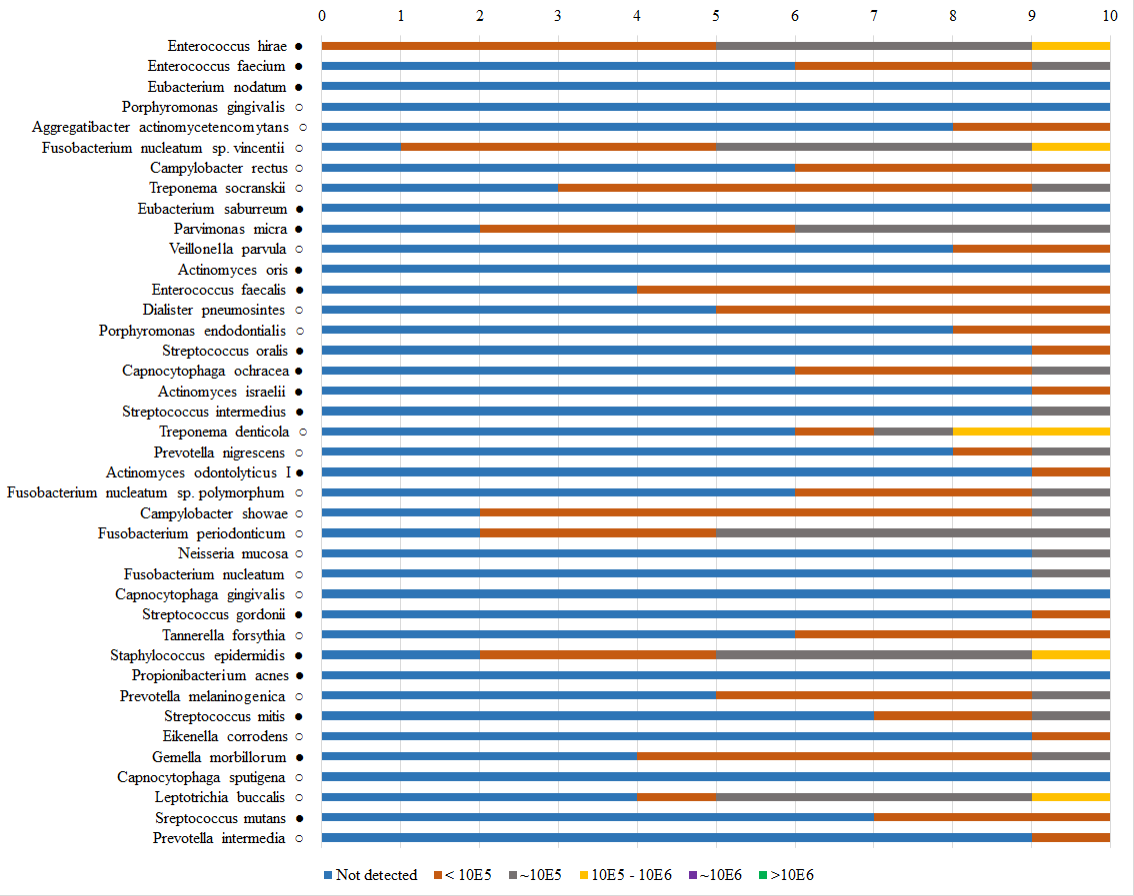


Figure 6. Microbial load of Gram-positive (●) and Gram-negative (○) species investigated in root canals after intracanal medication by using checkerboard DNA-DNA hybridization in teeth with irreversible pulpitis.

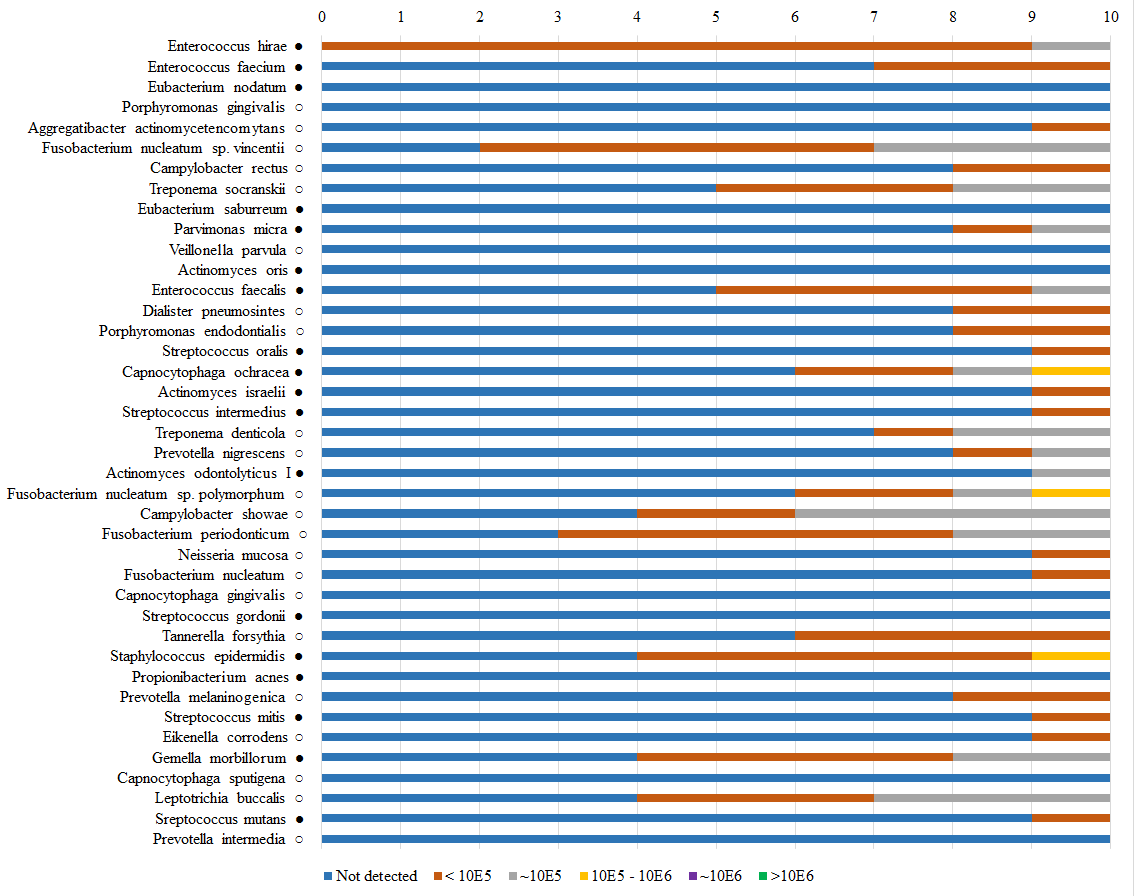
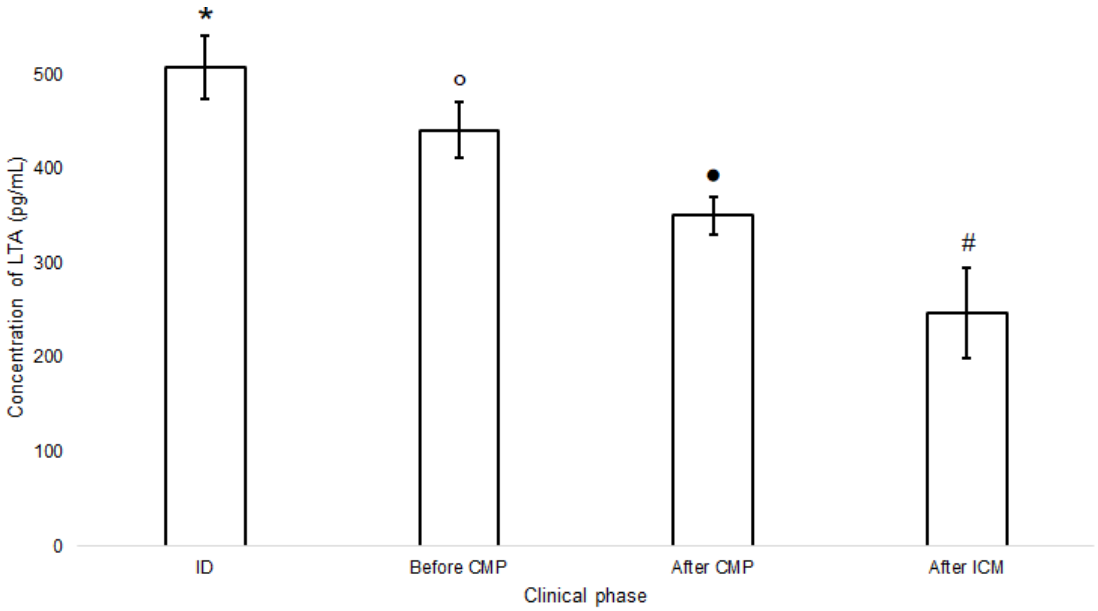


Figure 7. Mean (± standard deviation) of the levels of lipoteichoic acid in infected dentine and root canals at different phases of root canal treatment (before and after chemo-mechanical canal preparation, and after intracanal medication) in teeth with irreversible pulpitis.



2.2 EFFICACY OF 6% SODIUM HYPOCHLORITE ON INFECTIOUS CONTENT IN TEETH WITH IRREVERSIBLY INFLAMED PULP.

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Abstract

Introduction: The objective of this study was to monitor the effects of chemical-mechanical preparation performed with 6% sodium hypochlorite and calcium hydroxide-based intracanal medication on the levels and diversity of bacteria, endotoxins (LPS) and lipoteichoic acid (LTA) in root canals of teeth with symptomatic irreversible pulpitis.

Methods: Samples were collected from ten teeth with symptomatic irreversible pulpitis before chemo-mechanical preparation (S1), after chemo-mechanical preparation (S2) and after intracanal medication (S3). The levels of bacteria, LPS and LTA were assessed by using checkerboard DNA-DNA hybridisation, LAL Pyrogent 5000 and enzyme linked immunosorbent assay (ELISA), respectively. Wilcoxon's test, repeated measures ANOVA and Tukey's post-hoc test were used for statistical analysis at a significance level of 5%.

Results: Forty species were detected at S1. Two species were eliminated after chemo-mechanical preparation and five after intracanal medication. Resistant and pain-related species were detected in the root canals. Higher levels of LPS and LTA were detected at S1. Chemo-mechanical preparation was effective in reducing both LPS and LTA ($P < 0.05$). Intracanal medication produced additional reduction in the levels of LPS ($P > 0.05$) and LTA ($P < 0.05$).

Conclusion: It was demonstrated that Gram-positive and Gram-negative species were involved in the pathogenesis of teeth with symptomatic irreversible pulpitis. Chemo-mechanical preparation using 6% sodium hypochlorite and calcium hydroxide-based intracanal medication were effective in reducing the levels of bacteria, endotoxins and lipoteichoic acid in teeth with vital pulp and irreversibly inflamed pulp.

Keywords: Bacteria; Endodontics; endotoxins; lipoteichoic acid; sodium hypochlorite

Introduction

Dental caries is the main aetiological factor related to changes in dental pulp tissues. Microorganisms can damage the pulp by direct contact or by exposure to their virulence factors, initiating an inflammatory response[1]. Bacteria and their by-products invade dentinal tubules through caries, resulting in harmful stimuli even before any direct contact with the pulp[1-4].

There is an exacerbation of the inflammatory process with the leakage of infectious content from the caries to the pulp tissues, resulting in accumulation of immune cells and release of inflammatory mediators[5]. Consequently, the classic symptoms of symptomatic irreversible pulpitis with sharp, spontaneous, and lingering pain[6] occur. Furthermore, they worsen after lying down and over-the-counter analgesics are frequently ineffective.

Symptomatic irreversible pulpitis is clinically characterised by the presence of local inflammatory process, in which the host's defence is not capable of healing itself even when the causative agent is removed. Therefore, endodontic treatment is required to eliminate the symptoms and prevent the progression of the infection[1,7].

Recent studies have suggested a complex microbial profile in teeth with irreversible pulpitis[4,8]. Thus, important microbial virulence factors such as lipopolysaccharides (endotoxins, LPS) and lipoteichoic acids (LTA), which are major structural components of Gram-negative and Gram-positive bacteria, respectively, are expected[4,9-11].

Knowledge on the microbiota and its virulence factors in teeth with symptomatic irreversible pulpitis is of major importance for the development of novel strategies to enhance the effectiveness of the endodontic treatment[10]. To the best of the authors' knowledge, this is the first clinical study investigating the use of 6% sodium hypochlorite in teeth with vital pulp. Furthermore, only a few studies have evaluated the microbial aspects and concentrations of endotoxins and lipoteichoic acid in teeth with symptomatic irreversible pulpitis. Therefore, this study aimed to monitor the effects of chemical-mechanical preparation using 6% sodium hypochlorite and calcium hydroxide-based intracanal medication on the levels and diversity of bacteria, endotoxins and lipoteichoic acid in root canals of teeth with symptomatic irreversible

pulpitis.

Material and methods

Ethical Aspects

The Human Research Ethics Committee of the Piracicaba Dental School, State University of Campinas (UNICAMP), Piracicaba, SP, Brazil approved this observational study according to protocol CAAE 86140218.0.0000.5418. All volunteers signed an informed consent form prior to their participation.

Study Population

Ten teeth were included in the study, all from patients submitted to endodontic treatment due to symptomatic irreversible pulpitis based on criteria set by the American Association of Endodontists Consensus Conference on diagnostic terminology[6]. All volunteers presented with sharp, spontaneous and lingering pain (≥ 30 seconds after stimulus removal) and over-the-counter analgesics were ineffective for pain relief.

Inclusion and exclusion criteria are shown in Table 1.

Clinical Procedures and Sample Collection

All the clinical procedures and sample collection were performed by a single experienced operator as previously described[4,10].

Firstly, 2% chlorhexidine solution was used for disinfection of the patients' facial skin. Next, local anaesthesia (2% lidocaine with epinephrine at 1:100,000) was applied and teeth were isolated with rubber dam.

Rubber dam, clamp, crown, and surrounding structures were disinfected with 30% hydrogen peroxidase (v/v) for 30 seconds, followed by 2.5% sodium hypochlorite for the same period before neutralisation with 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil). To confirm the effectiveness of the disinfection of these sites, a swab sample was collected from the operative field and streaked onto a Petri dish containing 5% defibrinated sheep blood and fastidious anaerobe agar (LAB M,

Heywood, UK) before being incubated for up to 14 days anaerobically and aerobically[10,12]. After DNA extraction, polymerase chain reaction analysis was performed by using universal bacterial primers. In case of positive cultures or presence of DNA amplification products, the patient was excluded from the investigation.

Access cavity was performed in two stages by using a high-speed diamond bur with manual irrigation with sterile saline solution and syringe as the water supply was disconnected from the equipment. The first stage aimed to remove major contaminants. Prior to the second stage, the disinfection protocol was repeated as described above before accessing the pulp chamber. All procedures were performed under strict aseptic conditions.

Endodontic treatment was performed under magnification with a dental operating microscope (DF Vasconcellos S/A, São Paulo, SP, Brazil). The working length was determined based on pre-operative radiograph and checked with an electronic apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel) and a size 20 K-file (Dentsply Sirona, Ballaigues, Switzerland).

Initial sampling (baseline, S1) was performed after pulp extirpation with minimal instrumentation to allow insertion of a sterile paper point (size 20, Cell Pack; Dentsply Sirona, Ballaigues, Switzerland) into the full length of the root canal and left in position for 60 seconds. Then, the paper point was removed and placed into a sterile cryotube for analysis of endotoxin and lipoteichoic acid. Next, three sterile paper points (size 20, Cell Pack; Dentsply Sirona, Ballaigues, Switzerland) were inserted consecutively as described above and then transferred to a sterile tube containing 1 mL of pre-reduced transport medium (i.e., viability medium, Göteborg, anaerobically prepared and sterilised [VGMAIII]) for microbiological processing. The samples were stored at -80 °C for further analysis. In cases of multi-rooted teeth, samples were collected from the larger root canal to favour sample collection. When the root canals were dry, they were moistened with sterile saline solution to ensure appropriate microbial sampling.

Chemo-mechanical preparation was performed by using Reciproc R25 and R40 files (VDW, Munich, Germany) as recommended by the manufacturer. Reciprocating motion was applied in an in-and-out pecking motion (approximately 3-mm amplitude) with slight apical pressure generated by an electric motor (VDW Silver). After every

three pecking motions, the instrument was removed from the root canal and cleaned with sterile gauze. A size 20 K-file was used for checking the patency. The protocol was repeated until the Reciproc file reached the working length.

During the root canal preparation, 6% sodium hypochlorite was used as main irrigant (Drogal, Piracicaba, SP, Brazil). One mL of sodium hypochlorite was used prior to insertion of each instrument by using a 27-G needle attached to a syringe. Debris removal was performed with 5 mL of sterile saline solution immediately after the use of each endodontic file. Inactivation of sodium hypochlorite was performed with 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) for 60 seconds and then removed from the root canal by using 10 mL of sterile saline solution. A final rinse was performed with 3 mL of 17% ethylenediaminetetraacetic acid (EDTA), which was ultrasonically activated for 3 cycles of 20 seconds by using ultrasonic device (Satelec/Acteon, Mount Laurel, NJ, USA) and Irrisonic E1 tip (Helse Ultrasonic, Santa Rosa de Viterbo, SP, Brazil). EDTA was removed with 10 mL of sterile saline solution. A post-chemo-mechanical sample (S2) was obtained as described above. Subsequently, the root canals were dried with sterile paper points before the use of intracanal medication.

A freshly prepared calcium hydroxide-based intracanal medication was obtained by combining $Ca(OH)_2$ powder with 2% chlorhexidine gel. The paste was placed into the full working length with Lentulo spiral fillers (Dentsply Sirona). The intracanal medication was applied for a 30-day period. A sterilised cotton pellet was used to condense the $Ca(OH)_2$ paste at the level of the root canal orifice. The access cavity was sealed with a temporary cement of 2-mm thickness (Cimpat; Septodont, Saint-Maur-des-Fossés, France), followed by a light-cured composite resin (Filtek Z350 XT; 3M Dental Products, St Paul, MN, USA) in combination with a single bond adhesive (3M ESPE).

After the application period, the root canals were accessed aseptically, and the paste was removed by using 5 mL of sterile saline solution and a master apical file (size 40) plus two further files. In addition, 17% EDTA was ultrasonically activated as mentioned above for complete removal of the intracanal medicament. Then, the root canals were rinsed with sterile saline solution and $Ca(OH)_2$ was neutralised with 5 mL of 0.5% citric acid for 60 seconds before being removed with 10 mL of sterile saline solution. Post-intracanal medication sample (S3) was performed as described above

(S1 and S2).

A final rinse with 3 mL of 17% EDTA was performed as previously described. EDTA was removed with 10 mL of sterile saline solution. Then, the canals were dried with sterile paper points and filled with the single-cone technique and endodontic sealer Endométhasone N (Septodont, Saint-Maur-des-Fossés, France). Restoration of the access cavities was performed as above mentioned. Root canal treatment was performed even if the patients were not included in the study.

Checkerboard DNA-DNA Hybridisation

Checkerboard DNA-DNA hybridisation was used for microbial identification as previously reported elsewhere[10,13-15].

Presence, levels, and proportions of 40 bacterial species were determined by using the CB method (Table 2). Microbial DNA from American Type Culture Collection (ATCC) bacterial strains and endodontic samples were obtained according to the manufacturer's instructions. Probes were extracted and purified by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and the concentration of DNA was determined with a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE) with absorbance set at 260 nm.

The samples were boiled for 10 minutes and neutralised with 0.8 mL of 5 mol/L ammonium acetate. The released DNA was then placed into extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated on a 15−15 positively charged nylon membrane (Boehringer, Mannheim, Germany), and fixed to the membrane by incubation at 120 °C for 20 minutes. A Miniblotter 45 device (Immunetics, Cambridge, MA, USA) was used to hybridize the 40 digoxigenin-labeled whole-genomic DNA probes at right angles to the lanes of the collected clinical samples. Bound probes were detected by using phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent; Amersham Biosciences, Chicago, IL, USA). Signals were visually evaluated by comparison with two standards. These standards consisted of a mixture of 10^5 and 10^6 cells from each bacterium tested, which were placed in the outer two lanes of each membrane. The signals were coded into six different scores according to the following count levels: 0 = undetected; 1 = less than 10^5 cells; 2 = nearly 10^5 cells; 3 = between 10^5 and 10^6

cells; 4 = nearly 10^6 cells; and 5 = more than 10^6 cells.

The sensitivity of this assay was adjusted to permit the detection of 10^4 cells of a given species according to the concentration of each DNA probe.

Concentration of Endotoxins

Quantification of LPS levels was performed according to previous studies[10,15]. Turbidimetric test (Bio Whitaker, Inc., Walkersville, MD, USA) was used with LAL assay detection limit of 0.01-100 EU/mL for quantification of the concentrations of LPS in the different phases of the endodontic procedures (i.e. before and after root canal preparation, and after intracanal medication).

A standard curve was determined by using the LPS provided in the kit at a known concentration (i.e., 100 EU/mL) and then used to calculate the concentration of LPS in each collected sample. The concentrations of LPS in the standard curve were 0.01, 0.10, 1 and 10 EU/mL, according to the manufacturer's recommendations. The software calculates the LPS amounts for correction of the dilution factor.

The reactions were carried out in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA, USA) was incubated in a heating block set at 37°C throughout the experiment. Initially, the samples were suspended in 1 mL of LAL reagent water (supplied with the kit), agitated in a vortex mixer for 1 minute and diluted up to 10^{-1}. One hundred µL of the blank solution plus standard LPS solutions at the concentrations described above, along with 100 µL of each sample, were added in duplicate to the wells of the 96-well microplate.

The test procedure was performed according to the manufacturer's instruction. The LPS levels were measured individually by using an ELISA plate reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) operating at an absorbance of 340 nm. The mean absorbance value of the standard solutions was directly proportional to the concentration of LPS, with the concentration of unknown LPS being determined from the standard curve.

Concentration of Lipoteichoic Acid

Quantification of LTA levels was based on assays described in previous studies[10,16]. The concentrations of LTA in root canals in the different phases of the endodontic treatment were quantified by using the human LTA ELISA Kit (My BioSource, San Diego, CA, USA).

The standard, control and sample solutions were added to an ELISA multi-well plate, which had been pre-coated with specific monoclonal antibody for LTA supplied by the manufacturer. Anti-LTA antibodies labelled with biotin were added to bind to streptavidin conjugated with horseradish peroxidase (streptavidin-HRP), thus forming an immune complex. The plate was incubated for 1 hour at 37 °C and then washed for removal of unbound enzymes. A substrate solution containing hydrogen peroxide and chromogen was added to the reaction.

The LTA levels were evaluated by using an ELISA plate reader operating at 450 nm and the assay was normalised for negative control values. Each densitometric value, expressed as mean and standard deviation, was obtained from two independent experiments.

Statistical Analysis

Data were tabulated and statistically analysed by using SPSS software (SPSS Inc., Chicago, IL, USA), whereas the number of individuals was based on studies assessing the same parameters[4,10,15]. *Post-hoc* statistical power was calculated by using the G*Power software, version 3.1.9.7, resulting in $\alpha = 0.05$ and sample size = 10, with effect size according to Cohen[17]. The statistical power observed was $(1-\beta) = 0.80$ and Shapiro-Wilk's test was used for data normality. Wilcoxon's test was used for comparisons between the different phases of endodontic treatment. The levels of endotoxins and lipoteichoic acid were analysed by using repeated measures analysis of variance and Tukey's *post-hoc* test was used to assess differences in the different time-points. The significance level was set at 5% ($P < 0.05$).

RESULTS

Clinical Features

Figure 1 shows the flow diagram of the selection of volunteers for study eligibility.

The mean age of the patients was 35 ± 8.7 years old and the great majority were females (9/10). A total of eight molars (4 upper and 4 lower teeth) and two upper premolars were included. Restorations were fractured in seven teeth and caries were present in three ones with no previous restoration. Positive response to cold and electric testing was observed in all cases, including bleeding during access cavity preparation. Tenderness to percussion was reported by all participants, who also reported no symptom before root canal filling.

Microbial Profile

A total of 40 DNA probes were used in the checkerboard DNA-DNA hybridisation for characterisation of the microbial profile. Figures 2 and 3 show prevalence and microbial load of Gram-positive and Gram-negative species before and after chemo-mechanical preparation, and after intracanal medication, respectively.

Bacteria were present in all samples. A total of 260 strains were detected at the baseline, and after chemo-mechanical preparation the number of detected strains were significantly reduced to 215 ($P < 0.05$). After intracanal medication, 127 strains remained ($P < 0.05$).

All 40 species were detected at S1. The most prevalent species in the initial samples were *D. pneumosintes*, *F. nucleatum*, *N. mucosa*, *S. epidermidis* and *S. intermedius* (all 10/10). *A. odontolyticus*, *F. periodonticum* and *S. intermedius* showed the highest DNA load (> 10^6 cells).

After chemo-mechanical preparation (S2), two species were eliminated (*C. sputigena* and *P. gingivalis)*, whereas *D. pneumosintes*, *S. epidermidis* and *S. intermedius* (all 9/10), *C. gingivalis* and *N. mucosa* (all 8/10) predominated. *F. periodonticum* showed the highest DNA load (~ 10^6 cells).

However, after chemo-mechanical preparation (S3), five species were not detected (*C. sputigena*, *E. corrodens*, *E. saburreum*, *P. gingivalis* and *S. mitis*).

Quantification of Virulence Factors

Endotoxin

Figure 4 shows the concentrations of endotoxin in the different phases of endodontic treatment.

Endotoxins were present in all root canals. The initial values of endotoxins measured at the baseline were 0.38 ± 0.06 EU/mL.

Chemo-mechanical preparation was effective in reducing the levels of endotoxins ($P < 0.05$), whereas intracanal medication did not promote further reduction (S2 – S3, $P > 0.05$). Overall, reduction of endotoxins (S1 – S3) was 86.84%.

Lipoteichoic Acid

Lipoteichoic acid was observed in 100% of the root canals. The initial mean values of lipoteichoic acid were 423.52 ± 36.86 pg/mL (Figure 5).

After chemo-mechanical preparation, a significant reduction in the levels of lipoteichoic acid was observed ($P < 0.05$). Application of intracanal medication promoted significant reduction in the levels of lipoteichoic acid ($P < 0.05$). The overall reduction of LTA (S1 – S3) was 50.1%.

DISCUSSION

The microbiota of root canals of teeth with irreversible pulpitis was not completely elucidated, as it was considered that vital pulp tissues are free of infection or that the presence of bacteria is confined in the surface layers. However, recent studies discarded this hypothesis as a polymicrobial infection was observed[4,8]. Our results support these findings, since bacteria (Gram-positive and Gram-negative), endotoxins and lipoteichoic acid were detected in the root canals of teeth with irreversible pulpitis.

The checkerboard DNA-DNA hybridisation method has been successfully used to assess bacterial content in infected root canals[4,10,11,15,18]. It is important to highlight that the prevalence of cross-reaction test was shown to be low between different species of the same genera, with virtually no cross-reaction being observed, according to previous studies[10,15].

Forty species were detected, demonstrating a complex microbial community. In the initial samples, *F. nucleatum* was detected in 90% of the cases, which is a Gram-negative, strict anaerobic species frequently detected in primary endodontic infections[11,15]. However, *F. nucleatum* has been observed in teeth with post-treatment apical periodontitis[19] and in teeth with vital pulp and associated periodontal disease[10].

Importantly, it has been proposed that bacteria of the genera *Fusobacterium* (8.41%) and *Porphyromonas* (3.84%), both detected in this investigation and commonly associated with spontaneous pain, can progress into irreversible pulpitis and necrosis[8], suggesting the presence of ongoing infection.

Endotoxins from Gram-negative bacteria within the root canals are associated with symptomatology[20]. In the present study, we found the presence of *T. forsythia* (8/10; 3.07%), *F. nucleatum sp. vincentii*, *F. periodonticum*, *P. melaninogenica* (7/10; 2.69%), *T. socranskii* and *T. denticola* (6/10; 2.30%), all Gram-negative species. These bacteria are frequently associated with acute conditions and endodontic-periodontal lesions[10,12,21,22].

Gram-positive species were highly detectable, showing similarity with the microbiota of previously filled teeth[16,18,19]. The strains most associated with endodontic infections were *G. morbillorum* (8/10; 3.07%), *Enterococcus* spp. (10/10; 7.69%), *P. micra* (6/10; 2.30%) and *Actinomyces* spp. (6/10; 6.92%). As high levels of lipoteichoic acid were found in the root canals, a predominance of Gram-positive species was expected.

Chemo-mechanical preparation and intracanal medication reduced the levels of bacteria from the root canals, which agrees with previous studies[4,10,15,19]. Although the overall microbial reduction was 51.1%, it is worthy mention that molecular-based method does not distinguish between viable and unviable cells. This hypothesis could be confirmed by the absence of symptomatology after endodontic treatment.

Importantly, the root canal irrigant of choice was 6% sodium hypochlorite as it has broad antimicrobial activity and tissue dissolving capacity[23]. Additionally, the use of sodium hypochlorite has been reported to considerably reduce the levels of bacteria, endotoxins and lipoteichoic acid[15,16,24].

Endotoxin is the major virulence factor of Gram-negative bacteria. The immunomodulatory effect of lipid A initiates the inflammatory signalling pathways, with further inflammation, symptoms, and bone resorption[20,25].

The concentrations of endotoxin found in the root canals at the baseline (0.38 EU/mL) were lower than in teeth with symptomatic (19.1-519.0 EU/mL)[26,27] and asymptomatic (3.5-127.5 EU/mL)[15,26-28] primary infections or secondary/persistent endodontic infections (3.2-3.9 EU/mL)[24,27]. Our results detected higher levels of endotoxin than in teeth with vital pulps and associated periodontal disease (0.1 EU/mL)[10], a situation reportedly affecting the pulp tissues[29].

Notably, chemo-mechanical preparation was effective in lowering the levels of endotoxin within the root canals, a finding corroborated by previous study[15]. The levels of endotoxin were reduced after the use of calcium hydroxide-based intracanal medication, but with no statistical significance compared to samples obtained after chemo-mechanical preparation, a finding also corroborated by previous studies[4,10]. The overall reduction in the levels of LPS was compared to those reported in the literature, that is, from 55% to 90%[4,10,15,26-28].

Lipoteichoic acid, the main constituent of the Gram-positive bacteria cell wall[30], are widely present in cariogenic bacteria and can spread into the pulp tissues via dentinal tubules or direct contact, thus promoting an immune response[1,9]. Furthermore, lipoteichoic acid plays an important role in microbial adhesion to dentinal walls and biofilm formation[31,32], leading to invasion of dentinal tubules and difficult elimination.

The levels of lipoteichoic acid have been investigated in teeth presenting different clinical conditions[4,10,11,15,16]. In the present study, lipoteichoic acid was recovered from 100% of the root canals. However, chemo-mechanical preparation performed with 6% sodium hypochlorite and calcium hydroxide-based intracanal medication reduced the levels of lipoteichoic acid, which was corroborated by previous studies[15,16]. Conversely, lipoteichoic acids have been associated with difficult removal

of root dentine using sodium hypochlorite or chlorhexidine as main irrigating solution, demonstrating to be a clinical challenge[11,10,16].

Considerable reduction in the levels of lipoteichoic acid was achieved after intracanal medication. Calcium hydroxide inactivates lipoteichoic acids by modifying their structure and promoting deacetylation, which leads to ineffective binding to toll-like receptor 2 and minimises their inflammatory activity[33]. In fact, reduced levels of lipoteichoic acid were detected after intracanal medication[4,10,16].

The higher concentration of lipoteichoic acids compared to that of endotoxins suggests a predominance of Gram-positive species in teeth with irreversibly inflamed pulp. A drawback of this study is mostly due to the relatively low number of investigated species compared to high-throughput sequencing methods[8]. However, to overcome this limitation, the levels of endotoxin and lipoteichoic acid were quantified as they are important biological markers of the presence of Gram-negative and Gram-positive species, respectively. With this regard, the microbiota of teeth with irreversible pulpitis may have similarities with persistent/secondary endodontic infections due to the levels of endotoxin and lipoteichoic acid[11,16]. The use of sodium hypochlorite at higher concentrations was proposed as it improves pulp tissue dissolution[34] without interfering with post-operative pain compared to other concentrations of sodium hypochlorite and chlorhexidine[35].

This was a pioneer investigation conducted to monitor the levels of bacteria, endotoxins and lipoteichoic acids in teeth with symptomatic irreversible pulpitis in different phases of endodontic treatment performed with 6% sodium hypochlorite. The relevance of this study relies on the potential role of endotoxins and/or lipoteichoic acids in sensitising the hosts' immune system, which leads to clinical symptoms. Further studies are needed to evaluate the acceptable clinical levels of residual endotoxins and lipoteichoic acids. Importantly, it is known that endodontic treatment cannot completely remove all bacteria from the root canal system. With this regard, all procedures were performed under strict aseptic conditions and restoration was performed with light-curing composite to avoid coronal microleakage.

CONCLUSION

This study has demonstrated the involvement of Gram-positive and Gram-negative species in the pathogenesis of teeth with symptomatic irreversible pulpitis. Chemo-mechanical preparation with 6% sodium hypochlorite and calcium-hydroxide based intracanal medication were shown to be effective in reducing the levels of bacteria, endotoxins and lipoteichoic acids in teeth with vital pulp and irreversibly inflamed pulp.

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Tables and figures

Table 1. Inclusion and exclusion criteria for eligibility of the volunteers.

Criteria	
Inclusion	**Exclusion**
1. Prolonged response to cold testing with Endo-Ice (Hygenic Corp., Akron, OH, USA)	1. No response to cold testing
2. Positive response to electric testing (Odous De Deus, Belo Horizonte, MG, Brazil)	2. No response to electric testing
3. Bleeding on access cavity	3. No bleeding on access cavity
4. Mature root apex	4. Cracks
5. Absence of periapical lesion	5. Caries in the root surface
6. Coronal caries	6. Root-filled teeth
7. Allow appropriate isolation and restoration	7. Periapical lesion
	8. Non-restorable teeth
	9. Periodontal pockets > 3 mm
	10. Antibiotic therapy (< 3 months)
	11. Systemic disease
	12. Received intervention before being referred for endodontic treatment
	13. Patients who received intervention before being referred to endodontic treatment

Table 2. DNA probes used in the checkerboard DNA-DNA hybridisation method for microbial characterisation in root canals of teeth with symptomatic irreversible pulpitis.

