UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

FRANCINE CESARIO

Evaluation of physico-chemical and biological properties of calcium hydroxide associations

Avaliação de propriedades físico-químicas e biológicas de associações ao hidróxido de cálcio

BAURU
2019

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Tese constituída por artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutora em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Endodontia.

Orientador: Prof. Dr. Rodrigo Ricci Vivan

Versão Corrigida

BAURU

2019

Cesario, Francine
Evaluation of physico-chemical and biological properties of calcium hydroxide associations / Francine Cesario – Bauru, 2019.
73p. : il. ; 31cm.

Tese (Doutorado/Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. Dr. Rodrigo Ricci Vivan

Nota: A versão original desta tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru - FOB/USP.

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico esse trabalho a todos aqueles que, de alguma forma, contribuíram para sua idealização, realização e finalização, seja física ou moralmente. Sem vocês não seria possível.

“Nenhum homem é uma ilha…” - John Donne

AGRADECIMENTOS

À **Deus**, pela minha família e amigos, por guiar meus passos, mostrando a luz nos momentos de dificuldade e pela força para superar os obstáculos.

"Entregue seu caminho ao senhor, nele confie e ele agirá. Salmo 37 (36),5".

À minha Mãe, **Izilda**, a pessoa que mais admiro no mundo. Exemplo de dedicação, coragem, força e amor. Agradeço por todas as vezes em que sem hesitar sacrificou os seus sonhos para que eu possa realizar os meus, por estar ao meu lado e sempre me apoiar em toda e qualquer situação. Você é meu exemplo de vida, de mulher, mãe, esposa! EU TE AMO. **"Enquanto houver você do outro lado, desse aqui eu consigo me orientar"**

Ao meu Pai **Edvaldo** (*in memorian*), que construiu base principal da nossa família, saudades eternas! Te amo.

Ao meu irmão, **Fábio** obrigada por fazerem parte da minha vida.

Ao meu orientador **Prof. Dr. Rodrigo Ricci Vivan**, meu maior exemplo durante a graduação, por seu profissionalismo, empenho e dedicação à profissão e aos alunos, motivo por eu ter escolhido a endodontia como especialidade e o que acabou me levando a fazer a pós-graduação. Agradeço por ter me ensinado os primeiros passos na endodontia e na ciência através da iniciação científica, pela continuação desses ensinamentos até aqui e pelo incentivo a sempre buscar a ser mais e melhor em tudo o que faço. Minha gratidão e reconhecimento. Obrigada por tudo.

Ao **Prof. Dr. Marco Antonio Hungaro Duarte**, por todos os ensinamentos durante a pesquisa, por sempre estar à disposição para tirar dúvidas, pela realização da análise estatística.

Aos Professores do departamento de endodontia da FOB/USP, **Marco Antonio Hungaro Duarte**, **Flaviana Bombarda de Andrade**, **Ivaldo Gomes de Moraes (*In memorian*)**, **Clóvis Monteiro Bramante**, **Roberto Brandão Garcia** e **Norberti Bernardineli** por todo carinho, atenção, paciência e por todos os valiosos ensinamentos transmitidos durante a nossa convivência. Tenho uma imensa admiração, carinho e respeito.

Aos funcionários da Endodontia, **Suely Regina Bettio e Edimauro de Andrade**, por estar sempre dispostos a me ajudar, pela boa convivência por todos esses anos, muito obrigado pelo carinho.

Ao **Prof. Dr. Paulo Henrique Weckwerth**, obrigado por dispor as cepas bacterianas e pela ajuda com as minhas dúvidas na parte microbiológica do trabalho.

Ao **Prof. Dr. Rodrigo Cardoso de Oliveira**, pela disposição e ajuda durante a realização desta pesquisa no CIP.

À colega do CIP, **Cintia Tokuhara**, pelo auxílio para realização de parte dessa pesquisa, em ajudar a manusear os equipamentos e pela boa convivência.

À especialista em laboratório do centro integrado em pesquisa da FOB-USP, **Márcia Sirlene Zardin Graeff**, pela ajuda no Microscópio Confocal de Varredura a Laser. Obrigada pela paciência, em sempre estar disposta em me atender. Você foi fundamental para a realização dente trabalho.

À técnica de laboratório **Larissa Tercilia Grizzo** do departamento de bioquímica da FOB/USP, que realizou a leitura na liberação do cálcio. Você também fundamental para realização desta pesquisa.

Ao funcionário do CIP, **Marcelo Miranda Ribeiro Lopes** pela disponibilidade e inestimável ajuda a todo momento.

À amiga **Rafaela Fernandes Zancan**, agradeço não só por toda a ajuda durante a realização desse trabalho, que foi fundamental, mas por sua amizade sincera dentro e fora da faculdade, por todas as conversas e trocas de experiencias. Espero sempre ter sua amizade!

À amiga **Talita Tartari,** por sempre me ouvir, por todos os conselhos, por todas as dúvidas que me ajudou a sanar! Te admiro muito, espero levar sua amizade para sempre!