Microorganism	ATCC
Actinomyces israelii	12102
Actinomyces odontolyticus	17929
Actinomyces oris	43146
Aggregatibacter actinomycetemcomitans (a+b)	43718 + 29523
Campylobacter rectus	33238
Campylobacter showae	51146
Capnocytophaga gingivalis	33624
Capnocytophaga ochracea	33596
Capnocytophaga sputigena	33612
Dialister pneumosintes	G8427
Eikenella corrodens	23834
Enterococcus faecalis	29212
Enterococcus faecium	6569
Enterococcus hirae	10541
Eubacterium nodatum	33099
Eubacterium saburreum	33271
Fusobacterium nucleatum sp. *nucleatum*	25586
Fusobacterium nucleatum sp. *polymorphum*	10953
Fusobacterium nucleatum sp. *vincentii*	49256
Fusobacterium periodonticum	33693
Gemella morbillorum	27824
Leptotrichia buccalis	14201
Neisseria mucosa	19696
Parvimonas micra	33270
Porphyromonas endodontalis	35406
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Prevotella melaninogenica	25845
Prevotella nigrescens	33563
Propionibacterium acnes (I+II)	11827 + 11828

Selenomonas noxia	43541
Streptococcus gordonii	10558
Streptococcus intermedius	27335
Streptococcus mitis	49456
Streptococcus mutans	25175
Streptococcus oralis	35037
Tannerella forsythia	43037
Treponema denticola B1	Forsyth
Treponema socranskii S1	Forsyth
Veillonella parvula	10790

Figure 1. Flow diagram of the selection of volunteers for eligibility.

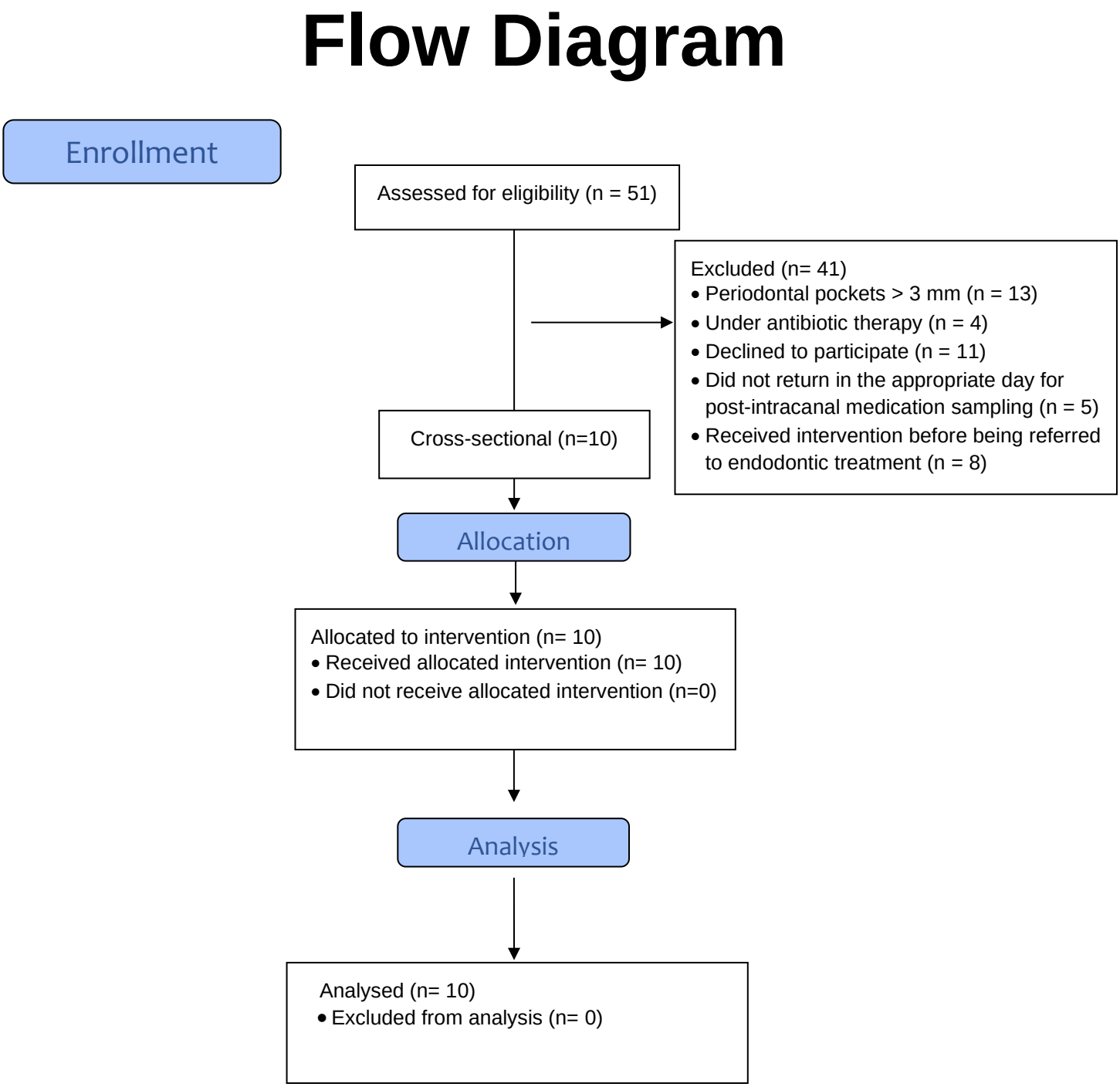


Figure 2. Prevalence and microbial diversity of Gram-positive species before chemo-mechanical preparation (S1), after chemo-mechanical preparation (S2) and after intracanal medication (S3) in teeth with symptomatic irreversible pulpitis.

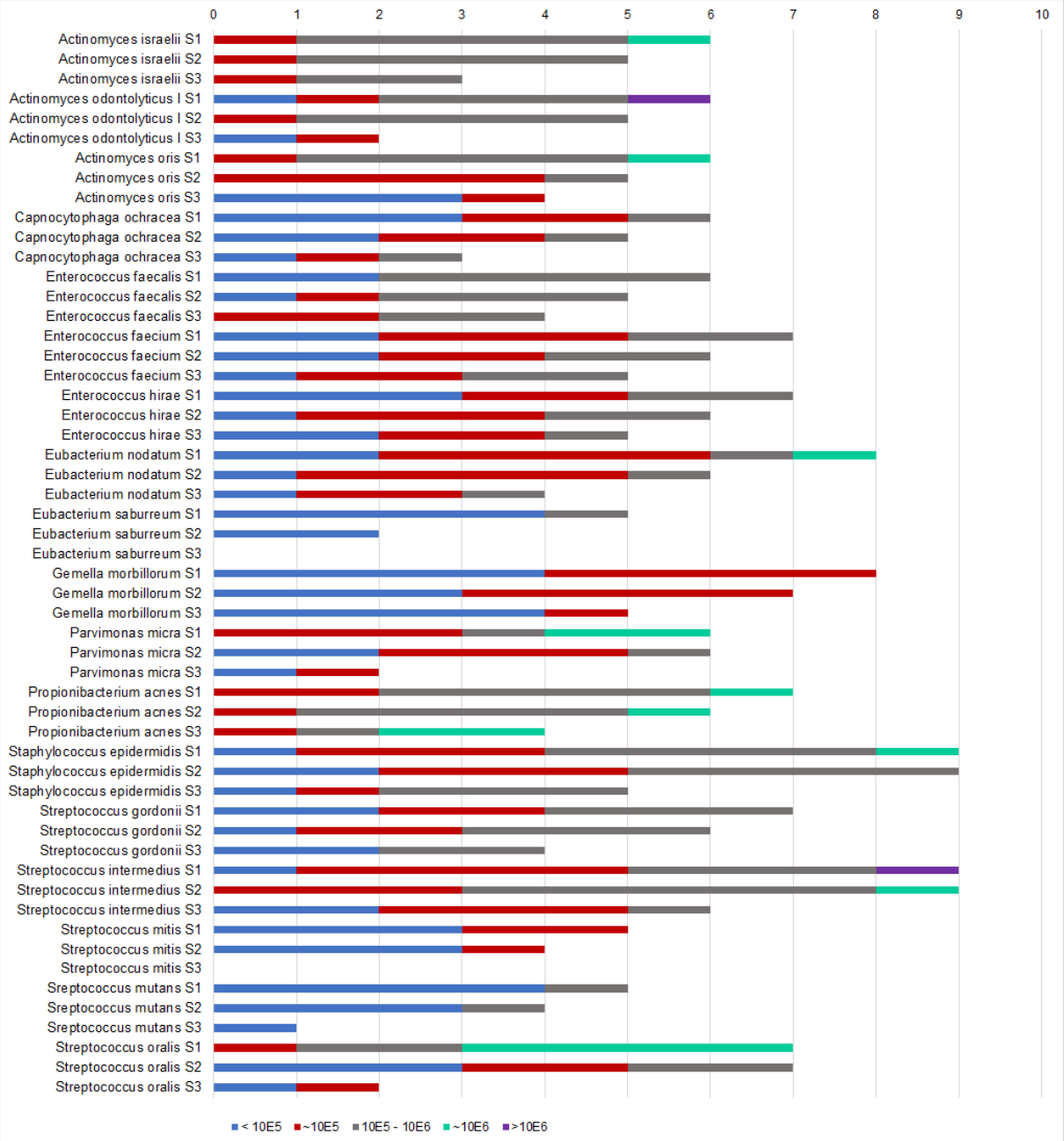


Figure 3. Prevalence and microbial diversity of Gram-negative species before chemo-mechanical preparation (S1), after chemo-mechanical preparation (S2) and after intracanal medication (S3) in teeth with symptomatic irreversible pulpitis.

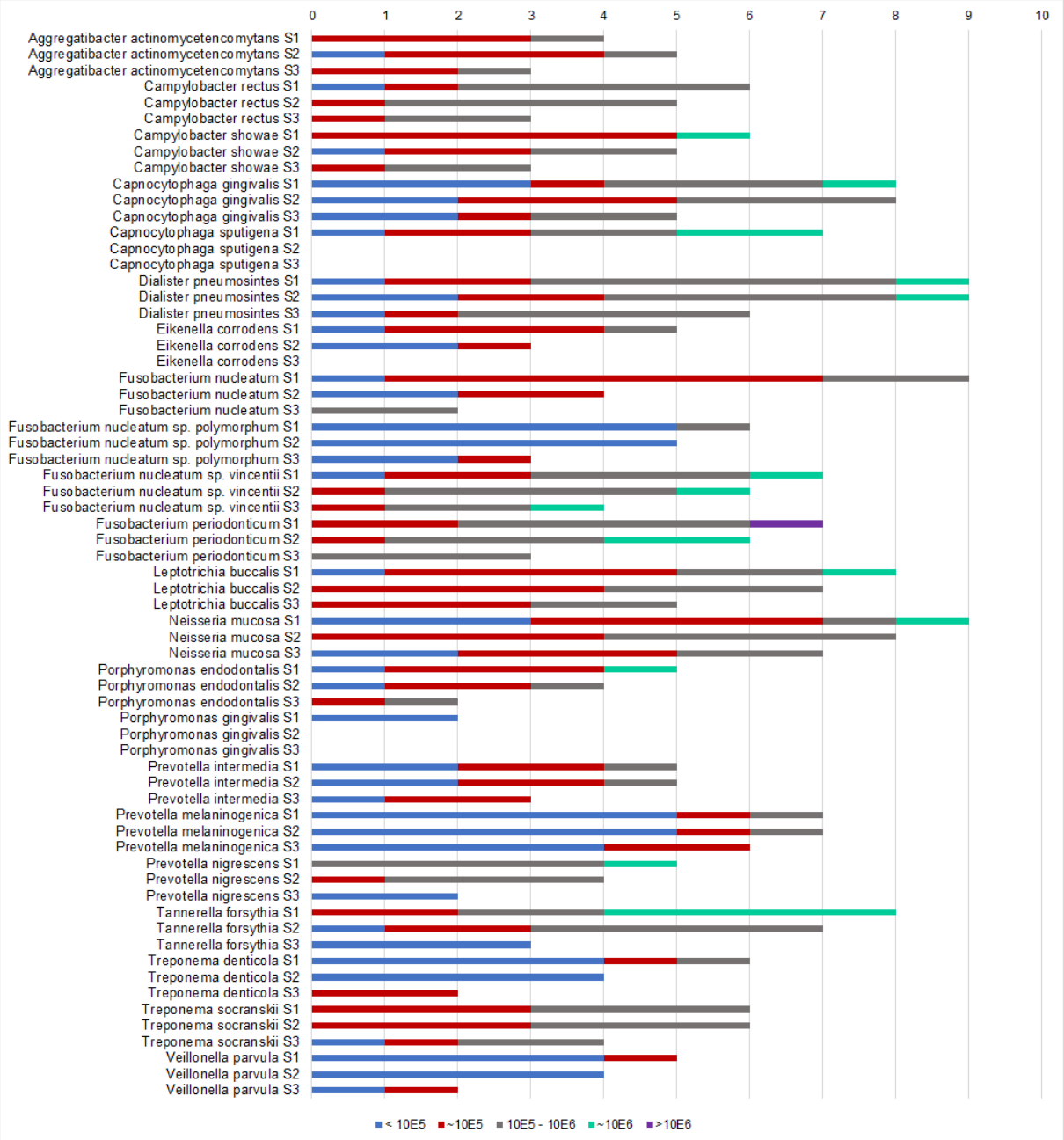


Figure 4. Concentration of endotoxins (EU/mL) before chemo-mechanical preparation (S1), after chemo-mechanical preparation (S2) and after intracanal medication (S2) in teeth with symptomatic irreversible pulpitis.

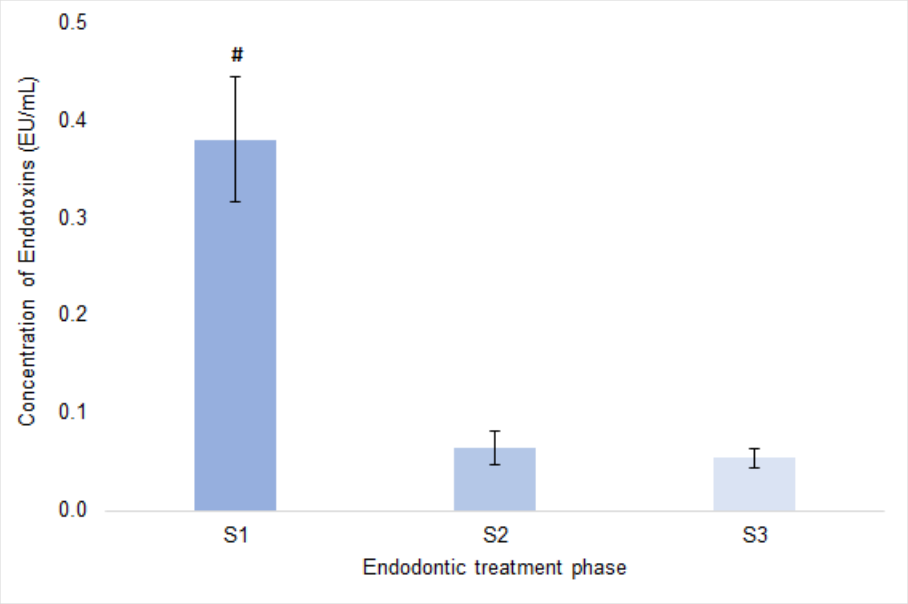
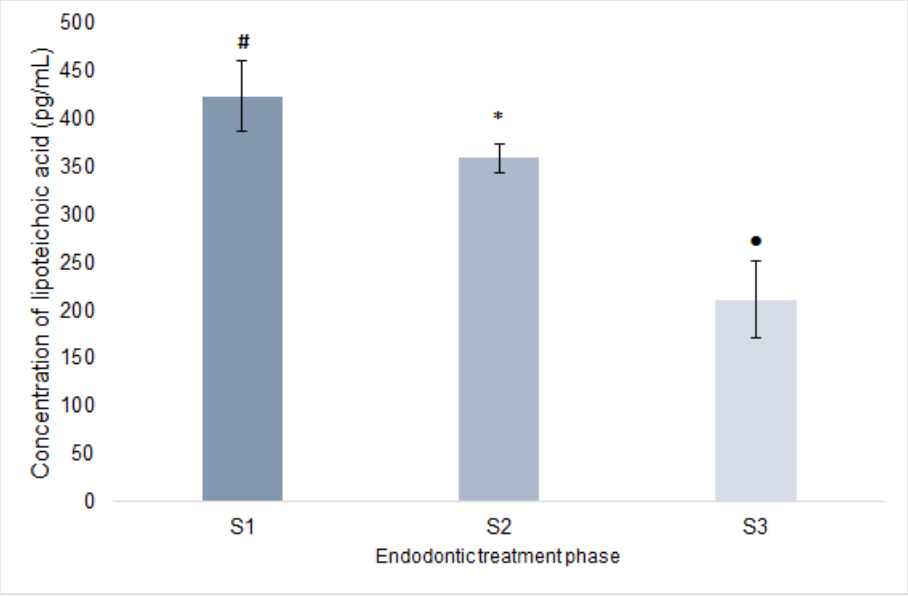


Figure 5. Concentration of lipoteichoic acids (pg/mL) before chemo-mechanical preparation (S1), after chemo-mechanical preparation (S2) and after intracanal medication (S2) in teeth with symptomatic irreversible pulpitis.



2.3 COMPARISON OF 2% CHLORHEXIDINE GEL AND 6% SODIUM HYPOCHLORITE ON INFLAMMATORY BIOMARKER LEVELS IN TEETH WITH SYMPTOMATIC IRREVERSIBLE PULPITIS

Rodrigo Arruda-Vasconcelos, Lidiane Mendes Louzada, Phillip L. Tomson, Henry F. Duncan, Josette Camilleri, Paul R. Cooper, Brenda P.F.A. Gomes.

Abstract

Aim This study investigated the levels of cytokines and matrix metalloproteinases (MMPs) in periapical tissues of teeth with symptomatic irreversible pulpitis after different stages of the root canal treatment performed with 2% chlorhexidine gel or 6% sodium hypochlorite.

Methodology Twenty teeth were included. Periapical tissues samples were collected before chemo-mechanical preparation (CMP)(baseline, S1), 48 hours after CMP (S2), and after intracanal medication (ICM)(S3). Levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1α,-1β,-10, MMP-2,-3,-8,-9 and -13 (pg/mL) were quantified by using Multiplex Immunoassay. Mann-Whitney and Wilcoxon tests were performed to verify statistical differences between the groups and different time-points, respectively. A significance level of 5% was set.

Results TNF-α and IL-10 were detected at higher values in periapical tissues (S1). At S2, decreased levels of IL-10 and TNF-α ($P < 0.05$) were evident in both groups, and no significant reduction in IL-1β concentrations. At S3, TNF-α were reduced in both groups ($P < 0.05$), with no significant modification in the levels of IL-α and 1IL-10. No significant modification was observed in the levels of IL-1β throughout the root canal treatment. For matrix metalloproteinases, MMP-2, -8 and -9 were present at higher levels at baseline. At S2, MMP-2 was decreased in both groups ($P < 0.05$) and MMP-8 in the chlorhexidine group ($P < 0.05$). MMP-9 was significantly reduced only in the sodium hypochlorite group. Increased levels of MMP-3 and -13 were observed. At S3, reduced levels of MMP-2, -3, -8 and -9 ($P < 0.05$) were detected, as well as increased concentrations of MMP-13 ($P < 0.05$).

Conclusion Root canal treatment had a positive effect on the levels of cytokines and matrix metalloproteinases, irrespective of the irrigant used (2% chlorhexidine gel or 6% sodium hypochlorite) during the chemo-mechanical preparation. Additionally, calcium hydroxide based intracanal medication was effective in keeping the inflammatory biomarkers at low levels in teeth with symptomatic irreversible pulpitis.

Keywords: Chlorhexidine, cytokine, inflammation, irreversible pulpitis, matrix metalloproteinase, sodium hypochlorite.

Introduction

Pulpitis has been characterized by the local accumulation of inflammatory mediators (*i.e.,* cytokines and chemokines) that contribute to the destructive and reparative processes in the pulp tissues (Cooper *et al.* 2010, Khorasani *et al.* 2020). The decision in performing root canal treatment in teeth with deep caries lesions are mostly based on symptomatic history reported by the patients, radiographic exams, and response to thermal and electric tests (Brizuela *et al.* 2020). Importantly, these information do not precisely inform the pulp tissues status, which is done only histologically (Seltzer *et al.* 1963). In this context, the diagnosis involving the quantification of different inflammatory biomarkers has received substantial attention.

Cytokines are polypeptides released by leucocytes, neutrophils, macrophages and structural cells, and these molecules are proposed as biomarkers of the inflammatory process (Elsalhy *et al.* 2013). Cytokines play an important role in the modulation of immune and inflammatory processes and are categorised as pro-inflammatory, which mediate increase inflammation, and anti-inflammatory, responsible for suppression of inflammation (Elsalhy *et al.* 2013).

Stimulated macrophages and other cells produce matrix metalloproteinases, promoting extracellular matrix destruction (Barbosa-Ribeiro *et al.* 2019). Matrix metalloproteinases belong to a family of calcium- and zinc-dependent endopeptidases and which can degrade extracellular matrix components, such as collagens, elastins, fibronectins, laminins, and proteoglycans (Mente *et al.* 2016). The majority of matrix metalloproteinases are originally produced as pro-enzymes which are secreted prior to their conversion into their active form (Sambandam & Neelakantan 2014). Recently, matrix metalloproteinases have been proposed to play a diagnostic role in the dental inflammatory processes (*i.e.,* pulpitis) (Mente *et al.* 2016).

A complex network between pro- and anti-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1α, -1β and -10, and a wide range of matrix metalloproteinases including gelatinase A (MMP-2), stromelysin-1 (MMP-3), collagenase-2 (MMP-8), gelatinase B (MMP-9) and collagenase-3 (MMP-13) are involved in inflammatory processes in the pulp and periapical tissues (Zehnder *et al.* 2003, Abd-Elmeguid *et al.* 2013, Elsalhy *et al.* 2013, Barbosa-Ribeiro *et al.* 2019,

Duque *et al.* 2019). Furthermore, the quantification of inflammatory biomarkers could indicate the stage of inflammation in teeth with irreversible pulpitis, leading to a more accurate decision-making in dental practice.

Thus, the aim of the present study was to investigate the levels of cytokines (TNF-α, IL-1α, -1β, and -10) and matrix metalloproteinases (MMP-2, -3, -8, -9 and -13) in periapical tissues of teeth with symptomatic irreversible pulpitis after different stages of the root canal treatment performed with 2% chlorhexidine gel or 6% sodium hypochlorite.

Materials and methods

Ethical aspects and patient selection

The Human Research Ethics Committee of the Piracicaba Dental School, State University of Campinas – UNICAMP, Piracicaba, SP, Brazil, approved this work (CAAE 86140218.0.0000.5418) describing the methods for sampling collection. Patients signed an informed consent form prior to their participation in the study.

This was an observational study. Twenty volunteers who attended the Dental Emergencies Services at the Piracicaba Dental School, State University of Campinas – UNICAMP with a requirement for root canal treatment due to symptomatic irreversible pulpitis were included in the study.

Symptomatic irreversible pulpitis was determined according to the criteria reported by the American Association of Endodontists Consensus Conference on diagnostic terminology (Levin *et al.* 2009). All volunteers presented with sharp, spontaneous, and lingering pain (often 30 seconds or longer after stimulus removal). Furthermore, they reported that over-the-counter analgesics were not effective.

Inclusion and exclusion criteria

Inclusion and exclusion criteria were based on patients' medical history as well as on clinical and pre-operatory radiographic exams at the initial consultation.

Inclusion criteria were as follows: a) healthy patients with no significant medical history; b) teeth with vital pulp; c) prolonged response to cold testing with Endo-Ice (1,1,1,2 tetrafluoroethane; Hygenic Corp., Akron, OH, USA); d) positive response to percussion test; e) presence of coronal carious lesion; f) presence of bleeding on access cavity; g) teeth that could be appropriately isolated and restored; h) mature root apices visible radiographically with absence of periapical lesion.

Exclusion criteria were as follows: a) volunteers who had received antibiotic treatment within the preceding 3 months; b) who had reported systemic disease starting with ASA 3 (American Society of Anaesthesiology); c) who had taken medications known to influence the immune response; d) teeth that could not be isolated using a rubber dam; e) teeth with no response to cold testing; f) no bleeding on access cavity preparation; g) root filled teeth; h) teeth with cracks; i) the presence of caries on the root surface; j) teeth with radiographic evidence of periapical lesions; h) teeth with periodontal pockets deeper than 3 mm. Patients who received intervention before being referred for endodontic treatment were not included in the study.

Clinical procedures and sample collection

Clinical procedures and sample collection were performed based on previous studies (Barbosa-Ribeiro *et al.* 2019, Duque *et al.* 2019, Louzada *et al.* 2020, Arruda-Vasconcelos *et al.* 2021) and conducted by one experienced operator in all cases.

Briefly, prior to intervention, the patient's facial skin was decontaminated with 2% chlorhexidine solution (Drogal, Piracicaba, SP, Brazil). After local anaesthesia (2% lidocaine with 1:100.000 epinephrine - Alphacaine, Nova DFL, Curicica, RJ, Brazil), the tooth was isolated with a rubber dam and light-curing gingival barrier (Top Dam, Joinville, SC, Brazil) to avoid infiltration of saliva.

The operatory field, crown and surrounding structures was disinfected with 30% hydrogen peroxide (v/v) for 30 s, followed by 5.25% sodium hypochlorite for the same period and then neutralised with 5% sodium thiosulfate. The disinfection of these sites was monitored by taking a swab sample, which was subsequently streaked on a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA – LAB M, Heywood, Lancashire, UK) before being incubated anaerobically and aerobically, respectively, for up to 14 days (Gomes *et al.* 2015, Duque *et al.* 2019, Louzada *et al.*

2020, Arruda-Vasconcelos *et al.* 2021). It was also followed by DNA extraction from the swab and polymerase chain reaction analysis using universal bacterial primers. If any positive cultures or the presence of DNA amplification products were detected, the patient was excluded from the study.

A 2-stage access cavity preparation was performed using a sterile high-speed diamond bur, with irrigation using sterile saline solution and syringe. The water supply was disconnected from the equipment and cooling was performed manually with a sterile saline solution. The first stage was carried out to remove all major contaminants. In the second stage of access cavity preparation, prior to accessing the pulp chamber, the disinfection protocol was repeated as described above. All procedures were performed under strict aseptic conditions.

The root canal treatment was performed under magnification using a dental operating microscope (DF Vasconcellos S/A, São Paulo, SP, Brazil). The working length was estimated by pre-operatory radiography and confirmed using both radiography and electronic apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). A size 25 K-file (Dentsply Sirona, Ballaigues, Switzerland) was used for apical patency to allow insertion of the paper points for periapical tissues sampling, after complete extirpation of the pulp tissues.

Initial sampling (baseline, S1) was based on previous studies (Barbosa-Ribeiro *et al.* 2019, Duque *et al.* 2019). In summary, two sterile absorbent paper points (size 20, Cell Pack; Dentsply Sirona, Ballaigues, Switzerland) were inserted into the periapical tissues (2 mm beyond the apex) and immobilised in position for 1 minute. Subsequently, the paper points were removed and placed in a sterile cryogenic tube and frozen at -80 °C until used in further analysis. Samples were collected from the wider root canal in multi-rooted teeth to favour sampling procedures.

The chemo-mechanical preparation was performed using Reciproc R25 and R40 files (VDW, Munich, Germany) according to the manufacturer's recommendations in a reciprocating motion generated by the engine-driven electric motor (VDW Silver, Munich, Germany). Reciproc files were used in an in-and-out pecking motion of approximately 3 mm in amplitude with slight apical pressure. After 3 pecking motions, the file was removed from the root canal and cleaned using a sterile gauze. Then, a

manual size 20 K-file was placed into the working length to confirm patency. It was repeated until the Reciproc file reached the working length (zero point displayed on the apex locator).

The 20 teeth were randomly assigned into 2 groups according to the root canal irrigant used.

• Group 1: 2% chlorhexidine gel (CHX)

• Group 2: 6% sodium hypochlorite (NaOCl)

Prior to the insertion of each instrument, the root canals were flooded with 2% CHX gel (Endogel; Essencial Pharma Ltd., Itapetininga, SP, Brazil) or 6% NaOCl (Drogal, Piracicaba, SP, Brazil) using a syringe (27-G needle). Subsequently, 5 mL of saline solution was used immediately after the use of each endodontic file to remove debris originated from the mechanical instrumentation. CHX was inactivated with a solution of 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin (Drogal, Piracicaba, SP, Brazil) and NaOCl was inactivated with a solution of 5 mL of 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) for 1 min. Elimination of neutralising agents from the root canals was performed using 5 mL of saline solution.

Final rinse was performed using 3 mL of 17% EDTA ultrasonically activated (3 cycles of 20 seconds) by using ultrasonic device (Satelec/Acteon, Mount Laurel, NJ, USA) with Irrisonic E1 tip (Helse Ultrasonic, Santa Rosa de Viterbo, SP, Brazil). Subsequently, 5 mL of saline solution was used to remove EDTA from the root canals. Subsequently, the canals were dried with sterile paper points, and a sterile cotton pellet was placed on the canal orifice. A 2-mm thickness of temporary cement (Cimpat; Septodont, Saint-Maur-des-Fossés, France) and light-cured composite resin (Filtek Z350 XT; 3M Dental Products, St Paul, MN, USA) combined with a single bond adhesive (3M ESPE) were used for access cavity sealing. The canals were left empty for 48 hours. Then, after removal of the restoration, a second sample (S2) was obtained as described above. The canals were re-instrumented; rinsed with 17% EDTA followed by saline solution as above described, and then dried with sterile paper points prior to the use of an intracanal medicament.

In all cases, freshly prepared calcium hydroxide-based intracanal medication (mixing calcium hydroxide powder with 2% chlorhexidine gel at a ratio of 1:1) was placed to the full working length using Lentulo spiral fillers (Dentsply Sirona, Ballaigues, Switzerland) (Louzada *et al.* 2020, Arruda-Vasconcelos *et al.* 2021). The intracanal medicament was applied for 30 days. A sterile cotton pellet was used to condense the paste at the level of the canal orifice. A 2-mm thickness of temporary cement (Cimpat; Septodont, Saint-Maur-des-Fossés, France) and light-cured composite resin (Filtek Z350 XT; 3M Dental Products, St Paul, MN, USA) combined with a single bond adhesive (3M ESPE) were used for access cavity restoration and sealing.

After the period of intracanal medication application, the root canal was aseptically accessed as previously described and the intracanal medicament was removed using a sterile saline solution (5 mL) and a master apical file (size 40) plus 2 further files. Additionally, 17% EDTA (3 mL) ultrasonically activated as describes previously, was used to completely remove the intracanal medicament. This procedure was performed under magnification to assure complete elimination of the intracanal medicament. The canals were then rinsed with sterile saline solution and calcium hydroxide was neutralized with 0.5% citric acid (5 mL) for 1 min and removed with 5 mL of sterile saline solution. The canals were dried with sterile paper points. Immediately after this procedure, a third sample (S3) was obtained in an identical manner to the S1 and S2 procedure.

For root canal treatment conclusion, a final rinse with ultrasonically activated 17% EDTA (3mL), as described previously, was performed. Irrigation with 5 mL of sterile saline was used for EDTA removal. The root canals were dried with sterile paper points and filled with a single Reciproc gutta-percha cone and Endométhasone N (Septodont, Saint-Maur-des-Fossés, France) sealer. Restoration of access cavities were carried out using a 2-mm layer of temporary cement (Cimpat; Septodont) and light-cured composite material (Filtek Z350; 3 M ESPE) in combination with single-bond adhesive.

Multiplex Immunoassay for quantification of cytokines and matrix metalloproteinases

The levels of cytokines (TNF-α, IL-1α, -1β and -10) and matrix metalloproteinases (MMP-2, -3, -8, -9 and -13) in pg/mL from the periapical tissues of teeth at different phases of root canal treatment were quantified using Luminex Human Magnetic Assay (R&D Systems, Minneapolis, MN, USA) kit and Multiplex Immunoassay (Bio-plex 200 - Bio-Rad Laboratories – Hercules, CA, USA) according to the manufacturer's protocol. Immediately before use, the samples were centrifugated at 16,000 x g for 4 minutes. All reagents used in this study were freshly prepared as described by the manufacturer. Briefly, 50 µL of standard was added to each well to generate a standard curve. Fifty µL of each sample was then placed in each well. Fifty µL of the microparticle cocktail was then added to each well of the microplate (R&D Systems, Minneapolis, MN, USA). The microplate was sealed and incubated for 2 h, at room temperature, in a microplate shaker (800 ± 50 rpm). The microplate was then washed 3 times with 100 µL of the Wash Buffer. After this procedure, 50 µL of Biotin-Antibody Cocktail was added to each well and incubated as previously described for 1 h. Then, the microplate was re-washed as described above. After this procedure, 50 µL of Streptavidin-PE was added to each well and samples were incubated for 30 min under the same conditions as described above. One hundred µL of Wash buffer was then added to each well and this was incubated for 2 min, at room temperature with agitation. Microplate readings were performed using a Bio-Rad analyser (Bio-Rad Laboratories – Hercules, CA, USA). Readings were tabulated and statistically analysed.

Statistical analysis

For the quantitative variables (levels of cytokines and matrix metalloproteinases), the Post Hoc statistical power was calculated using G*Power 3.1.9.7 for Windows. The calculation indicated that the sample size must be a minimum of ten individuals per group with α-type error = 0.05 and power β = 0.80. Data collected for the concentrations of cytokines and matrix metalloproteinases were tabulated in Excel spreadsheet (Microsoft, Redmond, WA, USA). Statistical analysis was performed by using the SPSS software for Windows (SPSS Inc., Chicago, IL, USA). Data normality was verified using a Shapiro-Wilk test. Mann-Whitney and Wilcoxon

tests were performed to verify statistical differences between the groups (2% CHX or 6% NaOCl) and different time-points (before and after chemo-mechanical preparation and after intracanal medication) respectively Significance levels were set at 5% ($P < 0.05$).

Results

Clinical features

A total of 98 volunteers was assessed for eligibility. Seventy-eight were excluded given the following reasons: Presented periodontal pockets > 3 mm (n = 20), were under antibiotic therapy (n = 9), declined to participate (n = 23), did not return in the appropriate day for post-intracanal medication sampling (n = 14) and received intervention before being referred to endodontic treatment (n = 12). Therefore, sampling procedures were performed in 20 teeth with symptomatic irreversible pulpitis.

Pulp vitality was confirmed by exacerbated and prolonged response to cold sensitivity testing. Electric pulp test was also performed and tested positive in all teeth. Bleeding on the coronal access confirmed pulp vitality. All patients were referred for root canal treatment due to spontaneous pain. No pain on palpation was recorded; however, tenderness to percussion was reported in all cases. Patients' mean age was 37.3 ± 8.94 with a total of 17 females and 3 males participating in the study.

The majority of the teeth analysed were molars (15; 8 upper and 7 lower), along with 4 pre-molars (3 upper and 1 lower) and 1 upper canine. In terms of the teeth included in the study, 12 were restored and presented with secondary caries lesion, and 8 had caries and no restoration.

Levels of Cytokines

Table 1 provides data on the levels of cytokines in the periapical tissues of teeth with symptomatic irreversible pulpitis at different phases of the root canal treatment.

In the initial periapical tissue samples (S1), TNF-α and IL-10 were present at higher values in both groups, followed by IL-1α and IL-1β. In S2, it was demonstrated

that there was a significant decrease in the levels of IL-10, IL-α and TNF-α, independently of the root canal irrigant used ($P < 0.05$). After the use of an intracanal medication (S3), there was a reduction in the levels of TNF-α in both groups ($P < 0.05$), which was not observed in the levels of IL-1α and IL-10 ($P > 0.05$). No significant modification was observed in the levels of IL-1β throughout the root canal treatment.

Levels of matrix metalloproteinases

Figure 1 shows the data on the levels of matrix metalloproteinases in the periapical tissues of teeth with symptomatic irreversible pulpitis at different stages of the root canal treatment.

In the initial periapical tissue samples (S1), MMP-2, -8 and 9 were present at higher levels in both groups. In S2, it was observed that there was a significant reduction in the levels of MMP-2 ($P < 0.05$) in both groups, and significant reduction of MMP-8 in the chlorhexidine group. The reduction in the level of MMP-9 was not significant in the chlorhexidine group but it was in the sodium hypochlorite group. Conversely, it was found that there were increased levels of MMP-3 and -13 in both groups ($P < 0.05$). After intracanal medication (S3), there were significant reductions in the levels of MMP-2, -3, -8 and -9 ($P < 0.05$). Significantly increased concentrations of MMP-13 were detected ($P < 0.05$).

Discussion

Only a limited number of studies have monitored the effects of the different phases of root canal treatment on the inflammatory content of teeth with symptomatic irreversible pulpitis, hence this was a motivating factor for conducting this clinical investigation.

The levels of cytokines and matrix metalloproteinases were investigated in the periapical tissues before chemo-mechanical preparation (baseline), 48 hours after chemo-mechanical preparation (S2), and after the use of a calcium hydroxide-based intracanal medication. The quantification of inflammatory mediators in the periapical tissues allowed to comprehend host’s response at different stages of root canal treatment in an inflammatory process.

Cytokine's findings

Different biomarkers have been proposed as indicators of the pathological condition of pulp tissues (Elsalhy *et al.* 2013). TNF-α is an important inflammatory mediator, which regulates the initial host response, including activation and expression of adhesion molecules, induction of other cytokines production and proliferation of immune cells (Elsalhy *et al.* 2013). On the other hand, IL-10, an anti-inflammatory cytokine, has an immunomodulatory role, inhibiting the production of macrophages and other cytokines synthesis, including TNF-α and IL-1β in a range of cell types (Markert 2003, Mosser & Zhang 2008, Elsalhy *et al.* 2013). In this context, it can be suggested that IL-10 prevents exacerbated inflammation response at early stages of the inflammatory process.

With regard to the periapical tissue samples, low levels of IL-1α and -1β were detected in all phases of the endodontic treatment, agreeing with previous study which observed lower levels of IL-1α and -1β compared with other cytokines (Zehnder *et al.* 2003). On the other hand, our findings diverged from those in which observed increased levels of IL-1β than TNF-α in teeth with irreversible pulpitis (Abd-Elmeguid *et al.* 2013).

Our findings could be explained by the fact that inflammation process principally remains confined within the pulp space; however, periapical alteration evidenced by positive response to percussion test may occur (Wolters *et al.* 2017). Other studies involving chronic inflammatory responses have reported higher levels of these cytokines (Martinho *et al.* 2018, Barbosa-Ribeiro *et al.* 2019, Duque *et al.* 2019).

Higher levels of TNF-α and IL-10 were observed at baseline; however, the chemo-mechanical preparation and intracanal medication provided significant reduction in the levels both cytokines. Our results are supported by previous study which reported higher levels of IL-10 in inflamed dental tissues compared with healthy tissues (Elsalhy *et al.* 2013).

The present study revealed that levels of TNF-α are lower compared with teeth with pulp necrosis and periapical lesion (Martinho *et al.* 2018), teeth with root canal treatment failure (Barbosa-Ribeiro *et al.* 2019) and teeth with endodontic-periodontal lesions (Duque *et al.* 2019). Our results are in agreement with these previously

reported findings in which the root canal treatment promoted reduction in the levels of TNF-α (Barbosa-Ribeiro *et al.* 2019, Duque *et al.* 2019).

Interleukin (IL)-1α and IL-1β are cytokines with similar molecule structure and which interact with the same receptor (Zehnder *et al.* 2003). Both cytokines have been associated with bone resorption (Zehnder *et al.* 2003, Rechenberg *et al.* 2016). Low levels of IL-1β were detected in all phases of the endodontic treatment, agreeing with previous studies which observed lower levels of IL-1β compared with other biomarkers (Zehnder *et al.* 2003). Our findings could be explained by the fact that inflammation process mostly remains confined within the pulp space; however, periapical alteration evidenced by positive response to percussion test may occur (Wolters *et al.* 2017), which can be an explanation to slight detection of IL-1α at baseline. Other studies involving chronic inflammatory responses have reported higher levels of these cytokines (Martinho *et al.* 2018, Barbosa-Ribeiro *et al.* 2019, Duque *et al.* 2019) than observed in the present study. CMP and ICM did not modified the levels of IL-1β compared with the baseline (S1).

Overall, it was not observed increased levels of cytokines after the endodontic procedures, suggesting that both chlorhexidine and sodium hypochlorite were not harmful to the periapical tissues.

Matrix metalloproteinases' findings

It is known that matrix metalloproteinases in part regulate the inflammation and destructive processes which occur in pulp tissues (Jain & Bahuguna 2015). Overall, the levels of MMP-2, -8 and -9 were higher in the early-stage samples compared with those obtained after performing chemo-mechanical preparation and/or after intracanal medication.

At baseline it is expected more inflammatory environment than after endodontic procedures, agreeing with previous studies that have found higher levels of matrix metalloproteinases in inflamed pulps compared with healthy pulps (Accorsi-Mendonça *et al.* 2013, Mente *et al.* 2016). Moreover, substances used during the root canal treatment including chlorhexidine, sodium hypochlorite and calcium hydroxide have been associated with inhibition of matrix metalloproteinase production (Jain & Bahuguna 2015, Barbosa-Ribeiro *et al.* 2019, Carvalho *et al.* 2020).

In contrast, after the chemo-mechanical preparation, it was observed a significantly increased levels of MMP-3 compared with samples obtained from initial samples. Literature has associated MMP-3 with the angiogenic response (Zheng *et al.* 2009). Furthermore, previous study has reported the anti-inflammatory effect of this molecule (Eba *et al.* 2012). After the use of a calcium hydroxide-based intracanal medication, it was observed that there was a reduction in the levels of MMP-3, giving similar levels to those initially detected. These data may relate to the balance between inflammation and healing processes.

Reduced levels of MMP-13 were observed in the initial samples; however, after the endodontic procedures the levels increased, especially after the use of intracanal medication. Our results were comparable with previous study that detected increased levels of MMP-13, suggesting this was due to the association with the healing process (Barbosa-Ribeiro *et al.* 2019).

Final considerations

Overall, the endodontic therapy irrespectively of the root canal irrigant used (chlorhexidine or sodium hypochlorite) modified the levels of cytokines and matrix metalloproteinases in the periapical tissues of teeth with symptomatic irreversible pulpitis.

A potential limitation of this study was the small volume sampled, which was not sufficient to quantify the concentration of total proteins. Similar condition was previously described (Zehnder *et al.* 2011, Geraldeli *et al.* 2012). Nevertheless, this data should be considered in future investigations.

This clinical investigation was undertaken to provide knowledge regarding the clinical levels of cytokines and matrix metalloproteinases in teeth with inflamed dental pulps at the different phases of root canal treatment in order to characterise host's inflammatory response.

Although the use of an intracanal medication in teeth with vital pulps is not mandatory, in some situations including emergency appointments, persistent bleeding, patients with management issues (*i.e.,* pregnancy, children and seniors) and lack of time for finalising root canal treatment, the use of intracanal medication is

recommended. Tissue regeneration occurs due to the equilibrium between the inflammation and repair processes promoted by a complex network involving different mediators of inflammation (Cooper *et al.* 2010, Elsalhy *et al.* 2013) in response to clinical procedures such as chemo-mechanical preparation and intracanal medication. The use of a calcium hydroxide based intracanal medication has several advantages, as it presents not only anti-inflammatory and antimicrobial properties; acts as chemical and physical barrier; but also maintains at low levels the cytokines and matrix metalloproteinases in the periapical tissues, as observed in this investigation and in a previous study (Tavares *et al.* 2013). Data also indicate that this can favour the treatment outcome regarding the inflammatory context in teeth presenting inflamed pulps.

Conclusion

Root canal treatment had a positive effect on the levels of cytokines and matrix metalloproteinases, irrespective of the irrigant used (2% chlorhexidine gel or 6% sodium hypochlorite) during the chemo-mechanical preparation. Additionally, calcium hydroxide based intracanal medication was effective in keeping the inflammatory biomarkers at low levels in teeth with symptomatic irreversible pulpitis.

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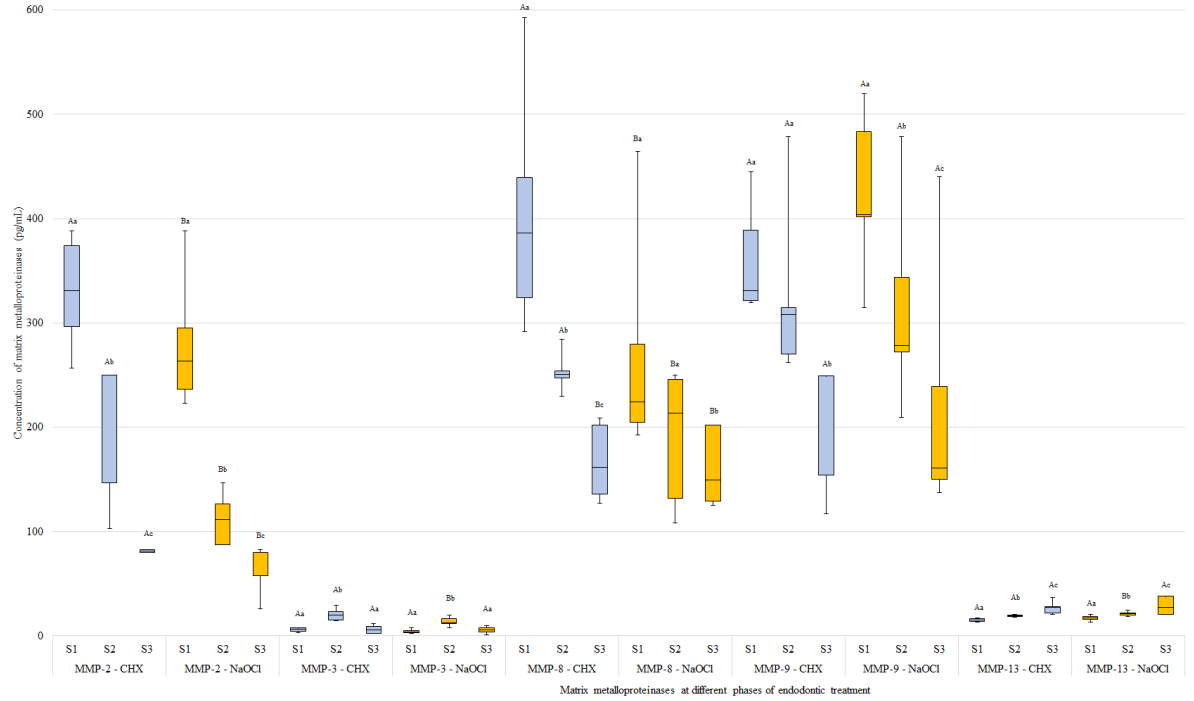
Figure and table legends

Table 1. Median (minimum – maximum) concentration of cytokines (pg/mL) in the periapical tissues at different phases of root canal treatment in teeth with irreversible pulpitis.

Cytokine	Endodontic treatment phase	Root canal irrigant	
		2% Chlorhexidine	6% Sodium hypochlorite
TNF-α	S1	4.74 (2.08 – 8.81) Ac	5.25 (2.98 – 8.99) Ac
	S2	0.71 (0.69 – 0.82) Ab	0.71 (0.70 – 1.19) Ab
	S3	0.46 (0.25 – 0.67) Aa	0.40 (0.23 – 0.71) Aa
IL-1α	S1	1.91 (0 – 4.82) Ab	0.96 (0 – 4.29) Ab
	S2	0 (0 – 3.78) Aa	0.32 (0 – 1.53) Aa
	S3	0 (0 – 1.53) Aa	0 (0 – 1.53) Aa
IL-1β	S1	0 (0 – 0.12) Aa	0 (0 – 0) Aa
	S2	0 (0 – 0) Aa	0 (0 – 0) Aa
	S3	0 (0 – 0) Aa	0 (0 – 0) Aa
IL-10	S1	4.12 (2.68 – 5.96) Ab	2.14 (1.42 – 16.27) Aa
	S2	0 (0 – 0) Aa	2.34 (0 – 11.06) Ba
	S3	0 (0 – 0) Aa	2.42 (0 – 8.56) Ba

S1: Before chemo-mechanical preparation; S2: 48 hours after chemo-mechanical preparation; S3: After intracanal medication. Different uppercase letters indicate a significant difference between the groups ($P < 0.05$). Different lowercase letters indicate a significant difference within the same group before and after chemo-mechanical preparation and after intracanal medication ($P < 0.05$).

Figure 1. Box plot showing the levels of matrix metalloproteinases (pg/mL) in the periapical tissues at different phases of endodontic treatment in teeth with irreversible pulpitis. Different uppercase letters indicate a significant difference between the groups (P < 0.05) by Mann-Whitney test. Different lowercase letters indicate a significant difference within the same group before and after chemo-mechanical preparation and after intracanal medication (P < 0.05) by Wilcoxon test.



2.4 EFFECTIVENESS OF TWO ROOT CANAL IRRIGANTS ON PAIN PERCEPTION, MICROBIAL ASPECTS AND LEVELS OF PROSTAGLANDIN E2 AND SUBSTANCE P IN TEETH WITH SYMPTOMATIC IRREVERSIBLE PULPITIS: A RANDOMISED CLINICAL TRIAL

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Abstract

Introduction: This study compared the effects of 2% CHX gel and 6% NaOCl used as root canal irrigants on the level of pain, microbial aspects and levels of prostaglandin E2 (PGE) and substance P in teeth with symptomatic irreversible pulpitis. Calcium hydroxide-based intracanal medication (ICM) was also evaluated.

Methods: Twenty teeth were randomly assigned into two groups. Samples were collected from root canals and periapical tissues before chemo-mechanical preparation (CMP), after microbial analysis, 48 hours after CMP (levels of PGE2 and substance P) and after ICM. Pain perception (visual analogue scale), microbial aspects (culture method and nested PCR) and levels of PGE2 and substance P (ELISA) were assessed. Statistical analysis was performed at significance level of 5%.

Results Pain perception was higher at baseline and significantly reduced after CMP (CHX and NaOCl groups). No pain was reported after ICM and both CHX and NaOCl were effective in reducing culturable bacteria in root canals. Low levels of bacteria were detected after ICM, with *E. faecalis* and *F. nucleatum* being the most detected species by using molecular method. Irrespectively of using CHX or NaOCl, microbial DNA was detected after CMP and ICM. Higher levels of PGE2 and substance P were detected at baseline and CMP performed with CHX and NaOCl did not significantly reduce their levels ($P > 0.05$). On the other hand, ICM was effective in lowering the levels of PGE2 and substance P.

Conclusions 2% CHX or 6% NaOCl were similar regarding clinical, microbiological, and inflammatory aspects. ICM helped control infection and attenuate inflammation by lowering the levels of PGE2 and substance P. Pain perception was reduced after endodontic treatment, regardless of the substance used.

Keywords: Bacteria, Chlorhexidine, Prostaglandin E2, Sodium hypochlorite, Substance P.

Introduction

Dental caries is the most common disease in the oral cavity and one of the most common chronic multifactorial diseases affecting the human population (Struzycka 2014, Sun *et al*. 2020), also being associated with different clinical features such as thermal sensitivity, spontaneous pain, and tenderness to percussion as it progresses (Levin *et al*. 2009, Sun *et al*. 2020).

Important impermeable barriers (*i.e.,* enamel and cementum) undergo demineralisation as dental caries progresses, thus allowing bacteria to move forward into the dentine-pulp complex (Hirsch *et al*. 2017). Due to the microbial invasion, accumulation of bacteria and inflammatory mediators is typically observed in the local of infection (Khorasani *et al*. 2020). The term symptomatic irreversible pulpitis refers to a severe degenerative process in the pulp tissue characterised by intense spontaneous pain or persistent pain after stimulus removal, which results in pulp necrosis if no treatment is performed (Levin *et al*. 2009).

A variety of biomarkers are related to inflammatory process and symptoms, including prostaglandin E2 (PGE2) and substance P. Several cells and tissues of the human body synthesise PGE2, which is considered the main prostaglandin in acute inflammation (Park *et al*. 2006). During the inflammatory process, different cells such as macrophages, fibroblasts and dendritic cells synthesise PGE2, which is also involved in the perception of pain by reducing the threshold of nociceptors and by compressing the nerve fibres through vasodilatation (Karataş *et al*. 2020). Furthermore, PGE2 has been associated with collagen degradation and increased vascular permeability (Martinho *et al*. 2011). Substance P is a neuropeptide released by nerve endings as a result of thermal, chemical and/or mechanical stimuli (Caviedes-Bucheli *et al*. 2008, 2009, Bıçakcı *et al*. 2016). This neuropeptide plays important role in inflammatory alterations, including vasodilatation, plasma extravasation and release of cytokines and prostaglandins (Awawdeh *et al*. 2002, Bamini *et al*. 2019, Arslan *et al*. 2020).

Endodontic treatment is clinically relevant for controlling infection, preventing development of apical periodontitis, or promoting its healing (Barbosa-Ribeiro *et al*. 2019). The use of chemical substances with antimicrobial activity is vital for microbial

reduction, which consequently attenuates inflammation and pain. Currently, sodium hypochlorite (NaOCl) has been widely used in endodontics at higher concentrations (*i.e.*, 6% to 8.25%) (Barbosa-Ribeiro *et al*. 2016, Demenech *et al*. 2021), mainly due to its antimicrobial activity and tissue-dissolving capacity. Chlorhexidine (CHX) has broad-spectrum antimicrobial activity and substantivity against Gram-positive and Gram-negative species (Gomes *et al*. 2013). However, there is no consensus in the literature about the best irrigating solution, that is, one with desirable properties but which has not yet been found. Furthermore, only a few studies used both types of irrigants in teeth with irreversible pulpitis. Therefore, the aim of this clinical study was to compare the effects of 2% CHX gel and 6% NaOCl on the reduction of perceived pain, microbial content, and levels of substance P and PGE2 in teeth with symptomatic irreversible pulpitis. Furthermore, this study also investigated the effects of a calcium hydroxide based-intracanal medication regarding all these aspects.

Materials and methods

This study was approved by the Human Research Ethics Committee of the Piracicaba Dental School, State University of Campinas (UNICAMP), Piracicaba (SP), Brazil, according to protocol number CAAE 86140218.0.0000.5418. All patients signed an informed consent form prior to their participation and the registry of cases included in this study can be found in the Brazilian Clinical Trials Registry (*ReBEC*; UTN U1111-1238-5402).

Study population

Patients attending the Dental Emergency Service of the State University of Campinas Piracicaba Dental School for endodontic treatment due to symptomatic irreversible pulpitis were invited to participate in the study. On admittance, the patients were randomly assigned to one or other group by flipping a coin. This randomisation process resulted in 47 teeth in the CHX group and 51 teeth in the NaOCl group. All clinical procedures and sample collections were performed by one experienced operator (RAV).

The criteria set by the American Association of Endodontists Consensus Conference on Diagnostic Terminology were used to determine the diagnosis of irreversible pulpitis (Levin *et al*. 2009).

The inclusion criteria were the following: presence of deep carious lesions, teeth with vital pulp allowing proper isolation with a rubber dam, prolonged and exacerbated response to cold testing using 1,1,1,2 tetrafluoroethane (Endo-Ice, Hygenic Corp., Akron, OH, USA) for more than 30 seconds, positive response to electric testing (Odous De Deus, Belo Horizonte, MG, Brazil), and bleeding in the access cavity.

The exclusion criteria were the following: root-filled teeth, teeth with necrotic pulps (*i.e.* negative response to cold and/or electric testing and no bleeding in the access cavity), teeth with caries on the root surface, presence of cracks, teeth allowing no isolation, teeth with periapical lesion radiographically evidenced, teeth with periodontal pockets ≥ 3 mm. Individuals taking medications known to influence the immune response, on antibiotic treatment within the preceding 3 months and reporting systemic disease starting with ASA 3 (American Society of Anaesthesiology) were not included. Patients who received any intervention or have taken analgesics prior to endodontic treatment were not included in the investigation either.

Clinical procedures

The methods for clinical procedures and sample collection were based on previous studies (Duque *et al*. 2019, Arruda-Vasconcelos *et al*. 2021).

Local anaesthesia

Briefly, prior to intervention, 2% CHX solution (Drogal, Piracicaba, SP, Brazil) was used for decontamination of the patient's facial skin. After local anaesthesia (2% lidocaine + epinephrine at 1:100.000), the teeth were isolated with rubber dam to prevent leakage of saliva.

Disinfection protocol

Disinfection of operative field, crown and surrounding structures was performed with 30% H_2O_2 (v/v) for 30 seconds, followed by 2.5% NaOCl (30 seconds) and subsequent neutralisation with 5% sodium thiosulfate. A swab sample from both

internal and external surfaces of the crown and surrounding structures was taken to check the disinfection.

The swab sample was streaked onto a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA – LAB M, Heywood, Lancashire, UK) before being incubated anaerobically and aerobically, respectively, for up to 14 days (Duque *et al*. 2019, Louzada *et al*. 2020). Next, DNA extraction from the swab and PCR analysis using universal bacterial primers were performed. If any positive culture or presence of DNA amplification product were obtained, then the patient was excluded from the study.

Endodontic procedures and sample collection

A two-stage access cavity preparation was performed by using a sterile high-speed diamond round bur and the water supply was disconnected from the equipment, with refrigeration/irrigation being performed manually with sterile distilled water and syringe. The first stage aimed to remove major contaminants, whereas the second stage involved disinfection, whose protocol was repeated as described above prior to accessing the pulp chamber. All procedures were performed under strict aseptic conditions.

Endodontic treatment was performed under high magnification with a dental operating microscope (DF Vasconcellos S/A, São Paulo, SP, Brazil). After removal of pulp tissue, the working length was radiographically determined and confirmed with an electronic apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel) and a K-file (size 20, Dentsply Sirona, Ballaigues, Switzerland).

Tables 1 and 2 show data on sampling moments, number of paper points, time required for sample collection, apical limit, storage media and temperature before analysis. Samples were collected from single and multi-rooted teeth, from larger root canals in case of the latter. Next, the samples were immediately transferred to laboratory for microbial culture processing.

Reciproc R25 and R40 files were used (VDW, Munich, Germany) according to the manufacturer’s instructions in a reciprocating motion generated by an electric motor (VDW Silver, Munich, Germany) during chemo-mechanical preparation.

Reciproc files were used in an in-and-out pecking motion (approximately 3 mm amplitude) with slight apical pressure. After three pecking motions, the file was removed from the root canal and cleaned with sterile gauze. Next, a manual size 20 K-file was placed into the working length for patency confirmation. This protocol was repeated until the Reciproc file reached the working length (zero displayed on the apex locator).

Prior to the insertion of each instrument, the root canals were flooded with 2% CHX gel or 6% NaOCl by using a syringe (27-G needle). After the use of each instrument, 5 mL of saline solution was used to remove debris from instrumentation. At the end of instrumentation, a solution containing 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin (Drogal, Piracicaba, SP, Brazil) was used for CHX neutralisation. NaOCl was inactivated with a solution containing 5 mL of 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) for 1 minute and the neutralising agents were eliminated with 10 mL of saline solution. Final rinse was performed with 3 mL of 17% ethylenediaminetetraacetic acid (EDTA) ultrasonically activated (3 cycles of 20 seconds) by using ultrasonic device (Satelec/Acteon, Mount Laurel, NJ, USA) with Irrisonic E1 tip (Helse Ultrasonic, Santa Rosa de Viterbo, SP, Brazil), and 10 mL of sterile saline solution was used for elimination of EDTA.

A calcium hydroxide-based intracanal medication was prepared by mixing $Ca(OH)_2$ powder with 2% CHX gel at a 1:1 ratio immediately before use. The intracanal medicament was placed in the full length of the root canal by using Lentulo spiral fillers positioned 2 mm from the apex (Dentsply Sirona, Ballaigues, Switzerland). A periapical radiograph was taken to ensure the correct positioning of the medicament, which remained for 30 days. The $Ca(OH)_2$ paste was condensed by using a sterilised cotton pellet at the level of the canal orifice. The access cavity was sealed with temporary cement of 2 mm thickness (Cimpat, Septodont, Saint-Maur-des-Fossés, France), followed by a light-cured composite resin (Filtek Z350 XT, 3M Dental Products, St Paul, MN, USA) in combination with a single bond adhesive (3M ESPE).

In the following appointment, the root canals were aseptically accessed as described above and the intracanal medicament was removed with 5 mL of sterile saline solution and master apical file (size 40) plus two other files. In addition, 17% EDTA was ultrasonically activated as previously described for complete removal of the

medicament. Then, the root canals were rinsed with 10 mL of sterile saline solution and $Ca(OH)_2$ was neutralised with 5 mL of 0.5% citric acid for 1 minute and removed with 10 mL of sterile saline solution.

Finally, the root canals were dried with sterile paper points and filled with a single Reciproc gutta-percha cone and endodontic sealer (Endométhasone N, Septodont, Saint-Maur-des-Fossés, France). The restoration of the access cavities was performed as described above.

Endodontic treatment was performed in all cases of indication, even when the patients were not included in the study or excluded from it.

Pain perception

Patients included in the study were asked to report their perception of pain in a visual analogue scale (VAS) (Jensen *et al*.,1986) according to the scores as follows:

- 0 = no pain
- 1 – 3 = mild pain
- 4 – 6 = moderate to severe pain
- 7 – 9 = very severe
- 10 = Worst pain possible

The reports on pain were taken in the first appointment, 48 hours after the first appointment and after intracanal medication.

Culture procedure

The method used to count the colony-forming units (CFUs) was based on previous studies (Martinho & Gomes 2008, Barbosa-Ribeiro *et al*. 2019, Aveiro *et al*. 2020). Briefly, the tubes containing the root canal samples and pre-reduced transport medium (VMGA III) were transported within 15 minutes to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) for bacterial culture analysis.

The tubes were shaken thoroughly for 60 seconds (Vortex; Marconi, Piracicaba, São Paulo, Brazil) and then serial 10-fold dilutions were made up to 10^{-4} in tubes containing fastidious anaerobe broth (FAB, Lab M, Bury, UK). Fifty µL of each serial dilution was plated onto 5% defibrinated sheep blood fastidious anaerobe agar (FAA, LAB M, Bury, UK) by using sterile plastic spreaders to isolate obligate anaerobes and

facultative anaerobes. The plates were anaerobically incubated at 37 °C for up to 14 days. After this period, CFUs were visually quantified in each plate.

Nested PCR

Nested PCR was performed according to previous study (Louzada *et al*. 2020). Briefly, bacterial DNA was extracted by using the QIAamp DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. After extraction, the DNA concentration was spectrophotometrically measured at 260 nm (Nanodrop 2000; Thermo Scientific, Wilmington, DE). The first DNA amplification was directed to regions covering ribosomal RNA genes 16S and 23S by using universal primers.

PCR reactions were run in a 50-µL volume, each containing 5 µL of buffer for PCR reaction (10X Reaction Buffer; Invitrogen, São Paulo, SP, Brazil), 2 µL of a mixture containing phosphate deoxyribonucleotides (2 mmol/L) (dNTPs, Invitrogen, São Paulo, SP, Brazil), 4 µL of magnesium chloride solution (25 mmol/L) (MgCl2, Invitrogen, São Paulo, SP, Brazil), 1 µL of forward and reverse primer solutions (20 mmol/L) (Invitrogen, São Paulo, SP, Brazil), 34.8 µL of ultrapure water free of deoxyribonuclease and ribonuclease, 0.12 µL of Taq polymerase enzyme (Taq Platinum, Invitrogen, São Paulo, SP, Brazil), and 2 µL of DNA extracted from the collected samples at a concentration of 40 ng/mL. The reaction consisted of initial denaturation at 95 °C for 2 minutes followed by 22 cycles with denaturation at 94 °C for 1 minute, annealing at 42 °C for 2 minutes, extension at 72 °C for 3 minutes, and then a final elongation at 72 °C for 10 minutes.

The universal PCR reaction products were analysed on 1% agarose gel electrophoresis (Invitrogen, São Paulo, SP, Brazil), stained with ethidium bromide, EDTA (pH 8.0) and 5 mg/mL of tris-borate-EDTA buffer (Invitrogen, São Paulo, SP, Brazil). A 1-kilobase molecular weight standard ladder (Invitrogen, São Paulo, SP, Brazil) was included in each gel, and after each run (60 V for 40 minutes), the bands were observed under ultraviolet light transillumination. Either positive or negative identification was based on the presence of clean bands of approximately 1500 base pairs.

A 1-µL aliquot of the first PCR reaction was used for the second one by using a species-specific primer (F) combined with L189R primer. The reactions were

processed in 25-µL samples, in which each contained 2.5 µL of buffer for PCR reaction (Invitrogen, São Paulo, SP, Brazil); 2.5 µL of a mixture of phosphate deoxyribonucleotides (2 mmol/L) (Invitrogen, São Paulo, SP, Brazil); 1.5 µL of sodium magnesium solution (25 mmol/L) (Invitrogen, São Paulo, SP, Brazil); 0.62 µL of a 100-mmol/L solution of forward and reverse primers each (20 mmol/L) (Invitrogen, São Paulo, SP, Brazil); 16.14 µL of ultrapure water, deoxyribonuclease and ribonuclease; 0.12 µL of Taq polymerase enzyme (Platinum, Invitrogen, São Paulo, SP, Brazil); and a 1-µL aliquot of the first reaction. The reaction consisted of initial denaturation at 95 °C for 2 minutes, 22 cycles with denaturation at 94 °C for 1 minute, annealing at 42 °C for 2 minutes and extension at 72 °C for 3 minutes, followed by a final extension at 72 °C for 10 minutes.

Specificity, size, direction, and sequence of the primers used in the PCR reaction are shown in Table 3.

Quantification of PGE2 and Substance P

The levels of PGE2 and substance P in the periapical tissues of teeth in different phases of endodontic treatment were quantified by using specific Quantikine® Human ELISA Kit (R&D Systems®, Minneapolis, MN, USA) and quantitative sandwich enzyme immunoassay technique according to the manufacturer's protocol.

Standard and sample solutions were added to an ELISA well plate (R&D Systems®, Minneapolis, MN, USA), which had been pre-coated with immobilised monoclonal antibody to PGE2, and substance P supplied by the manufacturer. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for each molecule was added to the wells, forming an immune complex. The plate was incubated for 60 minutes at room temperature on a shaker before washing to remove unbound enzymes. After another washing, a substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react. The colour development was stopped, and its intensity measured, thus revealing the concentrations of PGE2 and substance P. The levels of PGE2 and substance P were assessed with an ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm, and the negative control values were normalised. Each densitometric value, expressed as mean and standard deviation, was obtained from two independent experiments.

Statistical analysis

The resulting data were entered in Excel spreadsheet (Microsoft, US). Sample size was calculated with a power of 80% (β = 0.20), indicating a minimum sample size of 20 teeth (10 *per* group) and showing a 5% difference (α = 0.05) between the groups according to Cohen (1988). The calculations were made by using the G*Power 3.1.9.7 software for Windows. For non-parametric tests, corrections were made according to Prajapati *et al*. (2010). SPSS software for Windows (SPSS Inc., Chicago, IL, USA) was used for conducting the statistical analysis. Shapiro-Wilk's test was used to assess data normality. Non-parametric Mann-Whitney's and Wilcoxon's tests were used for comparisons between root canal irrigating solutions and different time-points, respectively. Friedman's test was used to assess statistical differences in the frequency of bacteria by using nested PCR and pain perception at different time-points. Significance level was set at 5% in all tests ($P < 0.05$).

Results

Clinical features

Figure 1 shows the flowchart for selection of the volunteers for eligibility in the study.

Pulp vitality was confirmed based on exacerbated and prolonged response to cold sensitivity testing. Electric pulp test was also used, and all teeth tested positive. Bleeding in the access cavity confirmed pulp vitality. All patients were referred for endodontic treatment due to spontaneous pain, and although no pain on palpation was reported, tenderness to percussion was found in all cases. Seventeen females and three males participated in the study, with a mean age of 37.3 ± 8.94 years old.

The majority of the teeth analysed were molars (8 upper and 7 lower teeth), whereas four were pre-molars (3 upper and 1 lower teeth) and 1 upper canine. In addition, 12 teeth had been restored and had secondary caries lesion, and eight had caries and no restoration.

At 6-month follow-up, all teeth responded normally to palpation and percussion

tests. Radiographic images revealed absence of periapical alterations and dental prophylaxis was performed as a preventive procedure, including coronal polishing.