Aos amigos que pós-graduação me deu **Mariana Borges**, **Denise Oda**, **Lyz Cristina Furquim Canali**, **Clarissa Rodrigues Teles, Bruno Martini Guimarães, Ericson Janólio de Camargo, Victor de Moraes Cruz, Raquel Midena,** agradeço todas as trocas de conhecimentos e boa convivência.

Aos meus colegas **Murilo Alcalde** e **Renan Furlan** que sempre estiveram dispostos a me ajudar e me ajudaram diversas vezes, dentro e fora da instituição.

Aos meus colegas **da pós-graduação** do departamento de Endodontia, agradeço por todos os bons momentos compartilhados, convivência agradável e conhecimentos que dividimos, desejo um caminho de saúde, felicidade e sucesso à todos.

Aos meus amigos **Aline Alves** e **Diego Magalhães**, meus irmãos por escolha, só tenho a agradecer por tudo que fizeram e fazem por mim e pela amizade sincera, amo vocês!

“Quem diz que não acredita em alma gêmea ainda não encontrou o amigo certo. Não há nenhuma prova de que duas pessoas são completamente iguais ou complementares além de uma amizade verdadeira. Mas não me refiro a qualquer tipo de amigos. Estou falando daqueles que leem pensamentos, que adivinham antes da gente falar, que topam qualquer aventura, que choram as nossas dores e comemoram, mais que nós mesmos, as nossas vitórias. Se tudo isso não servir de comprovação para a existência de “almas gêmeas”, me perdoa – eu também não acredito nelas. “

À minha amiga **Flavia Faria**, companheira de graduação para à vida, agradeço por tantos momentos que dividimos, de alegria e tristeza. Obrigada por ser minha parceira e por sempre estar ao meu lado em todas as situações.

As minhas amigas **Mariana Ferro**, **Fernanda Carvalho**, **Ana Luiza Miranda**, **Luana Campos**, **Amanda Camilo**, **Bruna Rapini**, **Julia Marques, Letícia Campos** e ao meu amigo **Iago Ladeira**, agradeço a amizade e companheirismo de sempre!! Amo vocês! “Um dos maiores prazeres concedidos aos homens sobre a terra é de reencontrar corações que simpatizam com o seu”.

Ao meu namorado **Rafael**, obrigada por tudo, desde o primeiro dia que eu te “reencontrei”. Tudo tem o tempo certo pra acontecer e nada acontece fora do tempo determinado por Deus.

Assim também agradeço a todos que direta ou indiretamente ajudaram para realização desse trabalho, muito obrigado! Que Deus os abençoe.

AGRADECIMENTOS INSTITUCIONAIS

À Faculdade de Odontologia de Bauru – USP na pessoa do diretor **Prof. Dr. Carlos Ferreira dos Santos** e do vice-diretor **Prof. Dr. Guilherme dos Reis Pereira Janson.**

À Comissão de Pós-Graduação da Faculdade de Odontologia de Bauru, na pessoa da Presidente **Prof. Dra. Izabel Regina Fischer Rubira de Bullen**.

Ao curso de Pós-Graduação em Ciências Odontológicas Aplicadas, na área de Concentração em Endodontia da Faculdade de Odontologia de Bauru – USP na pessoa de **Prof.Dr. Marco Antonio Hungaro Duarte**.

Ao Centro Integrado de Pesquisa (CIP), na pessoa de **Prof. Dr. Rodrigo Cardoso de Oliveira,** Coordenador por permitir o uso das dependências do laboratório para o desenvolvimento do projeto de pesquisa.

À Disciplina de Endodontia, do Departamento de Dentística, Endodontia e Materiais odontológicos, na pessoa do **Prof. Dr. Marco Antonio Hungaro Duarte** por permitir o uso das dependências e laboratório para o desenvolvimento do projeto de pesquisa.

À **Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES)**, pela concessão da bolsa de doutorado.

“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana”

Carl Jung.

RESUMO

"Avaliação de propriedades físico-químicas e biológicas de associações ao hidróxido de cálcio"