Pain perception

All patients presented with spontaneous pain, with CHX and NaOCl groups reporting median scores of 9.5 (8 – 10) and 10 (8 – 10) for pain perception, respectively. Significant reduction in the perception of pain was observed 48 hours after the first appointment in both groups ($P < 0.05$). Median values for S2 were 0 (0 - 2) in the CHX group and 0 (0 - 3) in the NaOCl group. No pain was reported after the use of intracanal medication in both groups (S2 – S3, $P > 0.05$).

Microbiological assessment

The samples included in this study showed neither positive culture, nor positive PCR bands, thus confirming that all procedures were performed under strict aseptic conditions.

CFU count

Table 4 shows the overall findings regarding colony forming units *per* millilitre (CFU/mL) in teeth with symptomatic irreversible pulpitis at different phases of endodontic treatment.

Culturable bacteria were recovered from all root canals at the baseline (S1). No significant differences in the number of CFU/mL were observed between the CHX and NaOCl groups ($P > 0.05$). Significant microbial reduction was observed after chemo-mechanical preparation in both groups with no difference between them ($P > 0.05$). Intracanal medication reduced the levels of bacteria within the root canals in both groups (S2-S3, $P > 0.05$).

Nested PCR

A total of 17 species-specific primers were investigated, with 16S rRNA universal reaction detecting the presence of microbial DNA in all samples. The most prevalent species detected at the baseline were *E. faecalis* (CHX: 10/10 and NaOCl: 10/10) and *F. nucleatum* (CHX: 8/10 and NaOCl: 6/10). *P. endodontalis*, *P. micra*, *F.*

alocis, *T forsythia, T. denticola* and *G. morbillorum* were also detected (Figure 2).

After chemo-mechanical preparation, there was a reduction in the detection of *E. faecalis* (CHX: 20% and NaOCl: 40%) and *F. nucleatum* (CHX: 37.05% and NaOCl: 50%) ($P < 0.05$). However, there was no significant reduction in the detection of *P. micra, G. morbillorum, F. alocis, T. forsythia* and *T. denticola* ($P > 0.05$). On the other hand, *P. endodontalis* was not detected at this stage.

Intracanal medication provided additional reduction against *F. nucleatum* while no detection of *F. alocis, P. endodontalis, T. forsythia* and *T. denticola* was occurred.

A. actinomycetemcomitans, *A. naeslundii*, *D. pneumosintes*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *P. tannerae*, *S. sobrinus*, and *T. socranskii* were not detected in teeth with irreversible pulpitis.

Levels of PGE2

The levels of PGE2 in the periapical tissues in the different stages of endodontic treatment performed in CHX and NaOCl groups are shown in Figure 3. The highest levels of PGE2 were observed at baseline (S1). Chemo-mechanical preparation performed with CHX or NaOCl reduced the levels of PGE2, with no significant difference compared to S1 ($P > 0.05$). In addition, intracanal medication significantly reduced the levels of PGE2 in both groups (S2 – S3, $P < 0.05$).

Levels of substance P

The levels of substance P in the periapical tissues in the different stages of endodontic treatment performed in CHX and NaOCl groups are shown in Figure 4. Higher levels of substance P were detected at baseline (S1). Although a reduction in the levels of substance P was observed after chemo-mechanical preparation, it was not statistically significant ($P > 0.05$) in both CHX and NaOCl groups. Intracanal medication was effective in lowering the levels of substance P in both groups (S2 – S3, $P < 0.05$).

Discussion

The invasion of bacteria into the dentine-pulp complex initiates an immune response, leading to vasodilatation, oedema, release of different inflammatory biomarkers and pain. With regard to the endodontic infection, irreversible pulpitis is situated at the beginning of the inflammatory process compared to other clinical conditions. Therefore, it is important to comprehend the predominant endodontic pathogens and host's response in the early stages of inflammatory process. This clinical study compared the effectiveness of CHX and NaOCl on microbial and inflammatory aspects in teeth with symptomatic irreversible pulpitis.

Traditional culture method was used because of its reliability for clinically monitoring the microbial load within the root canals in different stages of endodontic treatment (Barbosa-Ribeiro *et al*. 2019, Aveiro *et al*. 2020, Bícego-Pereira *et al*. 2020). However, this technique has drawbacks as it underestimates the microbial diversity and counts (Bícego-Pereira *et al*. 2020). Overall, our findings showed microbial growth in all initial root canal samples at a concentration of 10^2 CFU/mL. After chemo-mechanical preparation, it was observed a significant reduction in the level of culturable bacteria, irrespective of the root canal irrigant used, agreeing with previous findings (Valera *et al*. 2015, Barbosa-Ribeiro *et al*. 2016). Furthermore, literature shows that microbial elimination from root canals ranges from approximately 80% to over 99% (Martinho *et al*. 2008, Endo *et al*. 2012, Dornelles-Morgental *et al*. 2011, Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020). Intracanal medication did not promote additional effects as a considerable microbial reduction was observed after chemo-mechanical preparation using CHX and NaOCl, which agrees with previous findings (Barbosa-Ribeiro *et al*. 2016).

Nested-PCR method identified Gram-positive and Gram-negative species. Although this is a high-sensitive method for DNA detection, being successfully used for microbial profile investigation (Duque *et al*. 2019, Louzada *et al*. 2020, Barbosa-Ribeiro *et al*. 2020), it was not possible to identify whether the bacteria are viable or not. For this reason, CFU count was used as a complementary technique.

Enterococcus faecalis was detected in all initial samples (20/20). It is known that *E. faecalis* is frequently isolated from previously filled teeth (Barbosa-Ribeiro *et al*.

2020, Bícego-Pereira *et al*. 2020) and is highly resistant to endodontic procedures. However, this species has also been detected in different clinical situations, including teeth with primary endodontic infection, endodontic-periodontal lesions and symptomatic irreversible pulpitis (Gomes *et al*. 2015, Aveiro *et al*. 2020, Louzada *et al*. 2020, Arruda-Vasconcelos *et al*. 2021), in which different methods were used (*e.g.*, 16S rRNA gene sequencing and checkerboard DNA-DNA hybridization). Reduction, but not elimination, of *E. faecalis* was achieved by using both irrigating solutions, agreeing with previous findings (Gomes *et al*. 2015, Valera *et al*. 2015, Barbosa-Ribeiro *et al*. 2016, Aveiro *et al*. 2020, Louzada *et al*. 2020, Arruda-Vasconcelos *et al*. 2021).

Importantly, *Fusobacterium nucleatum* was detected in 70% of the samples, a higher prevalence than that previously found (41.7% - 50%) in a study conducted in teeth with vital pulps (Zargar *et al*. 2020). *F. nucleatum* is a Gram-negative species associated with swelling and pain (Gomes *et al*. 2004), in addition to participating in pulpal infections by causing dysregulation of inflammasome (Aral *et al*. 2020). *F. nucleatum* has adhesive characteristics which may favour co-aggregation with other microbial species (Cavalli *et al*. 2017). In fact, elimination of *F. nucleatum* was not fully achieved, irrespective of using CHX or NaOCl, a finding also corroborated by other studies (Barbosa-Ribeiro *et al*. 2020, Louzada *et al*. 2020).

Despite being detected at lower levels, it is worth pointing to the presence of bacteria belonging to the genuses *Filifactor*, *Gemella*, *Parvimonas*, *Porphyromonas*, *Tannerella* and *Treponema* in early stages of endodontic infection. Notably, these bacteria are closely related to long-standing infections and teeth with endodontic-periodontal lesions (Endo *et al*. 2013, de Brito *et al*. 2020, Barbosa-Ribeiro *et al*. 2020, Gomes *et al*. 2015, 2020, Louzada *et al*. 2020).

An inflammatory response initiates as a response to microbial invasion. Consequently, vasodilatation, oedema, accumulation of immune cells and release of biomarkers can be observed as a result (Awawdeh *et al*. 2002, Bamini *et al*. 2019, Arslan *et al*. 2020, Karataş *et al*. 2020). In teeth with pulpitis, spontaneous pain is a relevant clinical feature which should be considered for intervention. It is known that both prostaglandin E2 and substance P are involved in the perception of pain (Caviedes-Bucheli *et al*. 2008, 2009, Bıçakcı *et al*. 2016, Karataş *et al*. 2020).

The levels of PGE2 were higher in the initial samples of teeth with irreversible pulpitis. A previous study suggested that evaluation of PGE2 could be a diagnostic tool for pulpitis, as higher levels of PGE2 were observed in teeth with inflamed pulps (Petrini *et al*. 2012). Increased levels of PGE2 were also observed in teeth with apical periodontitis (Martinho *et al*. 2018, Karataş *et al*. 2020), commonly known for its inflammatory process potentially triggered by microorganisms. The results revealed that chemo-mechanical preparation performed with CHX or NaOCl reduced the levels of PGE2, but it was not statistically significant compared to baseline. On the other hand, the use of an intracanal medication was effective in lowering the levels of PGE2 in periapical tissues.

As for substance P, higher levels were found at baseline. These findings are in accordance with previous study in which the highest level of substance P was detected in teeth with symptomatic irreversible pulpitis (Sattari *et al*. 2010, Dincer *et al*. 2020). Furthermore, higher levels of salivary substance P were detected in teeth with symptomatic apical periodontitis than those observed in the control visit one week after endodontic treatment (Arslan *et al*. 2020), thus emphasizing the importance of this neuropeptide in inflammation and pain.

It has been proposed that both PGE2 and substance P are closely related to pain (Ma 2010). An increased perception of pain was reported by all volunteers at baseline. However, after endodontic procedures performed with CHX or NaOCl, the level of pain was considerably reduced. This finding suggests that lower levels of pain are related to microbial reduction and lower levels of inflammatory biomarkers. A higher concentration of NaOCl has not been associated with greater post-operative pain compared to CHX (Demenech *et al*. 2021), which supports our findings.

Overall, the present study successfully compared the effectiveness of NaOCl and CHX on the levels of bacteria, inflammatory mediators, and perception of pain in teeth with symptomatic irreversible pulpitis. Furthermore, it is worth pointing out that important endodontic pathogens related to clinical symptoms and endodontic treatment failure can be found in early stages of infection, this showing the need for a careful endodontic treatment for every type of infection. Notably, the use of 6% NaOCl did not negatively affect the clinical and periapical status compared to 2% CHX regarding the investigated parameters. Higher concentrations of NaOCl (*i.e.*, 6% or

8.25%) have been reported to be beneficial as it facilitates the dissolution of pulp tissues (Cullen *et al*. 2015), which may harbour bacteria and induce treatment failure. It is important to emphasize that chemical substances were removed from the root canals to decrease the formation of precipitations and neutralise their deleterious effects if in contact with periapical tissues. Such contact could interfere with the levels of biomarkers.

Overall, regardless of the root canal irrigant used, endodontic treatment led to a reduction in the levels of bacteria and consequently to a decrease in inflammation and pain.

Conclusions

Endodontic treatment by means of chemo-mechanical preparation performed with either 2% chlorhexidine gel or 6% sodium hypochlorite showed similar effects on microbial control and inflammatory mediator levels. Intracanal medication aided in the infection control and attenuation of inflammation by lowering the levels of PGE2 and substance P. Pain perception was reduced after endodontic treatment, regardless of the substance used.

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Figures and Tables

Table 1. Phases of the clinical sample collection.

Analysis	Before CMP	Immediately after CMP	48h after CMP	After ICM
Microbial Culture				
Nested PCR				
PGE2				
Substance P				

Table 2. Parameters used for sample collection.

Analysis	Number of paper points	Time (s)	Apical limit	Storage Media	Temperature of Storage (ºC)
Microbial Culture	3 (size 20, Dentsply Sirona)	60	Full length of the RC	VMGA III	-80
Nested PCR	2 (size 20, Dentsply Sirona)	60	Full length of the RC	Tris-EDTA buffer, pH 8	-80
PGE2	1 (size 20, Dentsply Sirona)	60	2 mm over apical foramen	Empty cryotube	-80
Substance P	1 (size 20, Dentsply Sirona)	60	2 mm over apical foramen	Empty cryotube	-80

Table 3. Binding region, specificity, synthesis direction and sequence of the primers used to detect target species in infected dentine and root canals of teeth with irreversible pulpitis in the different phases of the endodontic treatment using nested PCR.

Primer / Specificity	**Size (pb) / Direction**	**Sequence**
L422 / Universal Forward (23S)	422 / 5′→3′	GGAGTATTTAGCTT
785 / Universal Forward (16S)	785 / 5′→3′	GGATTAGATACCCTGGTAGTC
L189 / Universal Forward (23S)	1500 / 5′→3′	GGTACTTAGATGTTTCAGTTC
A. actinomycetemcomitans (16S)	1500 / 5′→3′	GAAGAAGAACTCAGAGATGGGTTT
A. naeslundii (16S)	1500 / 5′→3′	TGGAGACGGGGTTTCCTCCTTTGG
D. pneumosintes (16S)	1500 / 5′→3′	CCTTGACATTGATCGCAATCCATAGAAATAT
E. faecalis (16S)	1500 / 5′→3′	GTCGCTAGACCGCGAGGTCATGCA
F. alocis (16S)	1500 / 5′→3′	ACATACCAATGACAGCCTTTTAA
F. nucleatum (16S)	1500 / 5′→3′	TTCGGGGAAACCTAAAGACAGGTGG
G. morbillorum (16S)	1500 / 5′→3′	CGAGAGTCAGCCAACCTCATA
P. micra (16S)	1500 / 5′→3′	AACGAGAAGCGAGATAGAGATGTTA
P. endodontalis (16S)	1500 / 5′→3′	TTTAGATGATGGCAGATGAGAG
P. gingivalis (16S)	1500/ 5′→3′	CATCGGTAGTTGCTAACAGTTTTC

P. intermedia (16S)	1500 / 5´→3´	TGTTAGCGCCTTGCGCTA
P. nigrescens (16S)	1500 / 5´→3´	CGTTGGCCCTGCCTGCGG
P. tannerae (16S)	1500 / 5´→3´	CCAAGAGTGCGGAGTGCAGAGATGCGC
S. sobrinus	1500 / 5´→3´	TTTTTCTTCGGAACATCGGAG
T. forsythia (16S)	1500 / 5´→3´	TGCGATATAGTGTAAGCTCTACAG
T. denticola (16S)	1500 / 5´→3´	CAAGAGCAATGACATAGAGATATGG
T. socranskii (16S)	1500 / 5´→3´	ATGTACACTGGGCGTGTGCG

Table 4. Median (min-max) of colony forming units *per* millilitre (CFU/mL) in the different phases of endodontic treatment of teeth with symptomatic irreversible pulpitis.

Endodontic treatment phase	Overall	Negative Culture	Root canal Irrigant			
			CHX	Negative Culture	NaOCl	Negative Culture
S1	$3.90x10^2$ ($1.20x10^2$–$6.40x10^2$) a	0/20	$4.20x10^2$ ($1.40x10^2$–$5.4x10^2$) Ab	0/10	$3.70x10^2$ ($1.20x10^2$–$6.40x10^2$) Ab	0/10
S2	0 (0–80) b	16/20	0 (0–80) Aa	8/10	0 (0–80) Aa	8/10
S3	0 (0–20) b	17/20	0 (0–20) Aa	8/10	0 (0–20) Aa	9/10

S1: Before chemo-mechanical preparation; S2: After chemo-mechanical preparation; S3: After intracanal medication. CHX: 2% chlorhexidine gel; NaOCl: 6% sodium hypochlorite.

Figure 1. Flowchart of the selection of volunteers for eligibility.

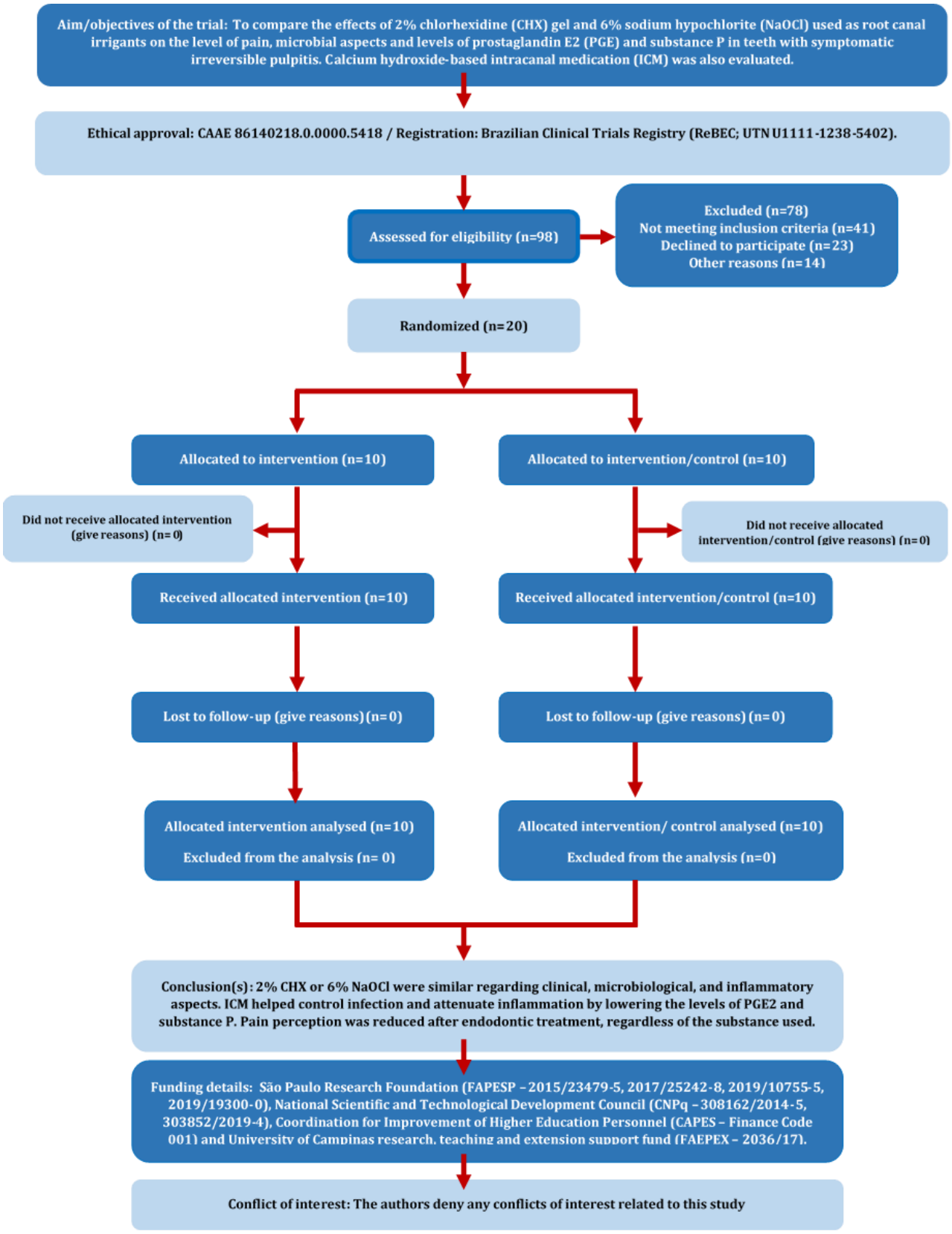


Figure 2. Prevalence and microbial diversity before (S1) and after (S2) chemo-mechanical preparation and after intracanal medication (S3) using 2% chlorhexidine gel (CHX) and 6% sodium hypochlorite (NaOCl) in teeth with symptomatic irreversible pulpitis.

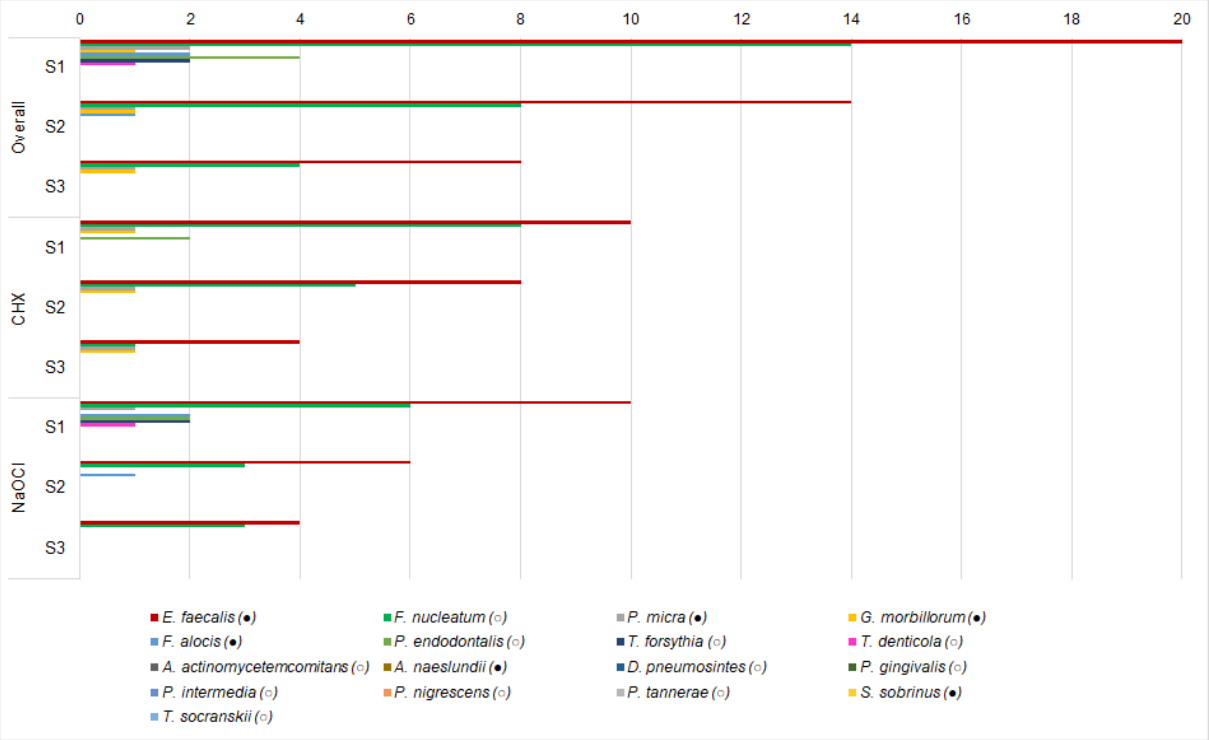


Figure 3. Concentrations of prostaglandin E2 (PGE2) in pg/mL before (S1) and after (S2) chemo-mechanical preparation and after intracanal medication (S3) using 2% chlorhexidine gel (CHX) and 6% sodium hypochlorite (NaOCl) in teeth with symptomatic irreversible pulpitis.

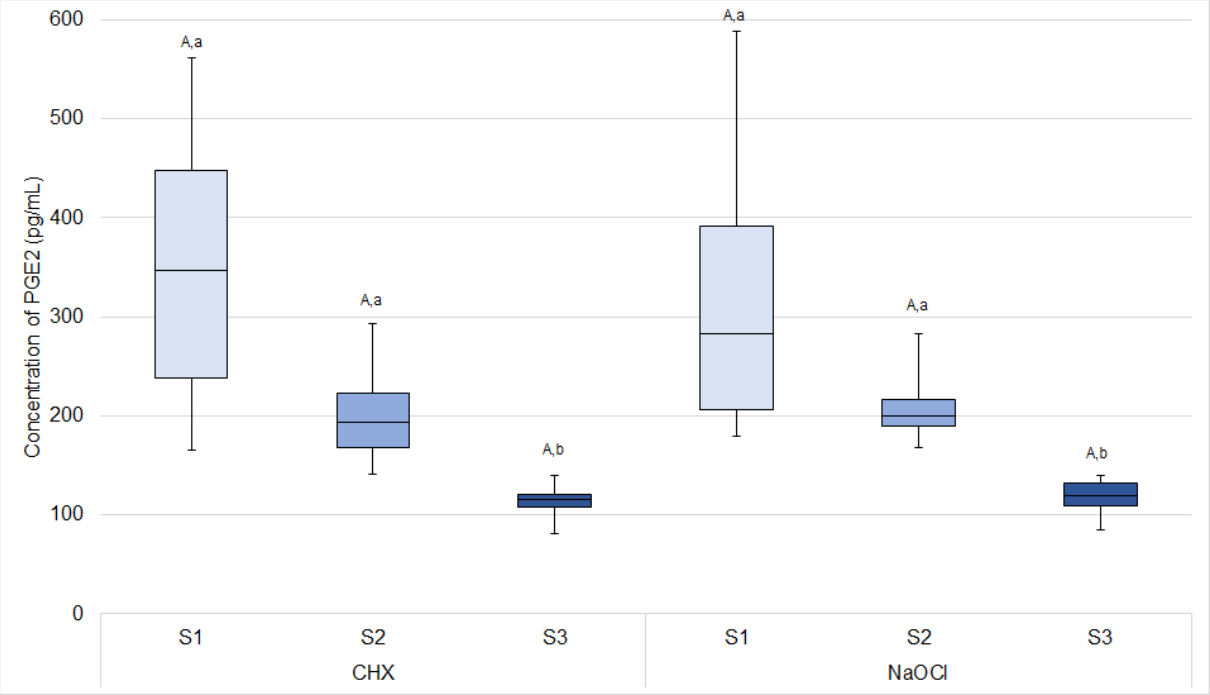
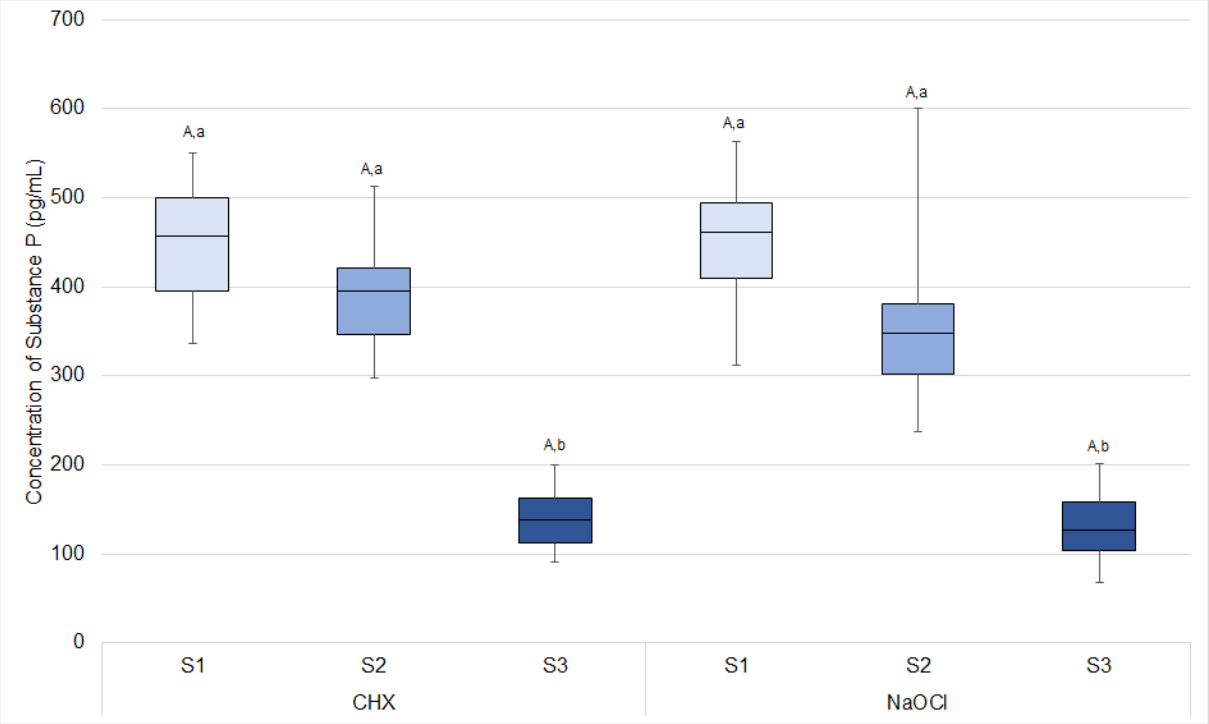


Figure 4. Concentrations of substance P in pg/mL before (S1) and after (S2) chemo-mechanical preparation and after intracanal medication (S3) using 2% chlorhexidine gel (CHX) and 6% sodium hypochlorite (NaOCl) in teeth with symptomatic irreversible pulpitis.



Capítulo II

2.5 APICALLY EXTRUDED DEBRIS USING PASSIVE ULTRASONIC IRRIGATION ASSOCIATED WITH DIFFERENT ROOT CANAL IRRIGANTS.

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SUMMARY

The present study evaluated the amount of apically extruded debris after chemo-mechanical preparation (CMP) associated with passive ultrasonic irrigation (PUI) using four different root canal irrigants, namely, 6% sodium hypochlorite (NaOCl), 2% chlorhexidine gel + saline solution (2% CHXg + SS), 2% chlorhexidine solution (2% CHXs) and SS alone. Sixty mandibular premolars with single straight root canals were selected and randomly assigned into 4 groups (n = 15) according to the root canal irrigant used as follows: G1 (PUI + NaOCl), G2 (PUI + CHXg + SS), G3 (PUI + CHXs) and G4 (PUI + SS). Reciproc® R25 files (25/.08) were used during CMP and the debris extruded from each tooth were collected in pre-weighted Eppendorf tubes and dried. The average weight of debris was assessed by using an analytical microbalance. Data were statistically analyzed by using ANOVA and post-hoc Tukey's test ($\alpha = 0.05$). Debris extrusion was observed in all groups, irrespective of the root canal irrigating, with 2% CHXg + SS being associated with lower debris extrusion compared to other irrigants ($p < 0.05$). No significant differences were observed between 6% NaOCl, 2% CHXs and SS. In conclusion, passive ultrasonic irrigation did not completely prevent apically extrusion of debris. PUI performed with 2% chlorhexidine gel + saline solution significantly minimized debris extrusion compared to 6% sodium hypochlorite, chlorhexidine solution and saline solution.

Keywords: Chlorhexidine, Debris extrusion, Passive ultrasonic irrigation, Reciproc, Sodium hypochlorite.

INTRODUCTION

Microbial reduction from root canals is of major relevance to achieve success in endodontic treatment. During the chemo-mechanical preparation (CMP), endodontic files and root canal irrigants are used to eliminate organic/inorganic tissues, which may harbor bacteria within the root canal system (1). As a consequence, organic and inorganic debris, bacteria and irrigants may extrude into the periapical tissues (2), resulting in severe pain (3).

Sodium hypochlorite (NaOCl) is the most used chemical substance during the root canal treatment (4). Chlorhexidine (CHX) has been recently proposed as an alternative to NaOCl due to its properties, such as lubricating action, broad spectrum, substantivity and lower toxicity compared to NaOCl (5,6). In addition, the rheological action of CHX-based gel maintains the debris in suspension (5), and as a result it prevents apical extrusion of debris (6).

In order to enhance the effectiveness of root canal irrigants, several studies have suggested its agitation. Passive ultrasonic irrigation (PUI) is a technique that relies on the ultrasonic activation of irrigants for efficient removal of debris and microorganisms (7). An ultrasonic tip is activated inside the root canal, along the working length (WL), and moved passively in up-and-down motions to prevent it from binding to the root canal walls (8).

PUI associated with different substances have been used to improve the effectiveness of the root canal treatment. However, literature is scanty regarding whether PUI can prevent debris extrusion. Therefore, the aim of the present study was to evaluate the amount of apically extruded debris after chemo-mechanical preparation associated with passive ultrasonic irrigation using four different root canal irrigants, namely: 6% sodium hypochlorite (NaOCl), 2% chlorhexidine gel + saline solution (2% CHXg + SS), 2% chlorhexidine solution (2% CHXs) and SS alone. The null hypothesis was that there were no differences in the amount of extruded debris between the irrigants tested.

MATERIAL AND METHODS

Sample Selection

The present study was approved by the Human Research Ethics Committee of the Piracicaba Dental School, State University of Campinas – UNICAMP, São Paulo, SP, Brazil.

Previous studies (9,10) were used to identify an effect size of 0.50 required to calculate the total sample size for this study. α-type error = 0.05 and power β = 0.80 were also input. A total of 48 samples were indicated as the minimum to observe differences between the systems (F test family, ANOVA, G*Power for Windows). A minimum of 12 teeth per group should be used. Therefore, 60 human mandibular premolars extracted for reasons not related to this study were selected. Prior to the experiments, the teeth were disinfected with 0.5% chloramine T, kept in distilled water at 4°C and used within 6 months after extraction. Soft tissue remnants and/or calculi on the external root surface were ultrasonically removed under constant and copious irrigation.

All the specimens selected for this study presented similar root length (19±1mm) confirmed by using a digital caliper (American Dental Systems, Vaterstetten, Germany). The inclusion criteria were as follows: single-rooted teeth with one straight root canal, one apical foramen with mature apex (radiographically confirmed) and absence of fractures, caries and resorptions. Digital radiographs from the buccolingual and mesiodistal angles were taken of each tooth to standardize the root canal anatomy and curvature. Image analysis software (AxioVision 4.5; Carl Zeiss Vision, Hallbergmoos, Germany) was used to evaluate the root canal anatomy and to measure the angle of curvature of each root canal. Teeth with single oval-shaped root canals, with a cross-section diameter ratio of ≥ 2.5:1 at 5 mm from the apex were included in the study. Curvature angle was measured at the coronal aspect of the apical third of the canal (11), and only those teeth with root canal curvature < 10° and initial apical size equivalent to a #15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) were selected (14).

Traditional endodontic access cavities were prepared on the occlusal surfaces by using high-speed diamond burs under copious water-cooling. To achieve apical patency, a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was introduced until its tip reached the apical foramen. This procedure was performed under an operating microscope (DFV Comercial e Ind. Ltda, Valença, RJ, Brazil) at 20X magnification. The working length (WL) was established at 1 mm short from the apical foramen and the foramen diameter of all teeth was standardized by using a #20 K-file (Dentsply Maillefer, Ballaigues, Switzerland) along the WL. After the initial apical diameter (0.20 mm) standardization, Reciproc R25 single-files were used for the mechanical instrumentation (not a tested variable in this study). Therefore, a compatible file with the initial diameter was used for minimum interference as possible in the passive ultrasonic irrigation results.

Root Canal Preparation

The specimens were randomly assigned into 4 experimental groups (n = 15) according to the root canal irrigating solution used during CMP. A computer algorithm (www.random.org) was used for specimen randomization as follows:

- G1 (PUI + 6% NaOCl)
- G2 (PUI + 2% CHXg + SS)
- G3 (PUI + CHXs)
- G4 (PUI + SS)

A single experienced and previously trained operator performed CMP and passive ultrasonic irrigation of all specimens. Reciproc® R25 files (25/0.08, VDW, Munich, Germany) were used with the RECIPROC ALL program (VDW) in a slow in-and-out pecking motion and 3-mm amplitude limit combined with brushing motion. After 3 pecking motions, the endodontic file was withdrawn from the root canal, cleaned and inspected before being reused (6).

Irrigation Protocol

All chemical substances were prepared by the same manufacturer (Drogal, Piracicaba, SP, Brazil) and used just after their manipulation.

Passive ultrasonic irrigation (PUI) was used as irrigation protocol in all groups. The root canal irrigant was delivered with a syringe and a 30-gauge needle (NaviTip, Ultradent Products Inc, South Jordan, UT, USA).

Initially, the root canal was rinsed with 5 mL saline solution for 1 minute in all groups. Immediately before CMP with R25 Reciproc files, the teeth were rinsed with 1 mL of each solution and after 3 pecking motions the root canals were again irrigated with 5 mL of each solution. A final irrigation was performed with 5 mL of each irrigating solution, and in the 2% CHXg + SS group, irrigation was completed with 5 mL of SS. As for group 2, before each preparation, the root canals were rinsed with 1 mL of CHXg.

The total volume used in groups 1, 3 and 4 was 35 mL. For group 2, 5 mL of 2% CHXg and 30 mL of SS were used for final volume standardization, as previously described.

Root canals were rinsed with each irrigating solution before 0.20-mm ultrasonic tip (0.01 taper) (E1, Helse, São Paulo, SP, Brazil) was placed 1 mm short of the WL, which was activated at a frequency cycle of 40-kHz *per* second for 30 seconds. After last irrigation, it was used for 1 minute (Obtura Spartan Endodontics, Algonquin, IL, USA).

Initially, the root canals were rinsed with 5 mL saline solution for 1 minute in all groups. Next, each tooth was filled with 1 mL of the correspondent irrigant solution (6% NaOCl, 2% CHXg, 2% CHXs or SS). After 3 pecking-motions, the root canals were rinsed with 5 mL of the correspondent irrigant solution, and in the 2% CHXg it was rinsed with SS. This procedure repeated until the R25 Reciproc file reached the WL.

Following, passive ultrasonic irrigation was carried out with ultrasonic tip E1 (Irrisonic, Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil) mounted on a piezoelectric ultrasonic unit (Piezon 150, Electron Medical Systems, Nyon, Switzerland). The ultrasonic tip was inserted 1 mm short of the WL and the irrigant present within the root canal space was passively activated using a power setting of 30% of the ultrasonic device. This procedure was performed in three cycles of 20 seconds each (total activation of 60 seconds). Short in-and-out motions (2–3 mm) were performed without touching lateral walls. Replenishment of the irrigant was

performed using conventional syringe/needle irrigation. The protocol was completed within 5 min using a total of 5 mL per tooth.

Debris Collection

The method used for the collection of apically extruded debris was adapted from previous studies (6,9,12,14).

Empty Eppendorf tubes were pre-weighted by using a 10^{-5} -g precision analytic microbalance (SP Labor, São Paulo, SP, Brazil). Three consecutive weights were obtained for each tube, and the mean value was considered to be its initial weight.

A 27-G needle was inserted alongside the stopper to be used as a drainage cannula and to equalize the air pressure inside and outside the tubes. Next, each stopper with tooth and needle was attached to its Eppendorf tube, and the tubes were fitted into vials.

The operator was blinded from seeing the root apex during the instrumentation procedures by a rubber dam overshadowing the vial.

Immediately after the instrumentation, the Eppendorf tube was removed from the vial. Each tooth was gently removed from the Eppendorf tube and the debris adhered to the root surface were collected by washing off the apex with 1 mL of distilled water into the Eppendorf tube. The tubes were stored in an incubator at 68°C for 5 days to evaporate the moisture before weighing the dried debris (14). Weighing was carried out again and three consecutive weights were obtained for each tube, and the mean was calculated. The dried weight of the extruded debris was calculated by subtracting the weight of the empty tube from that of the tube containing debris.

Statistical Analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA). Tukey’s post hoc test was used for multiple comparisons. The alpha-type error was set at 0.05.

RESULTS

Apical Extrusion of Debris

Table 1 provides an overview of the mean values and standard deviation in each group. Debris extrusion was observed in all groups, irrespective of the root canal irrigant used during CMP. The use of 2% CHXg + SS was associated with lower debris extrusion compared to their irrigants ($p < 0.05$). No significant differences were observed between 6% NaOCl, 2% CHXs and SS irrigation protocols.

DISCUSSION

The present study was undertaken to evaluate the amount of apically extruded debris after chemo-mechanical preparation associated with passive ultrasonic irrigation using four different root canal irrigants (6% NaOCl, 2% CHXg + SS, 2% CHXs and SS alone). PUI has been associated with different substances (i.e. sodium hypochlorite or Chlorhexidine) to enhance microbial reduction in the root canal system, consequently increasing the effectiveness of the endodontic therapy. In the present study, the use of 2% CHXg + SS minimized the apical extrusion of debris compared to other root canal irrigants tested ($p < 0.05$). Therefore, the null hypothesis was rejected.

For assessment of apically extruded debris, an already-established protocol described by Myers & Montgomery (12) was used in the presented study as well as elsewhere (6,9,14). Although this method was also recommended by Tanalp & Güngor (3), the limitation of the present study is related to the lack of an apparatus simulating the periodontal tissue. Thus, the foramina of the specimens were suspended in air (zero back pressure). The results obtained in our study may differ from a clinical study where the periodontium acts as a natural barrier, possibly limiting the extrusion of debris (6,9,15).

Teeth were carefully selected taking into consideration their type and length, standardization of the initial foramen diameter and working length, number of canals and canal curvature. This procedure guaranteed that debris extrusion was a result of the study variables (i.e., root canal irrigants). Mechanical instrumentation of the specimens was carried out by using reciprocating single files, as they allow less apical

extrusion of debris than conventional multiple-file rotary systems (6,16). Moreover, reciprocating single file systems are simple, faster and efficient compared to conventional rotary systems (2,6).

Most of the studies have evaluated different endodontic files and/or systems regarding the amount of apically extruded debris (9,17). With regard to assessment of the amount of extruded debris using different irrigating systems, only a few studies have been published (6,18). There are a few studies evaluating different root canal irrigation solutions for preventing debris extrusion. Barbosa-Ribeiro et al. (6) evaluated extruded debris by using positive (conventional irrigation) and negative pressure (EndoVac) irrigation systems in association with different irrigants. In our study, sodium hypochlorite and chlorhexidine (gel and solution) were used due to their great acceptability for use in the endodontic practice, whereas saline solution was used as control.

The use of 2% CHXg + SS prevents, though not completely, apical extrusion of debris compared to 6% NaOCl, 2% CHXs and SS alone ($p < 0.05$). The current results may be the consequence of the viscosity and rheological action of chlorhexidine in gel formulation, which keeps debris in suspension during CMP (5,6). Our findings agree with a previous study (6), which showed that 2% CHXg + SS was associated with less debris extrusion compared to 6% NaOCl, 2% CHXs and SS alone ($P < 0.05$) using conventional irrigation with syringe and needle. However, no difference was observed between the irrigating solutions using negative pressure irrigation system (EndoVac, SybronEndo, Orange, CA, USA), suggesting that mechanical action was the main responsible for preventing extrusion of debris.

Debridement of the root canal system with minimum extrusion of debris is one of the main goals of the endodontic treatment. Apical extrusion of organic and inorganic debris, bacteria and irrigants may cause damage to the periradicular tissues and result in severe pain, particularly in root canals with complex anatomies (2,6,19,20). The results of this work confirmed that PUI and the tested auxiliary chemical substances could not completely prevent extrusion of debris. Therefore, this study indicates a need for complementary irrigation approaches to enhance the cleaning of canals instrumented with single-files and minimizing the risks of pain due to the extrusion of toxic substances into the periapical region. Further studies should be conducted to

evaluate whether different cycles and frequency of the ultrasonic irrigation, device setting power and type of activation (passive or active) can prevent the amount of extruded debris. This is of clinical importance, since modern techniques for root canal treatment require less clinical time. Therefore, additional disinfection protocols are needed for better prognosis.

Although it was not possible to completely avoid the extrusion of debris through the apical foramen, it is mandatory the use of passive ultrasonic irrigation or other irrigation protocols to complement the disinfection of the root canal system and consequently increase the success rates of the endodontic therapy, clinically.

In conclusion, passive ultrasonic irrigation did not completely prevent apical extrusion of debris. However, PUI with 2% Chlorhexidine gel + saline solution was found to significantly reduce debris extrusion compared to 6% sodium hypochlorite, chlorhexidine solution and saline solution.

RESUMO

O presente estudo avaliou a quantidade de debris extruídos apicalmente após o preparo químico-mecânico (PQM) associado à irrigação ultrassônica passiva (IUP) em associação com quatro diferentes irrigantes – hipoclorito de sódio 6% (NaOCl), clorexidina 2% gel + solução salina (CLXg 2% + SS), solução de clorexidina 2% (CLXs 2%) e SS. Sessenta pré-molares inferiores com canais radiculares únicos e retos foram selecionados e aleatoriamente distribuídos em 4 grupos (n = 15) de acordo com o irrigante utilizado: G1 (IUP + NaOCl), G2 (IUP + CLXg + SS), G3 (IUP + CLXs) e G4 (IUP + SS). Limas Reciproc® R25 (25/.08) foram utilizadas durante o PQM e os debris extruídos de cada dente foram coletados em tubos Eppendorf pré-pesados e secos. O peso médio de debris foi avaliado através de microbalança analítica, e os dados foram analisados estatisticamente utilizando ANOVA e teste de Tukey post hoc ($\alpha = 0.05$). Extrusão de debris foi observada em todos os grupos, independente do irrigante. CHXg 2% + SS foram associados a menor extrusão de debris comparado aos demais irrigantes ($p < 0.05$). Não foram observadas diferenças estatisticamente significativas entre NaOCl 6%, CLXs 2% e SS. Concluindo, irrigação ultrassônica passiva não preveniu completamente a extrusão apical de debris, entretanto, IUP

realizada com CLXg 2% + SS minimiza significativamente a extrusão de debris comparado ao NaOCl 6%, CLXs 2% e SS.

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TABLE

Table 1. Mean and standard deviation (SD) of the amount of apically extruded during chemo-mechanical preparation associated with passive ultrasonic irrigation of each experimental group (in grams).

Root canal irrigants	Mean ± SD
6% Sodium hypochlorite	0.00422 ± 0.00154 B
2% Chlorhexidine gel + saline solution	0.00147 ± 0.00061 A
2% Chlorhexidine solution	0.00405 ± 0.00174 B
Saline solution	0.00539 ± 0.001921 B

Different superscript letters represent significant differences ($p < 0.05$).

2.6 LIBERATION OF TGF-β1 AND ANTIMICROBIAL ACTIVITY OF DIFFERENT PROTOCOLS FOR POTENTIAL USE IN REGENERATIVE ENDODONTIC PROCEDURES.

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Abstract

Aim To quantify the levels of TGF-β1 from root dentine matrix using different irrigation protocols and to evaluate the antimicrobial activity in order to optimise treatment strategies.

Methodology Seventy single-rooted teeth were included. Length of the teeth was standardised at 15 mm from the cementum-enamel junction and a 1.5mm ParaPost XT drill was used to standardise simulated immature apex. The protocols proposed were: I) 0.9g etidronic acid (HEDP) + 10 mL of 2% sodium hypochlorite (NaOCl); II) HEDP + 10 mL of distilled water (H_2O); III) 2% NaOCl + 2% CHX / $Ca(OH)_2$ as intracanal medication (ICM) + 17% EDTA; and IV) 2% CHX gel + ICM + 17% EDTA. Liberation of TGF-β1 was assessed by using ELISA and confocal laser scanning microscopy was used for evaluation of antimicrobial activity. Statistical analysis was set at 5%.

Results Groups treated with NaOCl resulted in significantly lower levels of TGF-β1 being released. The use of intracanal medicament and 17% EDTA significantly increased the liberation of TGF-β1 in the presence of NaOCl. The HEDP + H_2O and CHX + ICM + EDTA presented the higher levels of TGF-β1 ($P > 0.05$). For microbiological assessment, HEDP + H_2O resulted in the lowest antimicrobial activity ($P < 0.05$). The use of an intracanal medicament significantly enhanced the antimicrobial activity with no difference between either CHX or NaOCl as primary irrigant ($P > 0.05$).

Conclusion Despite high antimicrobial activity, sodium hypochlorite hampers the release of TGF-β1 in both one and two-visit protocols. Using this irrigant in revitalisation could therefore reduce the chance of creating a conducive environment for regenerative endodontic procedures. The protocols using a chelating agent (EDTA or HDEP) and chlorhexidine allowed for the liberation of TGF-β1 from dentine matrix and suitable antimicrobial activity, therefore these agents may provide a much better disinfection regime for revitalisation.

Keywords Chlorhexidine, dentine matrix, endodontics, revitalisation, sodium hypochlorite, TGF- β1

Introduction

Due to an increased biological understanding dental pulp regeneration has received substantial attention (Fouad *et al*. 2014) in recent years. Revitalisation has been suggested as biologically based procedures with the objective to replace damaged tissues such as dentine, cementum, and cells of the pulp-dentine complex (Hargreaves *et al*. 2013).

Growth factors, stem cells and scaffolds are key components required to successfully regenerate the pulp-dentin complex (Zeng *et al*. 2016). During tooth development, growth factors and other bioactive molecules are secreted by odontoblasts and sequestered in the dentin matrix; therefore, the dentine can act as a reservoir of growth factors for homeostasis and potential repair and regeneration (Goldberg *et al*. 2004, Tomson *et al*. 2007, Smith *et al*. 2012, Zeng *et al*. 2016, Widbiller *et al*. 2017). A range of growth factors have been detected in dentin extracellular matrix including transforming growth factor-beta 1 (TGF-β1) (Finkelman et al. 1990, Cassidy et al. 1997, Smith et al. 1998), vascular endothelial growth factor (VEGF), fibroblast growth factor - 2 (FGF-2) (Roberts-Clark & Smith 2000), insulin-like growth factor I and II (IGF-I and II) (Finkelman et al. 1990), bone morphogenetic protein (BMP) (Bessho et al. 1991) and others. TGF-β1 is a signalling molecule that induces cell proliferation, cell differentiation, chemotaxis and apoptosis in different types of cells (Kubiczkova *et al*, 2012, Niwa *et al*. 2018). It has been shown that these growth factors could be liberated on the damage to dentin caused by microbial acids in carious lesions (Smith & Cooper 2017), by insertion of pulp-capping materials (i.e., calcium hydroxide or mineral trioxide aggregate) (Graham et al. 2006, Tomson et al. 2007) or acid etch (Ferracane et al., 2013) or during repair processes to promote dentin regeneration (Tziafas *et al*. 2000, Smith & Lesot 2001).

Sodium hypochlorite (NaOCl) is widely used as the main root canal irrigant due to its antimicrobial activity and ability to dissolve necrotic tissues and the organic constituents of the smear layer (Zhender 2006). For revitalisation, lower concentrations of sodium hypochlorite are preferred as concentrations higher than 3% interfere with cell adhesion on dentine surface (Jung et al. 2019, Ferreira et al. 2020). Chlorhexidine (CHX) has been suggested as an alternative chemical substance due to its wide range of antimicrobial activity, substantivity, lower cytotoxicity than sodium hypochlorite,

lubricating properties and efficient clinical performance (Gomes *et al*. 2013). Chelating agents, such as ethylenediamine tetra acetic (EDTA) have been suggested for final irrigation to dissolve inorganic components of the smear layer that is created following root canal preparation as conventional root canal irrigant frequently do not have that ability (Zhender 2006, Johal *et al*. 2007). However, EDTA combined with sodium hypochlorite has been reported to reduce active chlorine, besides being cytotoxic (Grawehr et al. 2003, Ballal et al. 2009, Paulson et al. 2018).

The combination of etidronic acid (1-hydroxyethane 1,1-diphosphonic [HEDP]) with sodium hypochlorite has been suggested as it is a biocompatible “soft” chelator which provides less aggression to dentine than EDTA (Lottani *et al*. 2009, Paulson et al. 2018). HEDP when used alone has a weak chelating capacity when used alone (De-Deus *et al*. 2008), therefore, its combination with sodium hypochlorite as a single root canal irrigant as it reduces accumulation of mineralised tissues during the root canal treatment (Girard et al. 2005, Paulson et al. 2018).

It is known that the reduction/elimination of bacteria is a crucial step for a successful revitalisation. The knowledge of the behaviour of different root canal irrigant/intracanal medicaments on dentine surface is relevant to evaluate its potential to stimulate the release of growth factors from dentine matrix and, consequently, to promote healing of the dentin-pulp complex. Thus, the aims of the present study were to quantify the levels of TGF-β1 from root dentine matrix using different irrigation protocols and to evaluate the antimicrobial activity in order to optimise treatment strategies.

Material and methods

Specimen selection

Seventy teeth were selected from Birmingham School of Dentistry Research Tissue Bank following approval under generic ethics (14/EM/1128) with NHS trust R&D (reference BCHCDent374.ToothBank).

This study was divided into two parts:

1) Quantification of the levels of TGF-β1 from root dentine matrix using different irrigation protocols for potential use in revitalisation, and 2) Evaluation of antimicrobial activity of different irrigation protocols that could be used to enhance the biological actions in revitalisation.

Tooth selection

Non-carious extracted single-rooted maxillary canines were used in these studies (Part 1, n = 20 and Part 2, n = 50).

All teeth had had one straight root canal with no evidence of resorption confirmed radiographically and whilst visually it was confirmed there was one mature apical foramen, absence of fractures, and absence of caries. Teeth with a) severe curvature, b) with radiographic evidence of two canals, c) no crown and d) evidence of previously root canal treatment were excluded from the study.

Specimen standardisation

Teeth standardisation was the same in parts 1 and 2 of the experiment. Briefly, soft tissues, bone and calculus was removed using a scalpel and a Cavitron ultrasonic scaler (Denstply Sirona, OK, USA).

A single experienced operator performed all procedures. Conventional access cavities were prepared to access the root canal. The root canals were prepared using a crown-down technique. A size 10, 15 and 20 K-files (SybronEndo, Glendora, CA, USA), were used to initially negotiate the root canal. Subsequently, X1 to X5 ProTaper NEXT files (Dentsply Sirona, OK, USA) were used according to the manufacturer. In order to simulate an incompletely developed root create and a standardised internal diameter of the root canal for each tooth a 1.5mm ParaPost XT drill (Coltene Whaledent LTD, Burgess Hill, UK) was used. During the teeth preparation, a volume of 30 mL of saline solution was used to minimise the potential for heat generation; however, no active root canal irrigant were used during this phase of instrumentation.

The length of the teeth was standardised to 15 mm from the cementum-enamel junction to the apex of the tooth by using a carborundum disc attached to a low-speed

straight handpiece.

For the part 1, debris were removed from the root canals using a TePe interdental brush (Tepe Oral Hygiene Products, Somerset, UK). For the part 2, an ultrasonic bath with 17% EDTA was applied for 5 minutes.

Subsequently, teeth were coated with clear nail polish (Rimmel, London, UK) on the external surface to prevent the interaction of the irrigant with any exposed dentine present on the external surface of the tooth.

The apex was flattened with a diamond polishing disc (Horico, Berlin, Germany). The crowns of the teeth were left *in situ* to provide less irrigant loss when using a passive ultrasonic agitation technique. This approach also intends to replicate *in vivo* conditions.

Irrigating protocols

Irrigation protocols used in parts 1 and 2 are shown in table 1.

Treatment procedures

Sixty mL of the primary irrigant was applied and left to incubate at room temperature for 5 minutes. A ProTaper Next X5 file (Dentsply Sirona, OK, USA) was used for 30 seconds circumferentially around the internal surfaces of the tooth to simulate the chemo-mechanical procedures during root canal preparation, clinically. Subsequently, 1 minute of passive ultrasonic irrigation using an ultrasonic tip mounted on a piezoelectric ultrasonic unit (Sybron Endo, Glendora, CA, USA) with the power setting at 50% was applied to each tooth. After this procedure, in groups I and II the irrigant were removed with a micropipette (Appleton, Birmingham, UK) and 40 µL were store at -20 °C for further quantification of the levels of TGF-β1.

In group III, 5 mL of 5% sodium thiosulfate was used to neutralise sodium hypochlorite and then removed from the canal using 5 mL of saline solution prior intracanal medication application. In group IV, inactivation of chlorhexidine was performed with a solution of 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin for 1 min and then removed from the canal using 5 mL of saline solution was used to remove the primary irrigant and for volume standardisation. Then, the root canals were dried

with sterile paper points (Dentsply Sirona, OK, USA). In both groups, the canals were rinsed with 5 mL sterile saline and Ca(OH)2 was neutralized with 5 mL of 0.5% citric acid for 1 min and removed with 5 mL of sterile saline before application of 17% EDTA.

Treatment with an intracanal medication between the primary and secondary irrigant applications was used for 5 days. Subsequently, an endodontic sponge was placed coronally with a light bodied silicone (Henry Schein, NY, USA) which was used to seal the access. The teeth were incubated at 37 °C (Hybridisation Oven / Shaker, S1 20H; Stuart Scientific Ltd, Staffordshire, UK) in humidified conditions. After this period, exposure to a 60 µL of 17% EDTA only, the intention of potential growth factor release was delivered in a single application for 5 minutes with a 1-minute application of passive ultrasonic activation as previously described.

A total of 40 µL of the secondary irrigant were store at -20 °C for further quantification of the levels of TGF-β1.

Part 1: Quantification of TGF-β1 using Enzyme-Linked Immunosorbent Assay (ELISA)

The retrieved irrigant used in each protocol were thawed to reach room temperature prior to analysis. In total, 40 µL of each sample was diluted in 150 µl of PBS. Then, 30 µl of 1 M hydrochloric acid (Sigma, UK) was added and incubated at room temperature for 10 minutes to activate the TGF-β1. The reaction was neutralised with 30 µl of 1.2 M sodium hydroxide (Sigma, UK) and 0.5 M HEPES (Sigma, UK) and 50 µl of the resultant solution was assayed in duplicate using the Quantikine Human TGF-β1 ELISA kit (R&D Systems, UK) according to the manufacturer's instructions.

Absorbance readings at 450 nm were obtained using an ELX800 Universal Microplate Reader (Bio-Tek Instruments, USA) and concentrations determined by comparison with a standard curve prepared from a range of concentrations of pure recombinant human TGF-β1. TGF-β1 concentrations were determined by linear regression.

Part 2. Evaluation of the antimicrobial activity

The previously standardised teeth were placed individually in a 5 mL tubes (Eppendorf, Hamburg, Germany) containing 5 mL of Brain Heart Infusion broth (BHI;

Lab M, Bury, UK), and autoclaved at 121 °C for 15 min.

Preparation of inoculum and contamination of specimens

Inoculum preparation from human saliva was based on previous work from our group (Gomes et al. 2003). Briefly, 30 mL of saliva was collected from one volunteer at 8 AM on each day of exposure or solution replacement. The donor was instructed not to perform any oral hygiene procedures for 12 h before saliva collection. Salivary flow was stimulated by chewing 1 g of Parafilm (American National Can™, Menasha, WI, USA). The saliva was stored in a 100 mL container.

A total of 3 mL of saliva and BHI broth (3:1 ratio) was placed in contact with each tooth. and incubated at 37 °C under continuous agitation (170 RPM). The mixture was renewed every 72 h. The tubes were checked daily for BHI broth turbidity. The contamination procedure was performed for 21 days.

Microbiological assessment using confocal laser scanning microscopy (CLSM)

The viability of the biofilm was assessed by using the SYTO-9/propidium iodide assay (Live/Dead™ BacLight™; Invitrogen, Eugene, OR, USA). SYTO-9 is a green, fluorescent nucleic acid stain that labels both live and dead microorganisms; propidium iodide is a red fluorescent nucleic acid stain that only penetrates the cells with damaged membranes, labelling only dead microorganisms.

Following the endodontic treatment on each tooth was sectioned in a 1-mm slice and washed with 5 mL of phosphate-buffered saline (PBS). Subsequently, the slices were stained with 15 µL of each dye and incubated in dark environment for 15 min. The slices were washed again, and directly observed using an inverted CLSM (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). All slices were analysed, and images were obtained using 40X oil lens, 23 sections of 1 mm size of depth, at 1024 × 1024 pixels. Four confocal “stacks” of random areas were obtained for each sample.

All images obtained in CLSM were fragmented in stacks and converted into TIFF format by means of Leica Application Suite-Advanced Fluorescence software (Mannheim, Baden-Württemberg, Germany). For quantification of viable and non-viable bacteria purposes, ImageJ software (U. S. National Institutes of Health,

Bethesda, MA, USA) was used to calculate the percentage of live microorganisms detected after each protocol used.

Statistical analyses

The results obtained were tabulated in Excel spreadsheet (Microsoft; Redmond, WA, USA). Statistical analysis was performed using SPSS for Windows, version 19.0 (SPSS Inc, Chicago, IL, USA). Shapiro-Wilk test was used to verify data normality. For the quantification of TGF-β1, non-parametric Mann-Whitney U test was used. The percentage of live cells was analysed by using the Kruskal-Wallis followed by Dunn's post hoc test. Significance level was set at 5% in all tests.

Results

Quantification of TGF-β1

Liberation of TGF-β1 resulted following all treatment regimens (figure 1).

The groups treated with sodium hypochlorite resulted in significantly lower levels of TGF-β1 being released compared with treatment protocols that did not included NaOCl ($P < 0.05$). The use of an intracanal medicament and final irrigation with 17% EDTA significantly increased the liberation of TGF-β1 in the presence of sodium hypochlorite. The HEDP + H_2O and CHX + ICM + EDTA presented the higher levels of TGF-β1, with no significant differences between them ($P > 0.05$).

Microbiological assessment

Figure 2 shows representative images of microbial load within dentinal tubules after different treatment protocols using CSLM.

Table 2 provides the percentage of viable bacteria in all groups. The contamination of the teeth was confirmed using confocal laser scanning microscopy, detecting a total of 91.59% (86.50 – 95.39) viable bacteria for the positive control. When HEDP + H_2O was used as an irrigant regime it resulted in the lowest antimicrobial activity of all protocols tested ($P < 0.05$). The combination of 2% NaOCl to HEDP significantly increased antimicrobial activity. The use of an intracanal

medicament significantly enhanced the microbial activity with no difference discernible between either chlorhexidine or sodium hypochlorite ($P > 0.05$).

Discussion

The release of bioactive molecules such TGF-β1 from dentine matrix is a key factor in enhancing regeneration of the pulp tissues in the unoccupied root canal space. These biomolecules exert their function even at extremely low levels (picogram), modifying angiogenesis, cell recruitment, differentiation, immune defence, proliferation, and mineralization (Zhang *et al*. 2011, Smith *et al*. 2011, Galler *et al*. 2015). It is well established that efficient disinfection of the root canal system in a successfully revitalisation (Cooper *et al.* 2011). For regeneration techniques to work it is important to ensure that sufficient disinfection of the root canals space is carried out, however, this should not be at the expense of creating an environment that is not conducive to re-establishing vital tissue within this dead space.

Extracted teeth were used in these experiments due to their clinical relevance to assess the liberation of growth factors from root dentine (Galler *et al*. 2015, Widbiller *et al*. 2017), and evaluate the antimicrobial activity of different disinfection protocols within the dentinal tubules (Oda et al. 2019, Pereira et al. 2020). TGF-β1 was chosen as a surrogate marker of growth factor release due to its important role in revitalisation (Smith *et al*. 2017, Widbiller *et al*. 2017), including mediate signalling of odontoblast differentiation and mineralisation (Galler *et al*. 2015), and chemotactic effect on dental papilla-derived cells (Kwon *et al*. 2010).

Different root canal irrigant have been suggested for infection control in endodontics. Sodium hypochlorite is the most used irrigant due to its capacity to dissolve organic tissue and its well-established antimicrobial activity (Zhender 2006). On the other hand, for regenerative endodontic procedures, sodium hypochlorite has been associated to denaturation of growth factors (Gonçalves *et al*. 2016). Even in the presence of a chelating agent (i.e., HEDP), sodium hypochlorite seemed to denature the bioactive molecules. In this study, sodium hypochlorite promoted the lower levels of released of growth factors from dentine matrix compared with sodium hypochlorite-free protocols (i.e., HEDP + H_2O and CHX + ICM + EDTA). Previous study using blood

proteins observed that the reaction with sodium hypochlorite increases temperature; Furthermore, protein components were broken down into lower molecular weights fragments (Yamaguchi et al. 2001).

As an alternative to sodium hypochlorite, the use of chlorhexidine has been suggested during root canal treatment (Gomes *et al*. 2013) as it presents lower cytotoxicity than sodium hypochlorite, substantivity, a wide-range antimicrobial activity, lubricating properties and efficient clinical performance (Gomes *et al*. 2013). Furthermore, the gel-based formulation that could be used as an ICM shows rheological action, keeping debris originated from the instrumentation in suspension (Gomes *et al*. 2013) preventing from contaminated debris/cytotoxic irrigant extrusion (Arruda-Vasconcelos et al. 2019), which directly impart in the viability of cells in the periapical tissues. Interestingly, our findings revealed that the use of chlorhexidine promoted high levels of TGF-β1 release, which is consistent with previous study (Ferreira et al. 2020).

No significant differences were observed in TGF-β1 release in HEDP + H_2O and CHX + ICM + EDTA; however, it is important to highlight that chlorhexidine promoted greater microbial reduction than HEDP + H_2O, which is a key element for regenerative endodontic procedures (Verma et al. 2017, Cameron et al. 2019). Additionally, no significant differences in the microbiological status within dentinal tubules were observed between the NaOCl + ICM + EDTA and CHX + ICM + EDTA; however, the latter group was superior in promoting TGF- β1 release.

The release of growth factors from dentine matrix depends on the capacity of the root canal irrigant to chelate inorganic matter. With this regard, EDTA has been frequently used as a final rinse protocol to remove inorganic debris that could not be removed using conventional root canal irrigant (Pintor *et al*. 2016). In regenerative endodontic procedures, EDTA has been associated with high concentrations of TGF-β1 released from dentine matrix (Gonçalves *et al*. 2016, Widbiller *et al*. 2017). In the present study, etidronic acid was used as chelating agent, which allowed liberation of TGF-β1, agreeing with previous studies (Gonçalves *et al*. 2016, Widbiller *et al*. 2017).

With regard to intracanal medicaments in regenerative endodontic procedures, calcium hydroxide (Cotti *et al*. 2006) and a combination of antibiotics (Kontakiotis *et al*.

2015) have been proposed. It has been shown that antibiotic pastes (ciprofloxacin, metronidazole, and minocycline) at high concentrations has deleterious effect on stem cells survival (Ruparel *et al*. 2012). The results of these investigations show promise as chlorhexidine associated with calcium hydroxide promote increased levels of TGF-β1, and concomitantly reduce bacterial load from dentinal tubules. Although the levels of TGF-β1 were lower in the presence of sodium hypochlorite, a two-visit protocol, significantly increased the levels of TGF-β1 compared with sodium hypochlorite in combination with etidronic acid.

Overall, all protocols presented different behaviours regarding the liberation of biomolecules, being HEDP + H_2O and CHX + ICM + EDTA highly associated to the release of TGF-β1, whereas the presence of sodium hypochlorite inhibited its release from dentine matrix. Furthermore, the use of intracanal medication enhanced disinfection protocol within dentinal tubules compared to single visit protocols.

Regenerative endodontic procedures including revascularization, reinnervation, and restauration of protective function and tissue formation, mostly focus on young immature teeth (He et al. 2017, Ivica et al. 2020). Over 22 million root canal procedures are carried out annually in the United States on adult teeth (Raedel et al. 2015). It is known that traditional endodontic treatment presents high success rates; However, it only fills the empty pulpal space with a synthetic material without bringing back tooth's vitality. Revitalisation could be considered especially in cases of teeth with irreversible pulpitis, that although presents a complex microbiota (Arruda-Vasconcelos et al. 2021), infection duration is shorter than in teeth with pulp necrosis and apical periodontitis. Additionally, as the pulp is still vital (although incapable of self-regenerating) there is abundance of blood and consequently a variety of key cells for pulp regeneration. Our study has shed a light into different strategies that can be used in revitalisation. Further studies involving different protocols and/or evaluation of different biomolecules are needed for a better biological understanding, which may lead to a more predictable outcome.

Conclusions

Despite high antimicrobial activity, sodium hypochlorite hampers the release of TGF-β1 in both one and two visits protocols. Using this irrigant in revitalisation could therefore reduce the chance of creating a conducive environment for regenerative endodontic procedures. The protocols using a chelating agent (EDTA or HDEP) and chlorhexidine allowed for the liberation of TGF-β1 from dentine matrix and suitable antimicrobial activity, therefore these agents may provide a much better disinfection regime for revitalisation.

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Figures and tables legends

Table 1. Groups, protocols and respective manufacturers used in the study.

Group	Protocol	Manufacturer
I. HEDP + NaOCl	A mixture of 0.9 g of HEDP + 10 mL of 2% NaOCl as primary irrigating solution, simulating a single visit treatment	Dual Rinse® HEDP: Medcem GmbH, Vienna, Austria NaOCl: Drogal, Piracicaba, SP, Brazil
II. HEDP + H_2O	A mixture of 0.9 g HEDP + 10 mL of distilled water as primary irrigating solution, simulating a single visit treatment	Dual Rinse® HEDP: Medcem GmbH, Vienna, Austria
III. NaOCl + ICM + EDTA	2% NaOCl as primary irrigating solution, followed by application of 2% CHX + $Ca(OH)_2$ as ICM for 5 days, and 17% EDTA as secondary irrigating solution	NaOCl: Drogal, Piracicaba, SP, Brazil CHX: Essencial Pharma, Itapetininga, SP, Brazil $Ca(OH)_2$: BiodinâmicaTM, Ibiporã, PR, Brazil EDTA: Drogal, Piracicaba, SP, Brazil
IV. CHX + ICM + EDTA	2% CHX gel as primary irrigating solution, followed by application of 2% CHX + $Ca(OH)_2$ as ICM for 5 days, and 17% EDTA as secondary irrigating solution	CHX: Essencial Pharma, Itapetininga, SP, Brazil $Ca(OH)_2$: BiodinâmicaTM, Ibiporã, PR, Brazil EDTA: Drogal, Piracicaba, SP, Brazil

HEDP: Etidronic acid; NaOCl: Sodium hypochlorite; CHX: Chlorhexidine; $Ca(OH)_2$: Calcium hydroxide; ICM: Intracanal medication; EDTA: Ethylenediaminetetraacetic acid.

Table 2. Median (min – max) values of the percentage of viable bacteria after exposure to different irrigating protocols.

Group	% of viable bacteria
I. HEDP + NaOCl	39.84 (35.47 – 48.31) [A]
II. HEDP + H_2O	61.50 (57.43 – 68.08) [B]
III. NaOCl + ICM + EDTA	2.94 (1.74 – 4.36) [C]
IV. CHX + ICM + EDTA	3.99 (1.95 – 4.88) [C]
Positive control	91.59% (86.50 – 95.39) [D]

HEDP: Etidronic acid Dual Rinse®; NaOCl: Sodium hypochlorite; CHX: Chlorhexidine; ICM: Intracanal medication; EDTA: Ethylenediaminetetraacetic acid. *Different letters mean statistical significance between the groups by using the Kruskal-Wallis and Dunn's tests.

Figure 1. Box plot of the release of TGF-β1 from dentine matrix following different treatment regimes. Different letters represent significant differences among groups by Mann-Whitney test (P < 0.05).

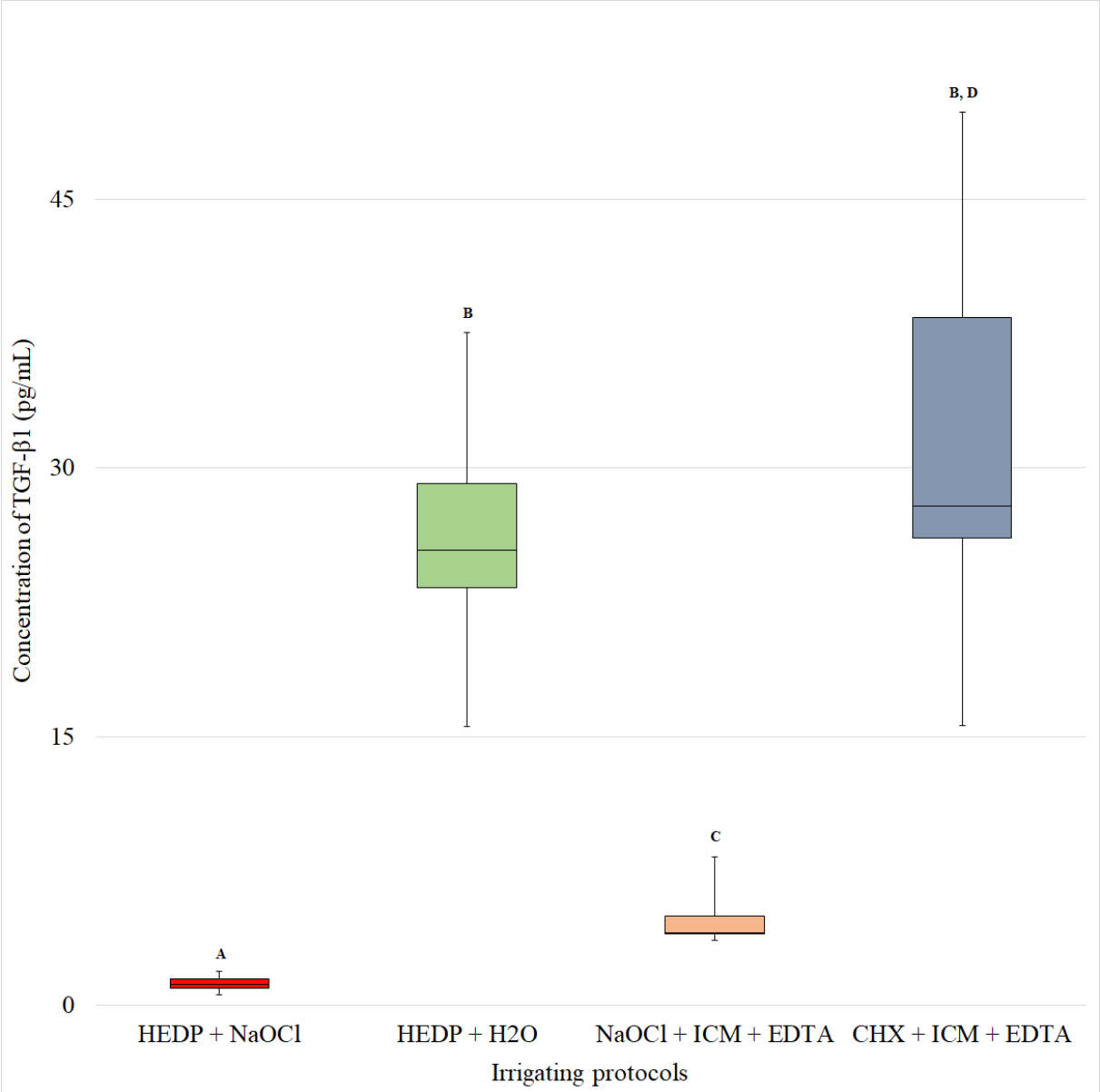
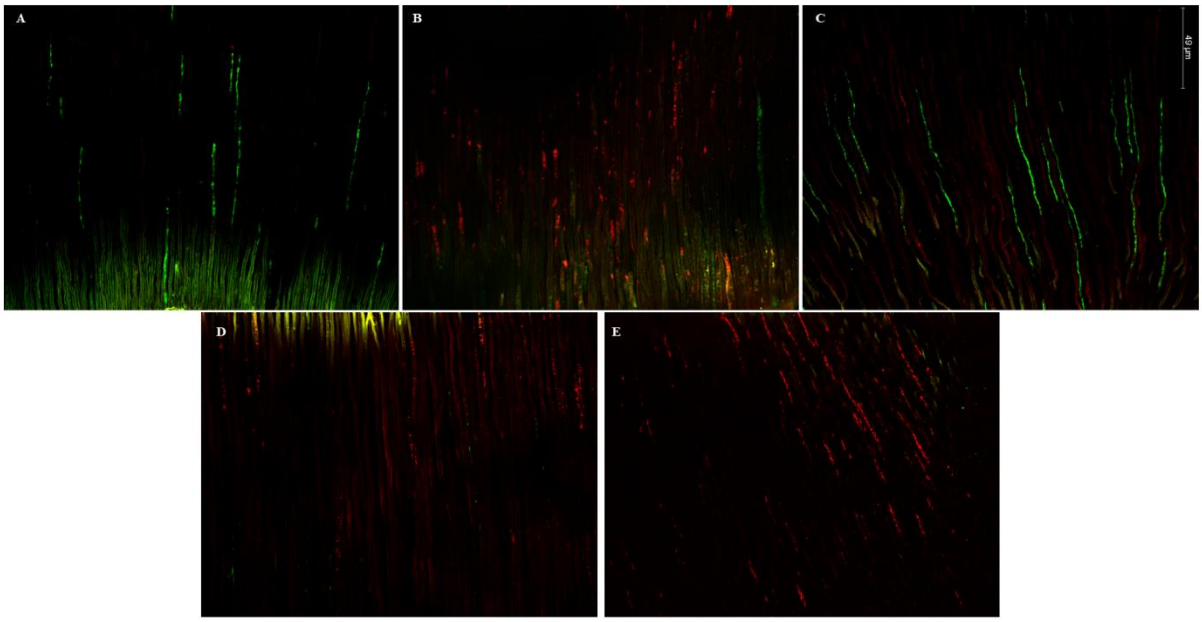


Figure 2. Microbiological status within the dentinal tubules showing viable (green) and non-viable (red) bacteria after the use of different protocols A) Control, B) HEDP + NaOCl, C) HEDP + H_2O, D) NaOCl + ICM + EDTA and E) CHX + ICM + EDTA.



Capítulo III

2.7 SARS-CoV-2 PANDEMIC: POTENTIAL RELATIONSHIP BETWEEN PSYCHIATRIC DISORDERS AND PULPITIS.

Rodrigo Arruda-Vasconcelos, Henrico B Strazzi-Sahyon, Lidiane M Louzada, Rebecca F Almeida-Gomes, Esdras G Alves-Silva, Paulo H dos Santos, Brenda PFA Gomes.

Dear editor,

In December 2019, the coronavirus outbreak officially started in Wuhan, Hubei, China and characterised as a pandemic by the World Health Organization (WHO) on 11th March 2020 when 6,315 people were infected[1]. Undoubtedly, this outbreak represents a public health emergency on a global scale[2]. On 27th February 2021, the WHO confirmed 112,902,746 cases and 2,508,679 deaths[3], with The United States of America (28,102,166 cases), India (11,079,979) and Brazil (10,390,461) as the countries with the highest prevalence of the virus[3]. Even after months into the pandemic, the world still faces ever increasing numbers of infections, with a recent record of 250,991 cases in a single day[3].

Several symptoms have been associated with the coronavirus disease (COVID-19) including fever, dry cough, absence of nasal congestion, persistent headache, anorexia, dizziness, asthenia (lack of energy and strength), chills, myalgia, conjunctivitis, diarrhoea and nausea/vomiting in the early stages, and the more distinctive symptoms including anosmia (loss of smell), ageusia (loss of taste), dyspnoea, (shortness of breath), chest pain, haemoptysis and vascular lesions in the later stages[4-9].

Lockdowns have been implemented as a measure to contain COVID-19 in several countries in order to minimise virus (SARS-CoV-2) transmission. As these measures were implemented, non-essential activities (i.e., pubs, restaurants, gyms, and stores) and those activities with the potential to cause agglomerations (i.e., schools and universities) were closed and travel restrictions were applied[10,11].

As a consequence of all the restrictive measures, individuals are susceptible to deleterious effects on their mental and physical health[11] and those presenting a history with symptoms of depression and anxiety may be more vulnerable. Previous studies have linked depression with inflammation, as detected by elevated levels of neutrophils, monocytes, $CD4^+$ T cells, interleukin (IL)-6, C-reactive protein (CRP) and prostaglandin E2 (PGE_2)[12,13]. Moreover, alterations in the tumour necrosis factor (TNF)-α system have also been reported to be associated with the development of psychiatric disorders and are related to changes in body weight, metabolism, and the endocrine system in psychiatric patients[14].

Symptomatic irreversible pulpitis refers to a severe degenerative process in the pulp tissue characterised by severe spontaneous pain or persistent pain after stimuli[15]. The most common aetiologic factor related to inflammation of the pulp tissues is caries. After microbial invasion, different cells including neutrophils, monocytes and T cells are recruited into the site of injury[16]. Studies have reported increased levels of TNF-α, IL-6 and CRP in teeth presenting irreversible pulpitis when compared to normal pulps[17-19]. The levels of PGE_2 in teeth with irreversible pulpitis have been associated with the degree of inflammation of the pulp tissue, higher in reversible pulpitis and lower in irreversible pulpitis, both conditions are characterised by exacerbated and prolonged pain[20].

In an Endodontic perspective, PGE_2 has been associated with several events including collagen degradation, vasodilatation and increasing vascular permeability[21]. IL-6 exhibits numerous biological effects and acts as a key mediator of the host response after tissue injury, infection, and inflammation[19]. It also triggers the up-regulation of adhesion molecules and stimulates angiogenesis leading to an increase in vascular permeability and oedema[19]. Additionally, IL-6 stimulates the production of CRP which is a member of the acute-phase reactants and belongs to a family of proteins involved in the innate immune response[22]. TNF-α is an important inflammatory mediator involved in the initial regulation of the host response, including the activation and expression of adhesion molecules, induction of the production of other cytokines and the proliferation of immune cells[19].

Several studies have detected an increase in the percentage of patients undergoing dental treatment during isolation/quarantine due to acute symptoms (i.e. pulpitis). A study compared the dental procedures in an oral emergency service in China in 2019 (1,537 patients) and 2020 (1,422 patients)[23]. Interestingly, the authors observed fewer total visits in 2020 when compared to 2019. On the other hand, a significant increase in the number of patients presenting acute symptoms including pulpitis was observed [2019: 278 cases (18.09%) ; 2020: 348 cases (24.5%)], acute apical periodontitis [2019: 143 cases (9.3%); 2020: 158 cases(11.1%)] and abscess [2019: 147 cases (9.56%); 2020: 184 cases(12.9%)]. A lower incidence in the need for procedures involving dental trauma, soft tissue injury and caries was observed[23].

Guo et al.[24] when evaluating the impact of COVID-9 on the utilization of emergency dental services (China), observed a reduction in the number of patients (1,567 pre-COVID-19 and 970 during COVID-19). However, the authors detected an increase in dental and oral infections from 51% (pre-COVID-19) to 71.9% (during COVID-19) and a reduction in dental trauma from 14.2% (pre-COVID-19) to 10.5% (during COVID-19).

Furthermore, another study conducted in China evaluated 96 patients seeking dental treatment and observed that most of them (56%) were admitted due to irreversible pulpitis[25]. Additionally, a study analysing over 1,500 patients over a five-week period during the pre-peak and peak period of COVID-19 in the U.K. identified pulpitis/periapical pathologies in 958 individuals (63.4%)[26].

Recently, a case-control study has associated periodontitis with increased risk of intensive care unit admission, mechanical ventilation and death of COVID-19[27], emphasizing the correlation between dental and health problems.

The development of the disease is related to the number and virulence of the microorganisms, and host response[28]. The biofilm formed in dental caries can destroy the tooth's protective barriers (i.e., enamel and dentine) and move towards the dental pulp causing little or no apparent symptoms. However, the host response is also influenced by external factors such as the mental disorders (including anxiety and depression) triggered by the fear of SARS-CoV-2 and the restrictive/isolation measures imposed during the outbreak. The truth is that outbreaks of infectious diseases, such as the current Coronavirus, can change all the habits of individuals and bring fear, which will affect their mental health, directly affecting their physical condition. In addition, the need for isolation ends up amplifying the problem.

While it is particularly important to stay informed and follow the authorities' recommendations, it is necessary to maintain healthy habits and the balance of emotions during this outbreak. The need for healthcare policies to provide psychological support, by presential or remote consults, should be assessed by the authorities. It could decrease the number of consultations due to the dental problems.

In conclusion, the increase in the number of patients seeking endodontic treatment due to acute symptomatology during the SARS-CoV-2 outbreak could be

related to the change of the individual´s habits and to the psychiatric disorders.

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The authors deny any conflicts of interest.

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3 DISCUSSÃO

O presente estudo foi submetido ao Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas. Os certificados de aprovação estão nos Anexos 1, 2 e 3. Todos os pacientes assinaram o Termo de Consentimento Livre e Esclarecido (TCLE) previamente a inclusão no estudo (Apêndice 1). A história médica e dados clínicos foram registrados em ficha específica (Apêndice 2).

3.1 Justificativa da pesquisa

Esta Tese foi dividida em 3 capítulos. Desta maneira, o capítulo I abordou o tema central nos artigos 1 a 4 relacionado a dentes com diagnóstico de pulpite irreversível sintomática. O capítulo II desta Tese englobou estudos ex vivo complementares ao primeiro capítulo. Por fim, o capítulo III esteve relacionado à pulpite em tempos atuais de pandemia.

Previamente à realização das coletas clínicas, estudo *ex vivo* foi conduzido com o objetivo de compreender o comportamento das substâncias que, posteriormente, seriam utilizadas na prática clínica em relação à extrusão de debris através do forame apical. Para tanto, a técnica de instrumentação dos canais radiculares foi planejada pensando no estudo clínico.

Apesar do tema principal deste estudo estar relacionado a dentes com pulpite irreversível, é importante salientar que as etapas do tratamento endodôntico se estendem para todas as condições relacionadas à prática clínica. Portanto, o artigo 5 desta Tese se encaixa em um amplo contexto.

A literatura científica é extensa no que se diz respeito a quantidade e qualidade dos estudos com o objetivo de monitorar os efeitos do tratamento endodôntico relacionados aos parâmetros clínicos, microbiológicos e imunológicos, especialmente em dentes com necrose pulpar e insucesso do tratamento endodôntico. Desde a instauração da linha de pesquisa “Microbiologia Aplicada à Endodontia” na Faculdade de Odontologia de Piracicaba – UNICAMP, diversos estudos foram realizados com esta finalidade. Entretanto, os estudos em dentes com polpas vitais (pulpite

irreversível) que têm como objetivo uma caracterização minuciosa referente a tal condição clínica são limitados. Portanto, a possibilidade de executar um estudo amplo, com o envolvimento de diversas variáveis, foi um fator motivador para a execução deste estudo. Este é o primeiro estudo da linha de pesquisa que se inicia em nosso laboratório (pulpite irreversível).

Ainda relacionado ao projeto de pesquisa proposto inicialmente, foi realizado um estudo *ex vivo* para quantificar os níveis de TGF-β1 liberados da matriz dentinária e avaliar a atividade antimicrobiana no interior dos túbulos dentinários através de diferentes protocolos de irrigação.

Por fim, é importante registrar o momento que estamos vivenciando com a epidemia do SARS-CoV-2. Com isso, fomos convidados a escrever sobre o tema "*The Global Response to the SARS-CoV-2 Pandemic from Endodontists and Researchers*".

3.2 Capítulo I - Estudos clínicos

3.2.1 Sujeitos da pesquisa e coletas das amostras clínicas

Foram incluídos neste estudo pacientes com diagnóstico de pulpite irreversível sintomática, baseados nos critérios da AAE (2013). Para tanto, a amostra foi constituída de pacientes portadores de dor aguda, espontânea e prolongada (30 segundos ou mais após a remoção do estímulo). Os voluntários relataram ineficácia ao uso de analgésicos.

O apêndice 3 mostra as características dos pacientes incluídos neste estudo. A amostra foi constituída de 20 pacientes, cada um contribuindo com um dente para a realização das coletas. A maioria dos voluntários foi do sexo feminino (85%). Doze pacientes (60%) eram menores de 40 anos de idade (média: 37,3 ± 8,9 anos). Apenas 2 dentes incluídos eram unirradiculares. A grande maioria dos dentes envolvidos era composta por molares. Apesar de não haver critérios para a exclusão de dentes multirradiculares, todas as coletas (inicial, pós preparo químico-mecânico e pós medicação intracanal) foram realizadas nos canais radiculares mais amplos de cada dente envolvido, conforme preconizado em estudo prévio (Gomes et al., 2015). Tal fato, permitiu atingir mais facilmente toda a extensão dos canais radiculares, sem que

houvesse instrumentação significativa previamente às coletas clínicas.

Todos os pacientes apresentaram restaurações fraturadas com cárie secundária ou cáries em dentes não restaurados previamente, dor espontânea no momento do atendimento e sensibilidade à percussão. Embora a resposta positiva ao teste de percussão possa indicar um comprometimento periapical, é possível que tal resposta possa ser devido a sensibilização do paciente que, geralmente tem sua qualidade de vida prejudicada em condições agudas, visto que a percepção de dor é um indicativo de qualidade de vida relatado pelos pacientes (Cimilli et al., 2012).

As coletas clínicas das amostras dos canais radiculares e tecidos periapicais foram realizadas com cones de papel absorvente estéreis/apirogênicos em toda a extensão do canal radicular e 2 mm além do forame apical, respectivamente (Gomes et al., 2015; Barbosa-Ribeiro et al., 2019; Duque et al., 2019; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021), e armazenados em tubos contendo meios específicos (Apêndice 4). De maneira geral, as coletas foram realizadas em quatro momentos distintos que permitiram o monitoramento do efeito do tratamento endodôntico em dentes com pulpite irreversível, sendo eles: I) antes do preparo químico-mecânico (baseline), II) após o preparo químico-mecânico, III) 48 horas após o preparo químico-mecânico e IV) após medicação intracanal aplicada por 30 dias (Apêndice 5).

3.2.2 Soluções irrigadoras – hipoclorito de sódio e clorexidina

Durante a realização do tratamento endodôntico é de fundamental importância a utilização de soluções irrigadoras com capacidade antimicrobiana. As principais soluções empregadas com esta finalidade são o hipoclorito de sódio e a clorexidina.

O hipoclorito de sódio é a solução irrigadora mais utilizada na prática endodôntica, uma vez que apresenta excelente propriedade antimicrobiana e dissolução tecidual (Siqueira Jr et al., 1998; Radcliffe et al., 2004; Zehnder, 2006; Stojicic et al., 2010). As concentrações de hipoclorito de sódio variam de 0,5% a 8,25% (Kuruvilla & Kamath, 1998; Ercan et al., 2004; Vianna et al., 2006; Barbosa-Ribeiro et al., 2016; Zandi et al., 2016, 2019; Demenech et al., 2021).

Por outro lado, a clorexidina tem sido sugerida como alternativa ao uso do hipoclorito de sódio devido ao seu amplo espectro antimicrobiano, substantividade, ação reológica e menor toxicidade comparada ao hipoclorito de sódio (Gomes et al., 2013).

Estudos não observaram diferenças significativas em relação a atividade antimicrobiana entre a clorexidina 2% e o hipoclorito de sódio em concentrações mais elevadas (5,25% e 6%) que a usualmente utilizada (2,5%) (Ercan et al., 2004, Gomes et al., 2013; Barbosa-Ribeiro et al., 2016, 2019; Zandi et al., 2016, 2019). Além disso, ambas têm sido associadas a redução dos níveis de endotoxinas e ácido lipoteicóico (Endo et al., 2012; Barbosa-Ribeiro et al., 2016; Neelakantan et al., 2019; Aveiro et al., 2020; Louzada et al., 2020).

Como ainda são poucos os estudos clínicos com o objetivo de monitorar os efeitos do tratamento endodôntico em dentes com pulpite irreversível, foi proposta a comparação entre clorexidina 2% gel e hipoclorito de sódio 6%. Embora o hipoclorito de sódio 2,5% seja a mais utilizado, a equipe está monitorando o efeito do tratamento endodôntico com o hipoclorito de sódio 6%, desta maneira, será possível o cruzamento de dados de diferentes condições clínicas com menos variáveis.

3.2.3 Medicação intracanal

O hidróxido de cálcio tem sido amplamente empregado como medicação intracanal. O principal objetivo desta manobra clínica é promover o controle microbiano e, consequentemente, reduzir os níveis de mediadores inflamatórios (Tavares et al. 2012; Martinho et al., 2018; Duque et al., 2019).

Apesar de apresentar boas propriedades antimicrobianas, alguns patógenos endodônticos são resistentes ao hidróxido de cálcio (Mohammadi & Dummer, 2011). Desta maneira, a associação a outras substâncias (i.e., clorexidina) tem sido sugerida (Gomes et al., 2003; Barbosa-Ribeiro et al., 2019; Duque et al., 2019; Uluköylü et al., 2019; Louzada et al., 2020).

Nossos resultados mostraram que o hidróxido de cálcio aplicado por 30 dias favoreceu o controle da infecção, e maior redução dos níveis de ácido lipoteicóico,

concordando com estudos prévios (Barbosa-Ribeiro et al., 2016; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021). Além disso, foi observado redução de biomarcadores inflamatórios, corroborando com a literatura (Tavares et al., 2012; Martinho et al., 2018; Duque et al. 2019).

Outro ponto relacionado ao tempo de aplicação da medicação intracanal é a possibilidade do enfraquecimento dentinário e, consequentemente, possibilidade de fratura quando empregada por longos períodos (Andreasen et al., 2002). Entretanto, estudo clínico recente não associou períodos prolongados (1 a 12 meses) de medicação à base de hidróxido de cálcio a risco aumentado de fratura radicular (Best et al., 2021).

Diversos estudos estão sendo conduzidos em nosso laboratório em diferentes condições clínicas (infecção endodôntica primária sintomática e assintomática, infecção endodôntica secundária/persistente e lesões endodôntico-periodontais) com o mesmo tempo de aplicação da medicação intracanal. No futuro, isto permitirá a comparação dos achados clínicos e, possivelmente, propor diferentes tempos para utilização de medicação intracanal.

3.3 Aspectos microbiológicos dos canais radiculares

As análises microbiológicas foram realizadas a partir de coletas realizadas em toda a extensão dos canais radiculares de dentes com pulpite irreversível sintomática.

3.3.1 Cultura microbiana

Métodos tradicionais de cultura microbiana têm sido empregados em diferentes estudos com o objetivo de monitorar os efeitos do tratamento endodôntico nos níveis bacterianos em diferentes condições clínicas (Martinho et al., 2008; Endo et al., 2012). Apesar de ser um método consolidado, a cultura microbiana possui limitações, tais como, subestimar a carga e a diversidade microbiana dos canais radiculares (Bícego-Pereira et al., 2020).

Nossos resultados mostraram um crescimento microbiano nas amostras iniciais dos canais radiculares na ordem de 10^2 UFC/mL. Entretanto, após a realizado do preparo químico-mecânico foi observado uma redução significativa nos níveis de bactérias, independentemente da utilização da clorexidina 2% gel ou hipoclorito de sódio 6%, concordando com achados prévios (Valera et al., 2015; Barbosa-Ribeiro et al., 2016). Além disso, a literatura mostra uma redução acentuada na carga bacteriana após a realização do preparo químico-mecânico, variando de 80% a mais de 99% (Martinho et al., 2008; Endo et al., 2012; Gomes et al., 2009; Dornelles-Morgental et al., 2011; Barbosa-Ribeiro et al., 2016; Aveiro et al., 2020).

A medicação intracanal não foi capaz de promover redução adicional de bactérias comparado às coletas realizadas após o preparo químico-mecânico, o que é consistente com a literatura (Barbosa-Ribeiro et al., 2016). Entretanto, deve-se levar em consideração a expressiva redução microbiana atingida após o preparo químico-mecânico.

3.3.2 Nested PCR

O Nested PCR é uma técnica sensível (Rotstein et al., 2006), com capacidade de identificar pequenas quantidades de DNA microbiano (Louzada, 2019). Diversos estudos realizados em nosso laboratório utilizaram tal técnica para monitorar os efeitos do tratamento endodôntico utilizando sempre as espécies apresentadas neste trabalho (Gomes et al., 2005; 2006ab, 2007; Montagner et al., 2010; Nóbrega et al., 2013; Endo et al., 2013; Rosa et al., 2015, Louzada, 2019).

Foram identificadas bactérias Gram-positivas e Gram-negativas em canais radiculares de dentes com pulpite irreversível.

Apesar de frequentemente isolado em casos de insucesso do tratamento endodôntico (Barbosa-Ribeiro et al., 2020ab; Bícego-Pereira et al., 2020) e apresentar grande resistência aos procedimentos endodônticos, *E. faecalis* foi detectado em todas as amostras iniciais de dentes com pulpite irreversível sintomática. Além disso, tal cepa também tem sido reportada em casos de infecção endodôntica primária e lesões endodôntico-periodontais (Aveiro et al., 2020; Louzada et al., 2020), utilizando

diferentes metodologias (sequenciamento genético e *checkerboard DNA-DNA hybridization*). *E. faecalis* apresenta capacidade de adesão às células do hospedeiro e a outras bactérias, favorecendo a formação de biofilme e difícil remoção (Ayre et al., 2018). Como observado em estudos anteriores e confirmado no estudo atual, *E. faecalis* foi detectado após o tratamento endodôntico, independente da solução irrigadora utilizada durante o preparo químico-mecânico (Valera et al., 2015; Barbosa-Ribeiro et al., 2016; Aveiro et al., 2020; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021).

Fusobacterium nucleatum foi detectado em 70% das amostras, concordando com estudos anteriores (Baumgartner et al., 2004; Khemaleelakul et al., 2006; Sakamoto et al., 2006). *F. nucleatum* é uma espécie Gram-negativa, associada a sintomatologia dolorosa (Gomes et al., 2004; Sakamoto et al., 2006). *F. nucleatum* apresenta características de adesão que podem favorecer a co-agregação com outras espécies microbianas (Cavali et al., 2017). A eliminação de *F. nucleatum* não foi alcançada após os procedimentos endodônticos, independentemente da substância química auxiliar (clorexidina ou hipoclorito de sódio) (Louzada et al., 2020, Barbosa-Ribeiro et al., 2020ab).

Apesar da menor prevalência, bactérias dos gêneros *Filifactor*, *Gemella*, *Parvimonas*, *Porphyromonas*, *Tannerella* and *Treponema* foram detectadas. Estes grupos microbianos são frequentemente encontrados em casos de infecções de longa duração e lesões endodôntico-periodontais (Endo et al., 2013; Signoretti et al., 2013; de Brito et al., 2020; Barbosa-Ribeiro et al., 2020; Gomes et al., 2020; Louzada et al., 2020).

3.3.3 *Checkerboard DNA-DNA hybridization*

O *checkerboard DNA-DNA hybridization* foi utilizado para a avaliação do perfil microbiológico na dentina infectada (cárie) e canais radiculares. Tal método tem sido empregado com esta finalidade em ambos os sítios (Rôças et al., 2015; Aveiro et al., 2020; Louzada et al., 2020; Machado et al., 2020; Arruda-Vasconcelos et al., 2021). Além disso, o *checkerboard DNA-DNA hybridization* permite a investigação concomitante de até 40 espécies microbianas (Socransky et al., 1994; Vianna et al.,

2008; Aveiro et al., 2020; Louzada et al., 2020; Arruda-Vasconcelos et al., 2021).

Diversas espécies microbianas foram detectadas na dentina infectada (37/40) e nos canais radiculares (38/40). Dentre as bactérias presentes em ambos os sítios, é possível destacar a presença de *E. faecalis*, micro-organismo anaeróbio facultativo, Gram-positivo, frequentemente isolado em casos de insucesso do tratamento endodôntico (Gomes et al., 2006, 2008; Barbosa-Ribeiro et al., 2016) em 100% e 80% das amostras da dentina infectada e canais radiculares, respectivamente. Além disso, bactérias dos gêneros, *Eubacterium*, *Fusobacterium*, *Parvimonas* e *Porphyromonas* relacionadas a dor foram encontradas, corroborando com estudos anteriores (Gomes et al., 1996, 2004).

Tanto o preparo químico-mecânico, quanto a medicação intracanal à base de hidróxido de cálcio promoveram redução nos níveis e diversidade de bactérias dos canais radiculares, como reportado previamente (Barbosa-Ribeiro et al. 2016, 2019; Duque et al., 2019; Aveiro et al., 2020; Louzada et al., 2020).

3.3.4 Fatores de virulência – Endotoxinas e ácido lipoteicóico

A natureza polimicrobiana da dentina infectada e dos canais radiculares de dentes com pulpite irreversível, composta por bactérias Gram-negativas e Gram-positivas, evidencia a presença de marcadores biológicos importantes, tais como endotoxinas e ácido lipoteicóico.

Altos níveis de endotoxinas foram associados a presença de sintomatologia (Jacinto et al., 2005; Vianna et al., 2007), reabsorção óssea e presença de inflamação (Jacinto et al., 2005; Vianna et al., 2007; Gomes et al., 2012; Martinho et al., 2017; Aveiro et al., 2020). Nossos resultados revelaram altos níveis de endotoxinas na dentina infectada comparado às amostras iniciais dos canais radiculares, evidenciando a cárie como fonte primária de micro-organismos.

Foi observado uma redução significativa de LPS dos canais radiculares após o preparo químico-mecânico realizado com clorexidina ou hipoclorito de sódio, concordando com a literatura atual (Duque et al., 2019; Aveiro et al., 2020). A medicação intracanal não promoveu redução significativa de LPS, corroborando com

achados de Louzada et al. (2020).

Em relação aos níveis de LTA, estes foram mais elevados na dentina infectada e amostras iniciais de canais radiculares. Tanto o preparo químico-mecânico quanto a medicação intracanal promoveram redução significativa nos níveis de LTA nos dois grupos investigados. A completa remoção de LTA de canais radiculares tem se mostrado desafiadora (Barbosa-Ribeiro et al., 2016; Aveiro et al., 2020; Louzada et al., 2020), devido a capacidade de adesão às paredes dentinárias (Ciardi et al., 1977; Hahn & Liewehr, 2007).

3.4 Aspectos imunológicos

Para a avaliação dos aspectos imunológicos, as coletas clínicas foram realizadas a partir dos tecidos periapicais de dentes com pulpite irreversível sintomática, como descrito por outros autores (Barbosa-Ribeiro et al., 2019; Duque et al., 2019).

3.4.1 Citocinas

A presença de bactérias e seus fatores de virulência estimulam a indução do processo inflamatório através da liberação de biomarcadores, tais como, TNF-α, IL-1α, IL-1β e metaloproteinases de matriz (Gomes & Herrera, 2018; Barbosa-Ribeiro et al., 2019).

Diferentes mediadores inflamatórios têm sido propostos como indicadores do processo inflamatório (Elsalhy et al., 2013). TNF-α é uma citocina pró-inflamatória que desempenha papel importante no início do processo inflamatório, principalmente através da indução de outras citocinas e proliferação de células do sistema imune (Elsalhy et al., 2013). Em contrapartida, IL-10 é uma citocina anti-inflamatória com efeito imunomodulador, inibindo a produção de outras citocinas, tais como, TNF-α e IL-1β (Markert, 2003; Mosser & Zhang, 2008; Elsalhy et al., 2013).

Maiores níveis de TNF-α e IL-10 foram observados nas amostras iniciais dos tecidos periapicais. Entretanto, o preparo químico-mecânico e a medicação intracanal promoveram significativa redução nos níveis de ambas as citocinas, concordando com outros estudos (Barbosa-Ribeiro et al., 2019; Duque et al., 2019).

Os níveis de IL-1α e IL-1β foram reduzidos em todas as fases do tratamento endodôntico, corroborando com estudo prévio (Zehnder et al., 2003). Contrariamente, nossos achados são divergentes de outro estudo que observou níveis aumentados de IL-1β comparado a TNF-α em dentes com pulpite irreversível (Abd-Elmeguid et al., 2013); entretanto, no estudo supracitado, as análises foram realizadas diretamente nos tecidos pulpares, ao invés de coletas clínicas dos tecidos periapicais.

3.4.2 Metaloproteinases de matriz

Sabe-se que as metaloproteinases de matriz parcialmente regulam os processos inflamatórios que ocorrem nos tecidos pulpares (Jain & Bahuguna, 2015). Os níveis de MMP-2, -8 e -9 se apresentaram mais elevados nas amostras iniciais quando comparadas àquelas realizadas após o preparo químico-mecânico e medicação intracanal. Por outro lado, após o preparo químico-mecânico, foi observado aumento dos níveis de MMP-3, que tem sido associada a angiogênese (Zheng et al., 2009) e efeitos anti-inflamatórios (Eba et al., 2012). Após a uso da medicação intracanal, os níveis de MMP-3 foram reduzidos a níveis similares aos encontrados inicialmente, sugerindo um equilíbrio entre os processos de inflamação e reparo (Cooper et al., 2010).

Em relação aos níveis de MMP-13, foram observados níveis progressivamente mais elevados nas diferentes etapas do tratamento endodôntico, sugerindo possível processo de reparo (Barbosa-Ribeiro et al., 2019).

3.5 Aspectos relacionados a dor

Para avaliação da percepção da dor e quantificação de biomarcadores relacionados a tal condição clínica, os pacientes foram instruídos a preencher uma

escala analógica de dor. Além disso, a quantificação dos níveis de PGE2 e substância P foi realizada a partir de amostras clínicas dos tecidos periapicais.

3.5.1 Aspectos relacionado à dor

O envolvimento da PGE2 e substância P nos aspectos relacionados à dor, tem sido proposto. Na consulta inicial, foram reportados maiores níveis de dor. Fato também observado em relação aos níveis de PGE2 e substância P. Entretanto, 48 horas após a realização dos procedimentos endodônticos com clorexidina 2% gel ou hipoclorito de sódio 6%, os níveis de dor foram substancialmente reduzidos. Apesar de não estatisticamente significativa, houve redução de PGE2 e substância P nos tecido periapicais (S2), sugerindo uma relação entre dor e estes biomarcadores.

A utilização do hipoclorito de sódio em alta concentração não foi relacionado com aumento de dor pós-operatória quando comparado ao hipoclorito de sódio, corroborando com estudo prévio (Demenech et al. 2021).

3.5.2 PGE2

Os níveis de PGE2 foram mais elevados nas amostras iniciais dos tecidos periapicais. PGE2 tem sido sugerido com um biomarcador para o diagnóstico de dentes com pulpite irreversível, uma vez que maiores níveis de PGE2 têm sido encontrados em dentes com polpas inflamadas comparado a dentes com polpa normal (Petrini et al., 2012). Outros autores observaram níveis elevados de PGE2 nas amostras iniciais de dentes com infecção endodôntica primária e lesão periapical evidenciada radiograficamente (Martinho et al., 2018; Karataş et al., 2020), situação clínica comumente associada a processo inflamatório provocado por micro-organismos.

Os níveis de PGE2 foram reduzidos após os preparo químico-mecânico, utilizando tanto a clorexidina ou hipoclorito. Apenas após uso de medicação intracanal foi observado redução significativa comparado à coleta inicial, sugerindo atenuação do processo inflamatório (Martinho et al., 2018; Karataş et al., 2020).

3.5.3 Substância P

Foram observados maiores níveis de substância P nas amostras iniciais dos tecidos periapicais. Estes resultados são compatíveis com estudos anteriores que detectaram maiores níveis de substância P em dentes com pulpite irreversível (Sattari et al., 2010; Akbal Dincer et al., 2020). Outro estudo também observou maiores níveis deste neuropeptídeo em dentes com lesão periapical sintomática, comparando amostras realizadas antes e após o tratamento endodôntico (Arslan et al., 2020).

Maior redução dos níveis de substância P foram observados após utilização da medicação intracanal, possivelmente por propiciar um microambiente menos inflamatório (Arslan et al., 2020; Bamini et al., 2020; Evangelin et al. 2020).

3.6 Capítulo II - Estudos *ex vivo*

3.6.1 – Extrusão apical de debris

Em relação ao estudo *ex vivo*, foi observada extrusão apical de debris em todos os grupos. O presente estudo se utilizou de técnica clássica para a quantificação de debris extruídos (Meyers & Montgomery, 1991). Entretanto, este estudo apresenta limitação, uma vez que o forame apical está suspenso no ar, sem que haja algum aparato para simular os tecidos periapicais, como descrito em outro estudo que utilizou gel de agarose a 1,5% (Alves et al., 2017).

Um achado interessante foi a menor quantidade de debris extruídos quando da utilização da clorexidina em gel. Provavelmente, estes resultados foram atingidos pela formulação da clorexidina que confere viscosidade e ação reológica, mantendo os debris em suspensão durante o preparo químico-mecânico, o que favorece a sua remoção dos canais radiculares (Gomes et al., 2013).

3.6.2 Biomoléculas - Fator de transformação do crescimento beta 1 (TGF- β1)

A liberação de biomoléculas da matriz dentinária (TGF-β1) é um fator importante para que ocorra regeneração dos tecidos pulpares, uma vez que estão

envolvidas nos processos de angiogênese, mineralização, recrutamento, proliferação e diferenciação celular (Zhang et al., 2011; Smith et al., 2011; Galler et al., 2015).

De maneira geral, os resultados obtidos neste estudo relevaram que maiores níveis de TGF-β1 foram observados em protocolos nos quais o hipoclorito de sódio não foi utilizado, uma vez que esta substância química é capaz de promover a desnaturação de proteínas (Yamaguchi et al., 2001). Além disso, a utilização de uma medicação intracanal promoveu maior desinfecção do sistema de canais radiculares, além de potencializar a liberação de TGF-β1 da matriz dentinária.

3.7 Capítulo III – Aspectos atuais

3.7.1 Aspectos relacionados à pandemia do SARS-CoV-2

Por fim, é importante registrar que estamos diante de um cenário completamente novo e desafiador devido à pandemia causada pelo SARS-CoV-2. A literatura tem associado presença de sintomas físicos (dores, fadiga, falta de ar, dentre outros) e aumento de mediadores inflamatórios (TNF-α, PGE2, IL-6 e proteína C reativa) em pacientes com desordens psicológicas. Ao mesmo passo, em alterações dentais agudas (pulpite), também são observados níveis aumentados de biomarcadores, como apresentado neste trabalho e em outros estudos relacionados (Elsalhy et al., 2013). Embora não existam estudos que comprovem a ligação entre as desordens psicológicas exacerbadas pela crise sanitária, diversos estudos observaram aumento nos casos agudos, tais como pulpite e abscesso, apesar de haver uma redução do número de atendimentos odontológicos neste período (Grossman et al., 2020; Guo et al., 2020; Yu et al., 2020).

3.8 Análises em andamento

Pensando nas próximas pesquisas, durante o Doutorado foram realizadas coletas clínicas adicionais para permitir a extração do máximo de informações clínicas possíveis dos pacientes participantes.

Para tanto, além das coletas dos canais iniciais e tecidos periapicais, foram realizadas coletas da cárie dental e fluido dentinário destes pacientes que, em futuro próximo, possibilitará a correlação entre os níveis bacterianos, de fatores de virulência, citocinas e metaloproteinases de matriz da cárie dental/fluido dentinário com os canais radiculares/tecidos periapicais.

Além dos dados obtidos nesta Tese de Doutorado, ainda existem análises em andamento que serão apresentadas no futuro, entre elas, a caracterização microbiológica através do *Next Generation Sequencing*. Esperamos com isso, no futuro, prever os níveis de biomarcadores presentes no canal radicular a partir das amostras coletadas na porção coronária do elemento dental.

3.9 Considerações finais

Apesar de o uso de uma medicação intracanal não ser mandatória em casos de dentes com polpas vitais, nossos resultados mostraram efeitos positivos em alguns parâmetros analisados, tais como na redução adicional de algumas espécies bacterianas, dos níveis de ácido lipoteicóico, e modificação dos níveis de citocinas, metaloproteinases de matriz, substância P e PGE2.

Em situações específicas, onde não é possível a obturação dos canais radiculares em sessão única, tais como, persistência de sangramento, pacientes com alterações sistêmicas, onde há necessidade de consultas mais curtas, falta de tempo por inabilidade do profissional ou baixa cooperação do paciente, a utilização de uma medicação intracanal pode melhorar os resultados do tratamento (Arruda-Vasconcelos et al., 2021).

Embora o processo infeccioso em dentes com pulpite irreversível apresente menor tempo de duração quando comparado a dentes com necrose pulpar e evidencia radiográfica de lesão periapical, o presente estudo mostrou a presença de endotoxinas, ácido lipoteicóico e bactérias relacionadas a sintomatologia clínica e insucesso do tratamento endodôntico. Diante deste fato, a realização de protocolos clínicos eficazes, visando a desinfecção do sistema de canais radiculares se faz necessário.

É importante ressaltar que micro-organismos resistentes, localizados em regiões de difícil acesso, podem sobreviver aos procedimentos endodônticos, e com isso, manter um processo infeccioso que poderá resultar em insucesso do tratamento endodôntico (Pinheiro et al., 2003). Para minimizar a ocorrência do insucesso do tratamento endodôntico, foram realizadas diversas manobras clínicas, como remoção de todo tecido cariado antes do tratamento endodôntico propriamente dito, realização do preparo químico-mecânico com substâncias químicas auxiliares com atividade antimicrobiana, irrigação ultrassônica passiva, com o objetivo de atingir áreas de difícil acesso, obturação adequada e selamento coronário em resina composta em todas as etapas do monitoramento.

O uso do hipoclorito de sódio em maior concentração tem sido relacionado com maior capacidade de dissolução do tecido pulpar (Cullen et al. 2015), sem que haja aumento de dor pós-operatória (Demenech et al. 2021). Com isso, o emprego do hipoclorito de sódio 6% pode ser útil na tentativa de promover eliminação de tecido pulpar remanescente que pode servir de substrato para o crescimento microbiano, contribuindo para o insucesso do tratamento endodôntico.

4 CONCLUSAO

De acordo com as metodologias propostas neste estudo, é possível concluir que:

Capítulo I

1) Dentes com pulpite irreversível apresentam um perfil microbiano complexo, com presença de bactérias Gram-positivas e Gram-negativas, anaeróbios facultativos e estritos. (Artigo 1)
2) O tratamento endodôntico realizado com clorexidina 2% gel ou hipoclorito de sódio 6% se mostrou efetivo na redução dos níveis de bactérias, endotoxinas e ácido lipoteicóico em dentes com pulpite irreversível. (Artigo 2)
3) O tratamento endodôntico apresentou efeito positivo nos níveis de citocinas e metaloproteinases de matriz, independentemente da substância química auxiliar utilizada (clorexidina ou hipoclorito). A medicação intracanal à base de hidróxido de cálcio foi efetivo na manutenção dos biomarcadores inflamatórios em níveis baixos. (Artigo 3).
4) O tratamento endodôntico por meio do preparo químico-mecânico (clorexidina 2% ou hipoclorito de sódio 6%) e medicação intracanal à base de hidróxido de cálcio foi efetivo no controle microbiano e reduziu os níveis de prostaglandina E2 e substância P. (Artigo 4)

Capítulo II

5) Houve extrusão apical de debris durante a técnica de irrigação ultrassônica passiva. O preparo químico-mecânico realizado com clorexidina 2% gel e solução salina foi o método mais para minimizar a extrusão de debris. (Artigo 5)
6) O hipoclorito de sódio dificulta a liberação de TGF-β1 da matriz dentinária, podendo reduzir as chances da criação de um microambiente propício para a regeneração pulpar. (Artigo 6)
7) A utilização de substâncias quelantes (EDTA ou HDEP) e clorexidina permitiu a liberação de TGF-β1 da matriz dentinária, além de apresentar atividade antimicrobiana. (Artigo 6)

Capítulo III

8) O aumento do número de pacientes em busca de tratamento endodôntico devido a sintomatologia aguda durante a pandemia do coronavírus pode estar relacionado a alterações psicológicas. (Artigo 7)

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APÊNDICE 1 – Termo de Consentimento Livre e Esclarecido

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TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

Gostaríamos de convidá-lo a participar da pesquisa intitulada:

"Monitoramento clínico, microbiológico, imunológico e biomolecular das diferentes condições pulpares e perirradiculares"

1 – Apresentação da pesquisa:

1.1 Identificação da instituição

Faculdade de Odontologia de Piracicaba – Universidade Estadual de Campinas
Endereço: Avenida Limeira, 901, Bairro Areião, CEP: 13414-903 – Piracicaba – SP.
Telefone: (19) 2106-5215 (Endodontia)

1.2 Responsável pela pesquisa

As informações contidas neste documento foram fornecidas pela orientadora Profª. Drª. Brenda Paula Figueiredo de Almeida Gomes e/ou pelos pesquisadores Emelly de Aveiro, Ezequiel Gabrielli, Juliana Delatorre Bronzato, Lidiane Mendes Louzada, Maria Eunice da Silva Davidian, Rodrigo Arruda Vasconcelos e Vito Madio Chiarelli Neto, responsáveis também pela apresentação e obtenção do consentimento.

1.3 Justificativa

a. Este trabalho visa investigar a presença bacteriana em casos de dentes com polpa vital e indicação protética (que servirá como controle), pulpite irreversível, abscesso periapical (agudo e crônico), dentes com envolvimento periodontal crônico e com lesão endo-periodontal combinada, dentes com insucesso do tratamento endodôntico com necessidade de retratamento ou de cirurgia parendodôntica. A realização desta pesquisa servirá para avaliar o efeito do tratamento na redução destas bactérias.
b. Investigar o conteúdo tóxico de bactérias, avaliando a contribuição destas moléculas no desenvolvimento de dor e presença de lesões no ápice do dente.
c. Pesquisas realizadas em nosso laboratório alertaram sobre o aumento da resistência à antibióticos de determinadas bactérias encontradas em canais radiculares de dentes com insucesso do tratamento endodôntico. Desta forma, é necessário um acompanhamento da resistência aos antibióticos destas bactérias.

2 – Informações sobre a pesquisa:

2.1 Objetivo geral

Avaliar a influência das bactérias e seus produtos tóxicos na indução de inflamação/infecção em dentes. E também avaliar o efeito do tratamento de canal na redução e/ou eliminação desse conteúdo em canais radiculares, objetivando analisar o efeito das diferentes formas de tratamento utilizadas.

Visa também investigar se há resistência ou não das bactérias aos antibióticos usados rotineiramente em endodontia

2.2 Procedimentos e metodologias

Seleção dos pacientes

Inicialmente serão analisadas as fichas dos pacientes cadastrados na Área de Endodontia da Faculdade de Odontologia de Piracicaba (FOP/UNICAMP) encaminhados aos Cursos de Atualização e Especialização. Ao final, serão selecionados 440 voluntários com idade entre 18 e 60 anos, de ambos os sexos e independente de raça. Os pacientes serão divididos em grupos de acordo com a condição do dente. Serão excluídos voluntários que trataram o dente a menos de 2 anos; que tenham usado antifúngicos e antibióticos em menos de 3 meses; dentes com fratura na raiz; dentes com extensa destruição; dificuldade de acesso ao canal radicular; e dentes sem restauração, seja temporária ou definitiva.

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Exame clínico (anamnese e exame físico)

Serão anotados dados pessoais dos pacientes tais como idade e gênero, história médica e dentária.

Aspectos clínicos e radiográficos

Ao iniciar o tratamento, durante o exame clínico inicial serão registradas informações sobre o dente, onde será verificada a presença/ausência de dor, natureza da dor, dor à percussão (horizontal e vertical) e à palpação. Também serão anotados dados tais como: presença/ausência de edema (inchaço), fístulas, mobilidade dental e bolsa periodontal. Todas essas características clínicas serão anotadas na ficha clínica de cada paciente.

Coleta de amostras

As coletas clínicas do canal radicular, bolsas periodontais e lesões periapicais serão realizadas na Clínica de Especialização da FOP/UNICAMP, e as amostras processadas no Laboratório de Microbiologia Endodôntica.

Serão utilizadas técnicas assépticas e instrumentos esterilizados. Haverá a remoção de cáries e restaurações defeituosas, assim como a realização do isolamento absoluto para não haver infiltração de saliva durante o procedimento.

Antes do atendimento, será realizada a descontaminação do rosto do paciente com clorexidina gel 2% e anestesia local na região do dente envolvido. Após a abertura do dente, será utilizado o localizador apical (Novapex, Forum Technologies, Rishon le-zion, Israel) para confirmar se o canal está totalmente acessível para o tratamento e obter o comprimento da raiz.

Para as coletas das amostras, cones de papel absorventes estéreis e apirogênicos serão introduzidos no dente, permanecendo nesta posição por 60 segundos.

A instrumentação do dente será realizada por limas acionadas a motor e manuais, possibilitando posterior obturação do canal radicular. Serão utilizadas substâncias químicas rotineiramente utilizadas no tratamento de canal para instrumentação dos dentes. Em alguns grupos serão colocados medicação intracanal durante 30 dias. Posteriormente, o dente será obturado e selado com restauração definitiva.

Para a coleta periodontal um cone será introduzido até o fundo da bolsa periodontal permanecendo por 1 minuto. Em seguida, esse cone será transferido para frascos de vidro apirogênicos e armazenados em freezer -20 °C.

Para coleta de lesões periapicais, as amostras serão coletadas após acesso cirúrgico durante cirurgia apical, com o uso de microscópio operatório DFVasconcellos, (DFV, São Paulo). Após assepsia do campo operatório com solução de iodo-povidine a 7,5% e aplicação de anestesia local, será feita incisão, levantamento de retalho e acesso ao ápice radicular com baixa rotação equipada com broca esférica carbide-tungstênio sob aplicação de solução salina fosfatada tamponada, estéril (PBS com ph de 7,4) para resfriamento. Então a lesão periapical será curetada.

2.3 Possibilidades de inclusão em grupo controle ou placebo

Alguns participantes poderão ser incluídos no grupo controle, pois também será realizado o tratamento de pacientes que apresentam indicação protética em dentes vivos (com o objetivo de instalar pinos na raiz do dente), para verificar-se as condições biológicas do dente sadio. Nesta situação a indicação estará bem adequada, não havendo recrutamento extra de voluntários, pois os pacientes já estarão encaminhados para a realização do tratamento endodôntico.

2.4 Métodos alternativos para obtenção da informação ou tratamento da condição

Não há formas alternativas de obtenção da informação desejada ou tratamento da condição.

2.5 Desconforto ou riscos esperados

A pesquisa tentará minimizar ao máximo possíveis desconfortos, principalmente aquele relacionado à dor pós-operatória, através de um monitoramento contínuo dos pacientes e a utilização de materiais e técnicas adequadas e seguras. Independentemente do grupo, todos os pacientes receberão um tratamento seguro e eficaz, para evitar dor e desconforto durante e após o procedimento clínico.

O tratamento será convencional, com a aplicação de anestesia local, isolamento absoluto, utilização de instrumentos estéreis e a utilização de equipamentos de proteção individual (óculos de proteção, máscaras, luvas, etc), tanto para o paciente quanto para o pesquisador.

Os instrumentos e substâncias químicas utilizadas são constantemente testados e aprovados em pesquisas científicas publicadas na área. Serão adotadas ainda, algumas combinações de medicação intracanal, principalmente nos casos onde se espera maior resistência bacteriana, a fim de combater a infecção dos canais radiculares.

Os procedimentos não acarretam risco de morte para os pacientes, assim como também não exige despesas extras, já que se trata de atendimento convencional realizado diariamente nesta Faculdade.

Qualquer desconforto ou risco são de natureza própria do tratamento endodôntico convencional, não havendo

APÊNDICE 1 – Termo de Consentimento Livre e Esclarecido (continuação).

desconforto ou risco relacionados as coletas da pesquisa. O processo é usualmente sem dor e, caso haja dor, esta não será devido à coleta de amostra e sim referente à inflamação/infecção nos canais radiculares e/ou tecidos perirradiculares. Os procedimentos desenvolvidos nesse trabalho estão em acordo com os tratamentos indicados e realizados atualmente na área de Endodontia da FOP/UNICAMP.

2.6 Benefícios do experimento

Não há benefício direto e específico pela participação na pesquisa.

2.7 Forma de acompanhamento e assistência ao participante:

Se houver dor fora dos dias marcados para o tratamento, o paciente receberá uma assistência imediata dos responsáveis pela pesquisa, assim o mesmo deverá entrar em contato através dos telefones locais descritos a seguir:
(0xx19) 2106 5215 (laboratório de Endodontia)
(0xx19) 98154-2449 (celular da pesquisadora Emelly de Aveiro)
(0xx54) 99929-8229 (celular do pesquisador Ezequiel Gabrielli)
(0xx19) 99699-4390 (celular da pesquisadora Juliana Delatorre Bronzato)
(0xx16) 99703-2333 (celular da pesquisadora Lidiane Mendes Louzada)
(0xx11)98279-3158/2640-2752 (celular da pesquisadora Maria Eunice da Silva Davidian)
(0xx19) 98244-7581 (celular do pesquisador Rodrigo Arruda Vasconcelos)
(0xx19) 99747-3677 (celular do pesquisador Vito Madio Chiarelli Neto)

O paciente também poderá ser atendido no Plantão de Emergência da FOP-UNICAMP conforme a disponibilidade deste serviço, que funciona normalmente de segunda à sexta-feira, de 8:00 às 12:00 h e de 13:30 às 17:30 h.

Para qualquer informação ou esclarecimento sobre o tratamento, o paciente poderá entrar em contato com os pesquisadores através dos telefones citados acima, e-mail: emelly.aveiro@gmail.com, e.gabriellisantin@gmail.com, mesdavidian@gmail.com, lidiane_mendes33@yahoo.com, julianadelatorre_@hotmail.com, vasconcelosra@yahoo.com, vitochiarelli@gmail.com, e bpgomes@fop.unicamp.br, assim como no endereço Rua: Regente Feijó, n° 639, centro.

O paciente tem toda a liberdade de pedir esclarecimentos sobre como serão feitos o tratamento e as coletas, antes e durante a pesquisa, podendo ou não concordar em participar da mesma. Caso recuse, seu tratamento será prosseguido normalmente, se o mesmo demonstrar interesse. Uma via assinada/rubricada deste termo (TCLE) será entregue ao voluntário, portanto o mesmo terá em mãos telefones e endereços para contato.

Apesar dos resultados serem divulgados publicamente para fins acadêmicos e científicos, será preservada a privacidade do indivíduo quanto aos dados confidenciais que possam a ser envolvidos na pesquisa.

ATENÇÃO: A sua participação em qualquer tipo de pesquisa é voluntária. Em caso de dúvida quanto aos seus direitos como voluntário da pesquisa, escreva para o Comitê de Ética em Pesquisa da FOP – UNICAMP, endereçado a Av. Limeira, 901 – Caixa Postal 52, CEP 13414-903, fone (19) 2106-5349, e-mail cep@fop.unicamp.br, na cidade de Piracicaba/SP.

3 – Garantias:

a- O voluntário tem a garantia de que receberá respostas a qualquer pergunta ou esclarecimento a respeito dos procedimentos, riscos, benefícios e outros assuntos relacionados à pesquisa.
b- Não haverá necessidade de deslocamentos ou procedimentos adicionais para coleta de amostra, além das necessárias para o tratamento endodôntico convencional (04 sessões) ou cirurgia paredodôntica. Não há previsão de ressarcimento, pois não há risco previsível pela participação na pesquisa.
c- O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo e assim do grupo de amostra, este não será penalizado e não haverá prejuízo ao seu tratamento, o qual será prosseguido normalmente.
d- Todos os dados coletados e qualquer informação referente a este estudo permanecerá confidencial, assegurando proteção à imagem, sigilo e respeitando valores sociais, culturais, morais, éticos e religiosos. Os resultados desta pesquisa serão divulgados apenas para fins científicos, porém a identidade dos voluntários não será divulgada.
e- Este termo de consentimento livre e esclarecido foi gerado em duas vias, sendo uma direcionada ao voluntário e outra ao pesquisador. Todas as páginas serão rubricadas pelos sujeitos da pesquisa e responsável.

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4 – Consentimento informado

Eu, _____, RG _____ certifico que tendo lido as informações acima e suficientemente esclarecido (a) de todos os itens pela Profª. Drª. Brenda Paula Figueiredo de Almeida Gomes, Emelly de Aveiro, Ezequiel Gabrielli, Juliana Delatorre Bronzato, Lidiane Mendes Louzada, Maria Eunice da Silva Davidian, Rodrigo Arruda Vasconcelos e Vito Madio Chiarelli Neto, estou plenamente de acordo com a realização do experimento. Assim, eu autorizo a execução da pesquisa, exposta acima, em mim.

Piracicaba, _____/_____/_____.

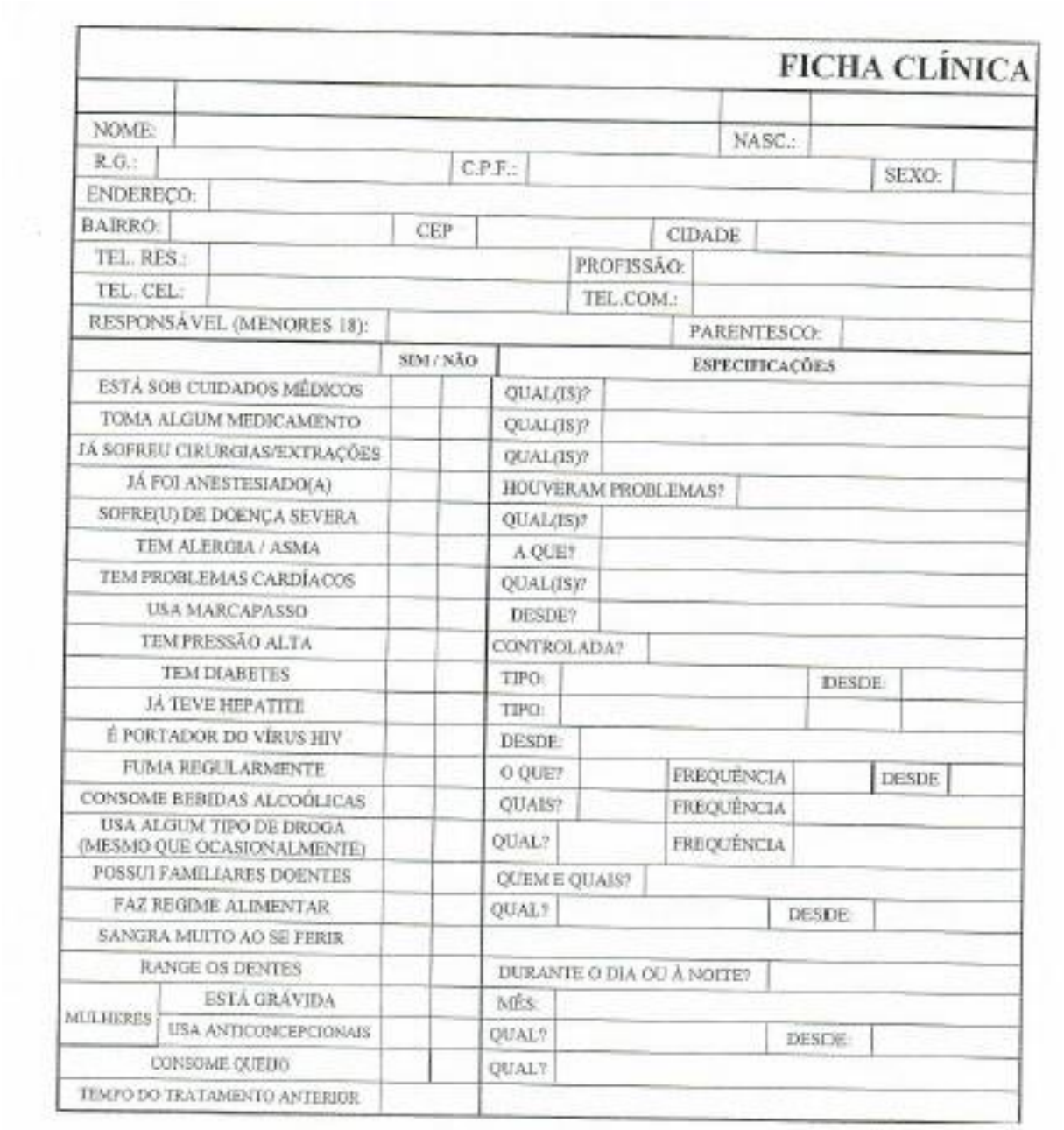
_____
Assinatura do Paciente

Nome:_____,
RG: _____

_____
Assinatura do Pesquisador

*Todas as páginas deverão ser rubricadas pelos sujeitos da pesquisa e responsável.

APÊNDICE 2. Ficha clínica para coleta de informações dos pacientes envolvidos na pesquisa.

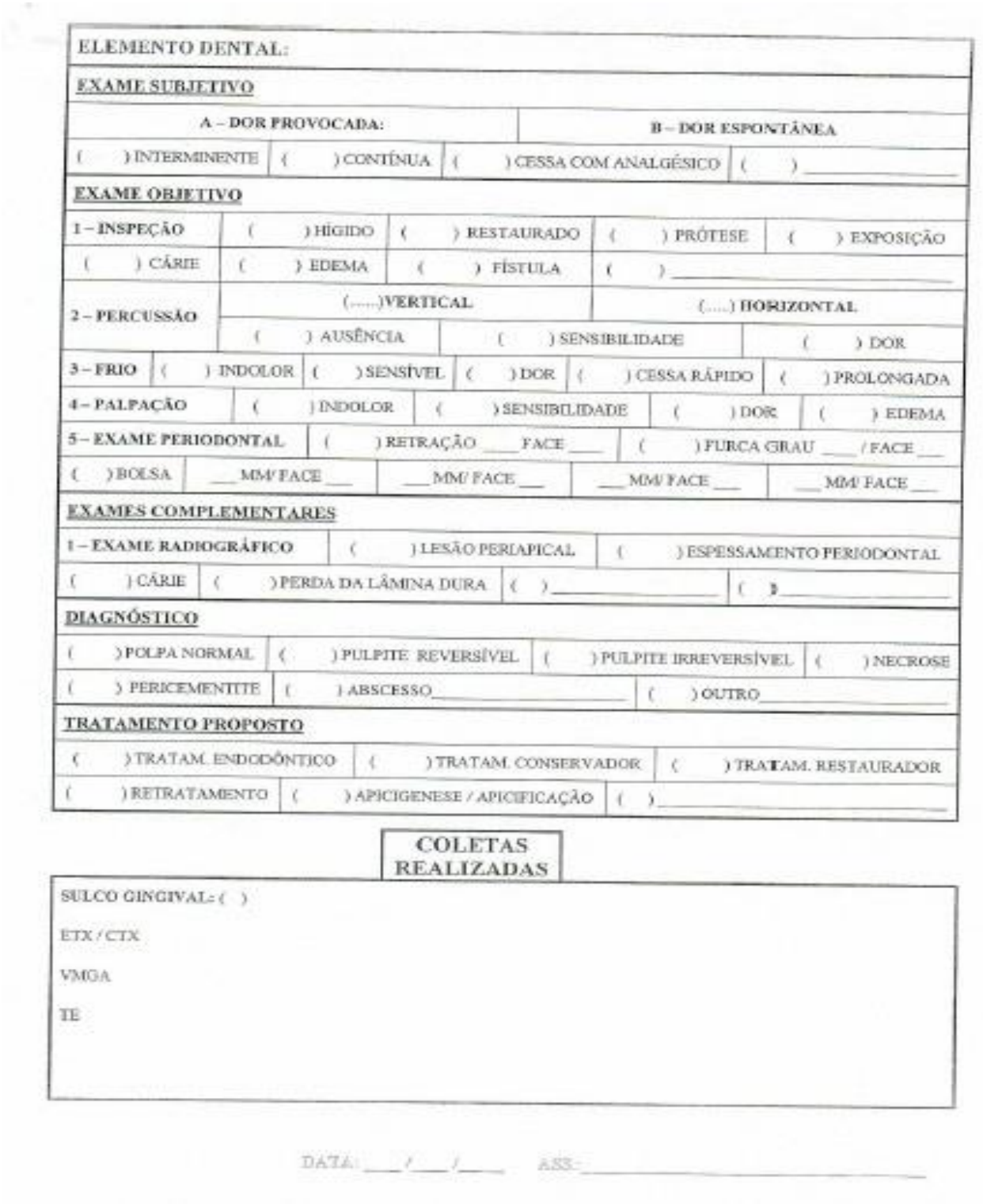


FICHA CLÍNICA

NOME:				NASC.:	
R.G.:		C.P.F.:		SEXO:	
ENDEREÇO:					
BAIRRO:		CEP		CIDADE	
TEL. RES.:			PROFISSÃO:		
TEL. CEL:			TEL.COM.:		
RESPONSÁVEL (MENORES 18):				PARENTESCO:	

	SIM / NÃO		ESPECIFICAÇÕES	
ESTÁ SOB CUIDADOS MÉDICOS			QUAL(IS)?	
TOMA ALGUM MEDICAMENTO			QUAL(IS)?	
JÁ SOFREU CIRURGIAS/EXTRAÇÕES			QUAL(IS)?	
JÁ FOI ANESTESIADO(A)			HOUVERAM PROBLEMAS?	
SOFRE(U) DE DOENÇA SEVERA			QUAL(IS)?	
TEM ALERGIA / ASMA			A QUE?	
TEM PROBLEMAS CARDÍACOS			QUAL(IS)?	
USA MARCAPASSO			DESDE?	
TEM PRESSÃO ALTA			CONTROLADA?	
TEM DIABETES			TIPO:	DESDE:
JÁ TEVE HEPATITE			TIPO:	
É PORTADOR DO VÍRUS HIV			DESDE:	
FUMA REGULARMENTE			O QUE?	FREQUÊNCIA / DESDE
CONSOME BEBIDAS ALCOÓLICAS			QUAIS?	FREQUÊNCIA
USA ALGUM TIPO DE DROGA (MESMO QUE OCASIONALMENTE)			QUAL?	FREQUÊNCIA
POSSUI FAMILIARES DOENTES			QUEM E QUAIS?	
FAZ REGIME ALIMENTAR			QUAL?	DESDE:
SANGRA MUITO AO SE FERIR				
RANGE OS DENTES			DURANTE O DIA OU À NOITE?	
MULHERES – ESTÁ GRÁVIDA			MÊS:	
MULHERES – USA ANTICONCEPCIONAIS			QUAL?	DESDE:
CONSOME QUEIJO			QUAL?	
TEMPO DO TRATAMENTO ANTERIOR				

APÊNDICE 2. Ficha clínica para coleta de informações dos pacientes envolvidos na pesquisa (continuação).



ELEMENTO DENTAL:				
EXAME SUBJETIVO				
A – DOR PROVOCADA:			**B – DOR ESPONTÂNEA**	
( ) INTERMINENTE	( ) CONTÍNUA	( ) CESSA COM ANALGÉSICO	( ) _____	
EXAME OBJETIVO				
1 – INSPEÇÃO	( ) HÍGIDO	( ) RESTAURADO	( ) PRÓTESE	( ) EXPOSIÇÃO
( ) CÁRIE	( ) EDEMA	( ) FÍSTULA	( ) _____	
2 – PERCUSSÃO	(.....) **VERTICAL**		(.....) **HORIZONTAL**	
	( ) AUSÊNCIA	( ) SENSIBILIDADE	( ) DOR	
3 – FRIO	( ) INDOLOR	( ) SENSÍVEL	( ) DOR	( ) CESSA RÁPIDO	( ) PROLONGADA
4 – PALPAÇÃO	( ) INDOLOR	( ) SENSIBILIDADE	( ) DOR	( ) EDEMA	
5 – EXAME PERIODONTAL	( ) RETRAÇÃO ____ FACE ____		( ) FURCA GRAU ____ / FACE ____	
( ) BOLSA	___ MM/ FACE ___	___ MM/ FACE ___	___ MM/ FACE ___	___ MM/ FACE ___
EXAMES COMPLEMENTARES				
1 – EXAME RADIOGRÁFICO	( ) LESÃO PERIAPICAL		( ) ESPESSAMENTO PERIODONTAL	
( ) CÁRIE	( ) PERDA DA LÂMINA DURA	( ) _____	( ) _____	
DIAGNÓSTICO				
( ) POLPA NORMAL	( ) PULPITE REVERSÍVEL	( ) PULPITE IRREVERSÍVEL	( ) NECROSE	
( ) PERICEMENTITE	( ) ABSCESSO_____		( ) OUTRO_____	
TRATAMENTO PROPOSTO				
( ) TRATAM. ENDODÔNTICO	( ) TRATAM. CONSERVADOR		( ) TRATAM. RESTAURADOR	
( ) RETRATAMENTO	( ) APICIGENESE / APICIFICAÇÃO		( ) _____	

COLETAS REALIZADAS

SULCO GINGIVAL: ( )

ETX / CTX

VMGA

TE

DATA: ___/___/____ ASS: _____

APÊNDICE 2. Ficha clínica para coleta de informações dos pacientes envolvidos na pesquisa (continuação).

FICHA DE PROCEDIMENTOS ENDODÔNTICOS

DENTE:

1-) ANÁLISE RADIOGRÁFICA

CONDUTO	COMPRIM. APARENTE	AMPLITUDE			CURVATURA			
		CONSTRITO	MÉDIO	AMPLO	VESTIB.	MESIAL	DISTAL	LINGUAL
		CONSTRITO	MÉDIO	AMPLO	VESTIB.	MESIAL	DISTAL	LINGUAL
		CONSTRITO	MÉDIO	AMPLO	VESTIB.	MESIAL	DISTAL	LINGUAL
		CONSTRITO	MÉDIO	AMPLO	VESTIB.	MESIAL	DISTAL	LINGUAL

2-) PREPARO QUÍMICO-MECÂNICO

DESCRIÇÃO DA TÉCNICA:

SUBSTÂNCIAS IRRIGADORAS:

CONDUTO	FORMATO	LIMA ANAT. INICIAL	CRF	LIMA ANAT. FINAL (FORAMINAL)

3-) OBTURAÇÃO

CONE: | CIMENTO: | RESTAURAÇÃO:

TÉCNICA DE OBTURAÇÃO:

4-) DIFICULDADES ENCONTRADAS:

5-) AUTO AVALIAÇÃO: EXCELENTE SATISFATÓRIO REGULAR IRREGULAR

6-) NOTA FINAL: | **VISTO:**

PROCEDIMENTOS EXECUTADOS

DATA	DENTES	DESCRIÇÃO	VISTO

APÊNDICE 3. Características clínicas dos pacientes incluídos no estudo.

Variável	Categoria	Total	Grupo	
			Clorexidina	Hipoclorito de sódio
Sexo	Masculino	3	2	1
	Feminino	17	8	9
Idade	≥ 40 anos	8	5	3
	≤ 40 anos	12	5	7
Dente	Unirradicular	2	2	0
	Birradicular	3	1	2
	Trirradicular	15	7	8
Localização do dente	Superior	12	6	4
	Inferior	8	4	6
Dor espontânea	Sim	20	10	10
	Não	0	0	0
Sensibilidade a percussão	Sim	20	10	10
	Não	0	0	0
Dor a palpação	Sim	0	0	0
	Não	20	10	10
Restauração fraturada / Cárie	Sim	20	10	10
	Não	0	0	0

APÊNDICE 4. Método de coleta e armazenamento das amostras.

Amostra	Instrumento	Qtd	Comprimento	Meio
LPS	Cone de papel	01	No ápice	Criotubo (estéril e apirogênicos)
LTA	Cone de papel	02	No ápice	Criotubo (estéril e apirogênicos)
CM / CB	Cone de papel	03	No ápice	Tubo tipo Eppendorf com 1,0 mL de Viability Medium Götemborg Ágar – VMGA III
Nested PCR	Cone de papel	02	No ápice	Tris-EDTA buffer, pH 8 (1,0 mL)
CI / MMP/ SP / PGE2	Cone de papel	01	Periápice (+2 mm)	Criotubo (estéreis e apirogênicos)

LPS: Endotoxina; LTA: Ácido lipoteicóico; CM: Cultura microbiana; CB: Checkerboard DNA-DNA hybridization; PCR: Reação em cadeia da polimerase; CI: Citocinas; MMP: Metaloproteinases de matriz; SP: Sunstância P; PGE2: Prostaglandina E2.

APÊNDICE 5. Momentos das coletas clínicas.

Análise	Coleta inicial	Coleta após PQM	Coleta 48h após PQM	Coleta após MIC (30 dias)
Endotoxinas				
Ácido lipoteicóico				
Cultura microbiana				
Checkerboard DNA-DNA hybridization				
Nested PCR				
TNF-α, IL-1α, IL-1β, IL-10				
MMP-2, -3, -8, -9, -13				
Substância P				
PGE2				

Coleta analisada | Coleta não analisada

APÊNDICE 6. Biografia do autor

Rodrigo Arruda Vasconcelos, nascido em 03 de abril de 1988, em Salvador, BA, Brasil.

2008 – 2013	Graduação em Odontologia Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas – UNICAMP.
2014 – 2016	Especialização em Endodontia Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista – UNESP.
2014 – 2016	Mestrado em Odontologia, área de Endodontia Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista – UNESP.
2016 – 2017	Estágio no Laboratório de Microbiologia aplicada à Endodontia Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas – UNICAMP.
2017 – 2021	Doutorado em Clínica Odontológica, área de Endodontia Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas – UNICAMP.
2019 – 2020	Estágio de Doutoramento na Birmingham Dental Hospital, School of Dentistry – University of Birmingham, Birmingham, Reino Unido.

Lista de publicações página:
http://lattes.cnpq.br/1121352334335266

Apêndice 7. Texto para divulgação da Tese de Doutorado

A linha de pesquisa Microbiologia Aplicada a Endodontia, sob coordenação da Prof.ª Dr.ª Brenda Paula Figueiredo de Almeida Gomes, teve início no ano de 1997. Entretanto, desde o período em que realizou o seu Doutoramento no Dental Hospital, da *University of Manchester*, UK, a coordenadora se dedicou a pesquisar sobre os principais micro-organismos presentes no interior dos canais radiculares e quais suas relações com presença de sinais e sintomas clínicos, tais como dor, exsudação, reabsorções ósseas, dentre outros. Com o desenvolvimento das técnicas moleculares de identificação microbiana, estas começaram a ser aplicadas nas pesquisas, juntamente com a cultura bacteriana usando técnicas anaeróbicas avançadas. Posteriormente incorporou a quantificação dos fatores de virulência tais como LPS e LTA, a quantificação de citocinas pro-inflamatórias, metaloproteinases e substância P, entre outros. Também é realizado o monitoramento clínico da efetividade de diversas etapas do tratamento endodôntico na redução dos níveis destas substâncias.

Até o momento diversas pesquisas foram realizadas, incluindo as de iniciação cientifica no ensino médio (PIBIC-EM), Iniciação cientifica na graduação, trabalhos de conclusão de curso de graduação (39), Dissertações de Mestrado (n=29), Teses de Doutorado (n=33), supervisões de Pós-Doutorado (n=10). Trabalhos são apresentados anualmente em congressos nacionais e internacionais, tendo como principal objetivo a difusão do conhecimento e divulgação dos trabalhos realizados pelo grupo. Diversos trabalhos foram premiados.

Desde a criação da linha de pesquisa Microbiologia Aplicada a Endodontia são objetos de pesquisa canais radiculares de dentes com infecções endodônticas primárias (sintomáticos ou assintomáticos), infecções secundárias/persistentes e lesões endo-periodontais. Diversos estudos clínicos foram publicados em revistas de alto impacto científico por este grupo de pesquisa.

Com o grande desenvolvimento da Endodontia Regenerativa, passamos a observar a necessidade de estudar polpas vitais para compreender os aspectos iniciais das alterações de origem endodôntica, tanto microbiológicas como imunológicas. Esta tese é o primeiro fruto desta nova linha de pesquisa e teve como

principal objetivo o monitoramento microbiológico e imunológico nas diferentes etapas do tratamento endodôntico de dentes com pulpite irreversível. Para tanto, foram utilizadas diversas metodologias consolidadas e inovadoras com comprovada eficácia. Resultados encontrados podem servir de guia para num futuro bem próximo, prever os níveis de biomarcadores presentes no canal radicular a partir das amostras coletadas na porção coronária do elemento dental.

A Tese foi dividida em segmentos, com o objetivo de responder questionamentos importante acerca de dentes com pulpite irreversível sintomática, a saber:

1) Monitoramento microbiológico em dentes com pulpite irreversível: apesar de existirem inúmeros estudos envolvendo infecções de longa duração, o mesmo não acontece em relação a pulpite, que envolve os aspectos iniciais da infecção. Utilizamos técnicas de cultura para contagem de bactérias viáveis presentes na cárie dental e no interior dos canais radiculares contar através da contagem de unidades formadoras de colônia por mililitro (CFU/mL). Também foram utilizadas técnicas moleculares como nested PCR, *Checkerboard DNA-DNA hybridization* e *Next Generation Sequencing* para estudar o perfil microbiano desta condição clínica.

Observamos que dentes com pulpite irreversível sintomática apresenta um perfil polimicrobiano, com presença de bactérias Gram-positivas, Gram-negativas, anaeróbios facultativos e estritos. Maiores números e diversidade microbiana foram observados na cárie e amostras iniciais dos canais radiculares. O tratamento endodôntico se mostrou efetivo na remoção de microrganismos dos canais radiculares. Além disso, foi possível detectar espécies relacionadas ao insucesso do tratamento endodôntico, tais como, *Enterococcus faecalis*, e relacionadas à sintomatologia, como *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp.

2) Monitoramento dos fatores de virulência (endotoxinas e ácido lipoteicóico) presentes nos quadros de pulpite irreversível.

Observamos que maiores níveis de endotoxinas e ácido lipoteicóico foram detectados nas amostras da cárie dental e amostras iniciais de canais radiculares. Nosso estudo revelou também que o preparo químico-mecânico, independente da substância química utilizada (clorexidina ou hipoclorito de sódio) foi eficaz na redução

dos níveis de endotoxinas e ácido lipoteicóico. Após uso da medicação intracanal, os níveis de ácido lipoteicóico foram significativamente inferiores àqueles detectados pós-preparo químico-mecânico.

3) Monitoramento dos níveis de citocinas (pro-inflamatórias e anti-inflamatórias) e metaloproteinases de matriz em quadros de pulpite irreversível com o objetivo de melhor compreender a complexidade inflamatória.

Observamos que o tratamento endodôntico foi eficaz em modificar os níveis de citocinas e metaloproteinases, favorecendo redução da inflamação e, consequentemente, proporcionando ambiente para o reparo.

4) Monitoramento da percepção de dor, e níveis de prostaglandina E2 e substância P, com o objetivo de observar mediadores químicos relacionados à sintomatologia do paciente.

Observamos que maiores níveis de PGE2 e substância P foram observados nas amostras iniciais dos tecidos periapicais. Após preparo químico-mecânico realizado com clorexidina ou hipoclorito de sódio foi observado redução dos níveis de ambos os marcadores, porém, não significativo. Apenas após uso de medicação intracanal foi observado redução significativa dos níveis de PGE2 e substância P.

5) Comparação clínica da atividade antimicrobiana das duas principais substâncias químicas auxiliares (NaOC 6% e clorexidina gel 2%) utilizadas no preparo químico-mecânico dos canais radiculares.

De maneira geral, tanto a clorexidina 2% gel quanto o hipoclorito de sódio 6% foram eficazes no controle microbiológico e de fatores de virulência, bem como modificação dos níveis de citocinas, metaloproteinases, PGE2 e substância P. Além disso, não houve diferenças em relação à percepção de dor relatada pelos pacientes. Estes achados sugerem um ambiente favorável ao hospedeiro independentemente da substância química auxiliar utilizada durante o tratamento endodôntico.

6) Estudo *ex* vivo, foi realizado com o objetivo de avaliar diferentes substâncias químicas na extrusão de debris extruídos apicalmente. Tais debris potencialmente contêm restos de bactérias que podem causar injúrias aos tecidos periapicais.

A partir dos resultados obtidos, identificamos que a clorexidina em gel é capaz de minimizar a extrusão de debris apicalmente, em razão de suas propriedades, como ação reológica, capaz de manter os debris em suspensão para que possam ser eliminados através da irrigação com solução fisiológica.

7) Estudo *ex vivo*, realizado durante o estágio no exterior com bolsa BEPE FAPESP (2019/10755-5) na *University of Birmingham*, Reino Unido, sob a supervisão de pesquisadores de renome internacional, tais como Dr. Phillip Tomson (*University of Birmingham*), Dr. Josette Camilleri (*University of Birmingham)*, Dr. Henry Duncan (*Trinity College Dublin*) e Prof. Paul Cooper (*University of Otago*).

Os resultados mostraram que protocolos realizados em duas sessões, com clorexidina 2% gel como substância primária, seguidos de medicação intracanal à base de hidróxido de cálcio, e EDTA 17% ao final do procedimento, foi altamente eficaz na liberação de fatores de crescimento (TGF-β1) da matriz dentinária, além de promover excelente controle microbiológico no sistema de canais radiculares. Com isso, tornando o ambiente propício para recrutamento de células tronco e, consequentemente favorecer os procedimentos regenerativos. Por outro lado, a utilização do hipoclorito de sódio como solução irrigadora inibiu a liberação de fatores de crescimento, apesar de apresentar efeito antimicrobiano satisfatório.

Ao longo dos 4 anos de Doutorado, as pesquisas realizadas tiveram o apoio da CAPES (*finance code* 001) e da FAPESP (2017/25242-8; BEPE 2019/10755-5). Também tiveram apoio dos auxílios a pesquisa da orientadora, através do Projeto Temático da FAPESP (2015/23479-5), do CNPq (308162/2014-5, 303852/2019-4) e da FAEPEX-UNICAMP (2036/17).

Pensando nas próximas pesquisas, durante o doutorado foram realizadas coletas clínicas adicionais para permitir a extração do máximo de informações clínicas possíveis dos pacientes participantes. Para tanto, além das coletas dos canais iniciais e tecidos periapicais, foram realizadas também coletas da cárie dental e fluido dentinário destes pacientes que, em futuro próximo, iremos correlacionar os níveis bacterianos, de fatores de virulência, citocinas e metaloproteinases de matriz da cárie / fluido dentinário com os canais radiculares / tecidos periapicais.

Outro ponto de grande relevância foi o estágio no exterior (bolsa BEPE FAPESP, 2019/10755-5) na *University of Birmingham*, Reino Unido, sob a supervisão de grandes nomes, tais como Dr. Phillip Tomson (*University of Birmingham*), Dr. Josette Camilleri (*University of Birmingham)*, Dr. Henry Duncan (*Trinity College Dublin*) e Prof. Paul Cooper (*University of Otago*). A experiência foi muito importante na construção da rede de pesquisas entre a UNICAMP e a UoB, assim como a aproximação com os pesquisadores. Além disso, tal experiência proporcionou conhecer nova cultura, novos valores, contato com o ambiente universitário, e amizades com pessoas de diversos países do mundo.

Além dos dados obtidos nesta Tese de Doutorado, ainda existem análises em andamento que serão apresentadas no futuro, entre elas, a caracterização microbiológica através do *Next Generation Sequencing*. Esperamos com isso, no futuro, prever os níveis de biomarcadores presentes no canal radicular a partir das amostras coletadas na porção coronária do elemento dental.

Até o momento, esta Tese teve publicações e outros artigos estão submetidos, ou em fase final para submissão. São eles:

Publicados:

1. Arruda-Vasconcelos R, Barbosa-Ribeiro M, Louzada LM, Mantovani GD, Gomes BP. Apically Extruded Debris Using Passive Ultrasonic Irrigation Associated with Different Root Canal Irrigants. Braz Dent J. 2019 Jul 22;30(4):363-367.

2. Arruda-Vasconcelos R, Louzada LM, Feres M, Tomson PL, Cooper PR, Gomes BPFA. Investigation of microbial profile, levels of endotoxin and lipoteichoic acid in teeth with symptomatic irreversible pulpitis: a clinical study. Int Endod J. 2021 Jan;54(1):46-60.

Enviados:

1. Rodrigo Arruda-Vasconcelos, Marlos Barbosa-Ribeiro, Lidiane M. Louzada, Beatriz I. N. Lemos, Adriana de-Jesus-Soares, Caio C. R. Ferraz, José F. A. Almeida, Marina A. Marciano, Brenda P. F. A. Gomes. Efficacy of 6% Sodium Hypochlorite on Infectious Content of Teeth with Irreversibly Inflamed Pulp. Journal of Endodontics. (2021).

2. Rodrigo Arruda-Vasconcelos, Lidiane M. Louzada, Marlos Barbosa-Ribeiro, Adriana de-Jesus-Soares, Caio C. R. Ferraz, José F. A. Almeida, Marina A. Marciano, Brenda P. F. A. Gomes. Effectiveness of Two Root Canal Irrigants on Pain Perception, Microbial Aspects and Levels of Prostaglandin E2 and Substance P in Teeth with Symptomatic Irreversible Pulpitis: A Randomised Clinical Trial. (International Endodontic Journal).

3. Rodrigo Arruda-Vasconcelos, Lidiane M. Louzada, Henry F. Duncan, Josette Camilleri, Phillip L. Tomson, Paul R. Cooper, Brenda P. F. A. Gomes. Comparison of 2% chlorhexidine gel and 6% sodium hypochlorite on inflammatory biomarker levels in teeth with symptomatic irreversible pulpitis: A randomised Clinical Trial. (International Endodontic Journal).

4. Rodrigo Arruda-Vasconcelos, Lidiane M Louzada1, Giovanna D Mantovani, Satnam S Virdee, David JB Green, Henry F Duncan, Josette Camilleri, Paul R Cooper, Brenda PFA Gomes, Phillip L Tomson. Liberation of TGF-β1 and antimicrobial activity of different protocols for potential use in revitalisation. (International Endodontic Journal). 2021.

Além disso, os trabalhos resultantes desta tese foram apresentados em congressos, ganhando diversos prêmios.

1. Vencedor (1º Lugar) na modalidade Pesquisa / Revisão Sistemática, Pós-Graduação / Profissional – Categoria Oral (online) – Monitoramento microbiológico e imunológico em dentes com pulpite irreversível sintomática, 34º Congresso Odontológico de Bauru (COB). Bauru/SP. 2021.

2. Menção Honrosa em 2º Lugar na modalidade Profissional / Apresentação Oral Online - Investigação clínica dos aspectos microbiológico e imunológico em dentes com pulpite irreversível sintomática, XIX Jornada Acadêmica e V Congresso Nacional de Odontologia da UNIRP. São José do Rio Preto/SP. 2020.

3. Menção Honrosa na modalidade Painel Prêmio Myaki Issao - PI0499 - Investigação de bactérias e níveis de endotoxinas em dentes com pulpite irreversível nas diferentes etapas do tratamento endodôntico, 37ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica – Divisão Brasileira da IADR. 2020.

4. Menção Honrosa na modalidade Painel Científico - Efeito do tratamento endodôntico no perfil microbiológico e imunológico em dentes com pulpite irreversível: estudo clínico, III Jornada Acadêmica Prof. Adair Luiz Stefanello Busato, curso de Odontologia ULBRA Canoas/RS. 2020.

5. 1º Lugar na modalidade Apresentação Oral – Pesquisa Científica – Investigação clínica de bactérias e quantificação de lipopolissacarídeos em dentes com pulpite irreversível, IV Jornada Odontológica do Centro Universitário Euro-Americano Edição Digital. Brasília/DF. 2020.

6. 1º Lugar na modalidade Painel Clínico – Pós-Graduação - Investigação do conteúdo infeccioso e inflamatório em dentes com pulpite irreversível, Congresso Universitário Brasileiro de Odontologia FOUSP. São Paulo/SP. 2020.

7. 2º Lugar na modalidade Painel Pesquisa Científica – Caracterização microbiológica e imunológica em dentes com pulpite irreversível, III Jornada Odontológica do Centro Universitário UniRuy. Salvador/BA. 2020.

8. 3º Lugar na modalidade Painel Pesquisa Científica - Monitoramento do conteúdo infeccioso em dentes com pulpite irreversível, III Jornada Odontológica do Centro Universitário UniRuy. Salvador/BA.

ANEXO 1 – Certificado do Comitê de Ética em Pesquisa em Seres Humanos da FOP-UNICAMP (artigos 1, 2, 3 e 4).

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "Monitoramento clínico, microbiológico, imunológico e biomolecular das diferentes condições pulpares e perirradiculares", CAAE 86140218.0.0000.5418-Versão: 4, dos pesquisadores Brenda Paula Figueiredo de Almeida Gomes, Emelly de Aveiro, Juliana Delatorre Bronzato, Ezequiel Gabrielli, Lidiane Mendes Louzada, Maria Eunice da Silva Davidian, Vito Madio Chiarelli Neto e Rodrigo Arruda Vasconcelos satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 10/08/2018.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "Clinical, microbiological, immunological and biomolecular monitoring of different pulp and periradicular conditions", CAAE 86140218.0.0000.5418-Version: 4, of the researcher's Brenda Paula Figueiredo de Almeida Gomes, Emelly de Aveiro, Juliana Delatorre Bronzato, Ezequiel Gabrielli, Lidiane Mendes Louzada, Maria Eunice da Silva Davidian, Vito Madio Chiarelli Neto and Rodrigo Arruda Vasconcelos, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on August, 10 2018.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

ANEXO 2 – Certificado do Comitê de Ética em Pesquisa em Seres Humanos da FOP-UNICAMP (artigo 5).

FACULDADE DE ODONTOLOGIA DE PIRACICABA - UNICAMP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito do preparo químico-mecânico com diferentes protocolos de irrigação e contato direto de substâncias químicas na redução microbiana e da extrusão apical de debris : estudo "ex vivo"

Pesquisador: Brenda Paula Figueiredo de Almeida Gomes

Área Temática:

Versão: 3

CAAE: 48274215.6.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.334.535

Apresentação do Projeto:

Trata-se de estudo in vitro que irá avaliar a eficácia de diferentes técnicas de irrigação endodôntica e substâncias químicas em reduzir Enterococcus faecalis (principal bactéria envolvida no insucesso endodôntico) e debris extruídos apicalmente de dentes submetidos ao tratamento endodôntico; e verificar a efetividade de diferentes substâncias químicas utilizadas na endodontia na viabilidade desta bactéria. O estudo será dividido em 2 partes (I e II) totalizando 63 dentes pré-molares inferiores humanos indicados para extração por motivo ortodôntico com as coroas seccionadas; ápices completamente formados; e ausência de curvaturas acentuadas na porção radicular e fratura. PARTE I - trata-se da avaliação quanto à técnica de irrigação e substância química auxiliar (n = 45). Os espécimes terão o comprimento dos dentes padronizados em 16 mm. Impurezas e remanescentes de ligamento periodontal serão removidos da porção radicular com fresas diamantadas. Para o preparo dos espécimes, será obtido da patência com limas K #25 e remoção passiva de remanescentes pulpares no interior do canal radicular, os quais serão submetidos a um banho em cuba ultrassônica contendo EDTA 17% por 10 minutos, seguidos de um banho de hipoclorito de sódio a 5,25% por 10 minutos e um banho de fosfato tamponado para eliminar os resíduos dos banhos precedentes. Após este procedimento, os espécimes serão banhados por 10 minutos em água destilada para a eliminação de smear Layer produzida durante

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ANEXO 2 – Certificado do Comitê de Ética em Pesquisa em Seres Humanos da FOP-UNICAMP (artigo 5) (continuação).

FACULDADE DE ODONTOLOGIA DE PIRACICABA - UNICAMP

Continuação do Parecer: 1.334.535

Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 24 de Novembro de 2015

Assinado por:
Felippe Bevilacqua Prado
(Coordenador)

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ANEXO 3 – Certificado do Comitê de Ética em Pesquisa em Seres Humanos da FOP-UNICAMP (artigo 6).

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da atividade antimicrobiana de soluções irrigadoras com potencial utilização na terapia endodôntica

Pesquisador: Brenda Paula Figueiredo de Almeida Gomes

Área Temática:

Versão: 3

CAAE: 34237020.7.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO
CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTIFICO E TECNOLOGICO-CNPQ

DADOS DO PARECER

Número do Parecer: 4.204.789

Apresentação do Projeto:

Transcrição editada do conteúdo do registro do protocolo e dos arquivos anexados à Plataforma Brasil

A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui BRENDA PAULA FIGUEIREDO DE ALMEIDA GOMES (Cirurgiã-Dentista, Especialista, Docente Titular do Departamento de Odontologia Restauradora, Pesquisadora responsável, Orientadora), GIOVANNA DORNELAS MANTOVANI (Graduanda no curso de Odontologia da FOP/Unicamp), LIDIANE MENDES LOUZADA (Cirurgiã-Dentista, Doutoranda no PPG em Clínica Odontológica, Área de Endodontiada, da FOP-UNICAMP) e RODRIGO ARRUDA VASCONCELOS (Cirurgião-Dentista,, Doutorando no PPG em Clínica Odontológica, Área de Endodontia da FOP-UNICAMP), o que é confirmado na declaração dos pesquisadores e na PB.

Delineamento da pesquisa: Trata-se de estudo laboratorial, in vitro, comparativo, que envolverá 45 dentes incisivos inferiores humanos obtidos em doação de um dentista que os extraiu em seu consultório por motivos clínicos e independentes da pesquisa e um doador de saliva adulto, pesquisador neste protocolo. O estudo tem como objetivo avaliar a efetividade de diferentes substâncias químicas auxiliares tais como: ácido etidrônico (HEDP), hipoclorito de sódio (NaOCl),

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ANEXO 3 – Certificado do Comitê de Ética em Pesquisa em Seres Humanos da FOP-UNICAMP (artigo 6) (continuação).

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

Continuação do Parecer: 4.204.789

Infraestrutura	Autoriz_uso_de_equipamentos_e_infraestrutura.pdf	25/06/2020 18:06:24	Almeida Gomes	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaracao_Biorrepositorio.pdf	25/06/2020 17:57:10	Brenda Paula Figueiredo de Almeida Gomes	Aceito
Declaração de Pesquisadores	Declaracao_Pesquisadores.pdf	25/06/2020 17:46:37	Brenda Paula Figueiredo de Almeida Gomes	Aceito
Folha de Rosto	1Folhaderosto.pdf	25/06/2020 17:38:12	Brenda Paula Figueiredo de Almeida Gomes	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 11 de Agosto de 2020

Assinado por:
jacks jorge junior
(Coordenador(a))

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ANEXO 4 – Comprovante de submissão do artigo “Efficacy of 6% sodium hypochlorite on infectious content of teeth with irreversibly inflamed pulp” ao periódico Journal of Endodontics.

Brenda P F A Gomes <bpgomes@unicamp.br>
Para: rodrigo vasc

ter., 18 de mai. às 15:42

---------- Forwarded message ---------
De: **The Journal of Endodontics** <em@editorialmanager.com>
Date: ter., 18 de mai. de 2021 às 15:41
Subject: Submission Confirmation for Efficacy of 6% Sodium Hypochlorite on Infectious Content of Teeth with Irreversibly Inflamed Pulp
To: BRENDA P F A GOMES <bpgomes@fop.unicamp.br>

Dear Dr. GOMES,

Your submission entitled "Efficacy of 6% Sodium Hypochlorite on Infectious Content of Teeth with Irreversibly Inflamed Pulp" has been received by the Journal of Endodontics.

You will be able to check on the progress of your paper by logging on to the Journal of Endodontics web site as an author.

The URL is https://www.editorialmanager.com/joe/

Your username is: bpgomes@fop.unicamp.br

Can't remember your password?
To reset your password please try to sign in and click 'continue'. On the next screen click the 'forgot password' link and follow the steps to reset your password.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to the Journal of Endodontics.

Kind regards,

Journal of Endodontics

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: https://www.editorialmanager.com/joe/login.asp?a=r). Please contact the publication office if you have any questions.

ANEXO 5 – Comprovante de submissão do artigo “Effectiveness of two root canal irrigants on pain perception, microbial aspects, and levels of prostaglandin E2 and substance P in teeth with symptomatic irreversible pulpitis: A randomised clinical trial” ao periódico International Endodontic Journal.

International Endodontic Journal

Home

Author

Review

Submission Confirmation

Print

Thank you for your submission

Submitted to
International Endodontic Journal

Manuscript ID
IEJ-21-00355

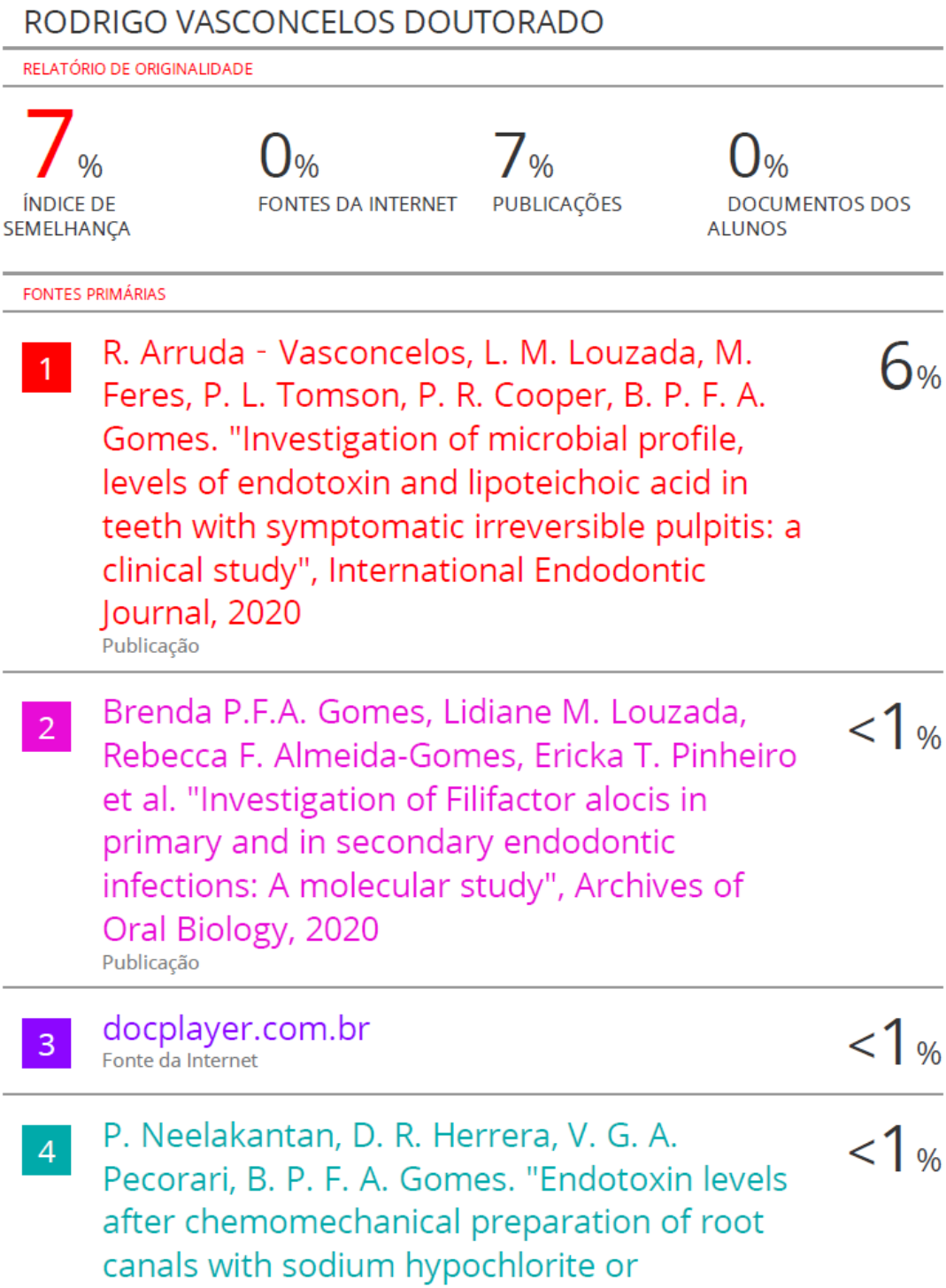
Title
Effectiveness of Two Root Canal Irrigants on Pain Perception, Microbial Aspects and Levels of Prostaglandin E2 and Substance P in Teeth with Symptomatic Irreversible Pulpitis: A Randomised Clinical Trial

Authors
Arruda-Vasconcelos, Rodrigo
Louzada, Lidiane
Barbosa-Ribeiro, Marlos
De-Jesus-Soares, Adriana
Ferraz, Caio
Almeida, José
Marciano, Marina
Gomes, Brenda

Date Submitted
23-May-2021

Author Dashboard

Anexo 6 – Comprovante TURNITIN.



RODRIGO VASCONCELOS DOUTORADO

RELATÓRIO DE ORIGINALIDADE

7%	0%	7%	0%
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FONTES PRIMÁRIAS

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2	Brenda P.F.A. Gomes, Lidiane M. Louzada, Rebecca F. Almeida-Gomes, Ericka T. Pinheiro et al. "Investigation of Filifactor alocis in primary and in secondary endodontic infections: A molecular study", Archives of Oral Biology, 2020 Publicação	<1%
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