O objetivo desse estudo foi avaliar diversas propriedades de pastas que associaram AINEs e antibióticos ao hidróxido de cálcio (CH). Foram analisados cinco grupos, sendo: G1: CH + propilenoglicol; G2: CH + 5% de diclofenaco de sódio + propilenoglicol; G3: CH + 5% de amoxicilina + propilenoglicol; G4: CH + 5% de ibuprofeno + propilenoglicol; e G5: CH + 5% de metronidazol + propilenoglicol. Canais radiculares de dentes de acrílico (n = 10/grupo) foram preenchidos com as pastas, e escaneados por microtomografia computadorizada antes (inicial) e após 7, 15 e 30 dias de imersão em água ultrapura para avaliar a solubilidade das pastas. O pH e a liberação de íons cálcio nessa água foram determinados por meio de um pHmetro e um espectrofotômetro de absorção atômica, respectivamente. Foi induzido um biofilme de *E. Faecalis* e um biofilme misto de *E. faecalis* e *Pseudomonas aueruginosa* em blocos de dentina (n = 4/ biofilme). Os blocos foram então distribuídos e imersos nas pastas experimentais por 7 dias para determinar a ação antimicrobiana. Para avaliar a adesão microbiana, blocos de dentina bovina foram acondicionados em placas de petri e em seguida foram cobertos com as pastas e levados para estufa a 37ºC, por 7 dias. Após, foi realizada a contaminação dos espécimes pelo período de uma hora utilizando-se a bactéria *E. faecalis.* Por meio do corante live/dead e de um microscópio confocal de varredura a laser imagens foram capturadas e a porcentagem de células bacterianas viáveis e não viáveis determinada. As pastas foram também inseridas em placas contendo macrófagos aderidos por 24 horas. A análise de produção de óxido nítrico (NO) por essas células foi realizada pela reação de Greiss. Os dados foram comparados estatisticamente ($\alpha < 0.05$). Os resultados mostraram que a maior liberação de íons hidroxila foi observado no período de 30 dias para o grupo G1 ($P < 0.05$). A liberação de íons cálcio foi maior no grupo G5 no período de 7 dias ($P < 0,05$). Todos os grupos tiveram perda de massa semelhantes e ação antimicrobiana contra biofilme misto ($P > 0,05$). No biofilme de *E. faecalis* a maior ação antimicrobiana foi observada no grupo G5 ($P < 0,05$) seguida do G4 ($P < 0,05$), assim como para adesão de M.O e liberação de NO. Todos os grupos foram diferentes estatisticamente do controle

positivo em todos os testes (P <0.05). As associações de AINEs com o hidróxido de cálcio não interferiram no pH, liberação de íons cálcio e solubilidade. As associações de AINEs e antibióticos contribuíram para ação antimicrobiana da pasta de hidróxido de cálcio. O uso do metronidazol aumentou a adesão de células bacterianas de *E. faecalis* à dentina e a produção de óxido nítrico por macrófagos.

Palavras-chave: hidróxido de cálcio, diclofenaco de sódio, ibuprofeno, biofilme.

ABSTRACT

"Evaluation of physical-chemical and biological properties of calcium hydroxide associations"

This study aimed to evaluate various properties of pastes that associated NSAIDs and antibiotics with calcium hydroxide (CH). Five groups were analyzed: G1: CH + propylene glycol; G 2: CH + 5% sodium diclofenac + propylene glycol; G3: CH + 5% amoxicillin + propylene glycol; G4: CH + 5% ibuprofen + propylene glycol; and G5: CH + 5% metronidazole + propylene glycol. Root canals of acrylic teeth (n = 10 / group) were filled with the pastes and scanned by computerized microtomography before (initial) and after 7, 15 and 30 days of immersion in ultrapure water to evaluate the solubility of the pastes. The pH and calcium ion release in this water were determined through a pH meter and an atomic absorption spectrophotometer, respectively. An *E. Faecalis* biofilm and a mixed *E. faecalis* and *Pseudomonas aueruginosa* biofilm were induced in dentin blocks (n = 4 / biofilm). The blocks were then distributed and immersed in the experimental pastes for 7 days to determine antimicrobial action. To evaluate microbial adhesion, bovine dentin blocks were placed in petri dishes and then covered with pastes and placed in a greenhouse at 37ºC for 7 days. Afterward, the specimens were contaminated for one hour using the bacterium *E. faecalis*. Through the live/dead dye and a confocal laser scanning, microscope images were captured and the percentage of viable and non-viable bacterial cells determined. The pastes were also inserted into plates containing macrophages adhered for 24 hours. The analysis of nitric oxide (NO) production by these cells was performed by Greiss reaction. Data were statistically compared (α <0.05). The results showed that the highest release of hydroxyl ions was observed within 30 days for group G1 (P <0.05). The release of calcium ions was higher in group G5 within 7 days (P <0.05). All groups had a similar mass loss and antimicrobial action against mixed biofilm (P> 0.05). In *E. faecalis* biofilm the highest antimicrobial action was observed in group G5 (P <0.05) followed by G4 (P <0.05), as well as for adhesion of M.O and release of NO. All groups were statistically different from the positive control in all tests (P <0.05). Associations of NSAIDs with calcium hydroxide did not interfere with pH, calcium ion release and solubility. Combinations of NSAIDs and antibiotics contributed to the antimicrobial action of

calcium hydroxide paste. The use of metronidazole increased the adhesion of *E. faecalis* bacterial cells to dentin and the production of nitric oxide by macrophages.

Keywords: calcium hydroxide, sodium diclofenac, ibuprofen, biofilm.

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

LISTA DE SÍMBOLOS

ATCC	*American type culture collection*
BHI	*Brain Heart Infusion*
CH	*Calcium hidroxyde*
CO_2	*Carbon dioxide*
EDTA	*Ethylenediaminetetraacetic acid*
E. faecalis	*Enterococcus faecalis*
LPS	Lipopolissararídeo
LTA	*Lipoteichoic acid*
MDM	*Monocyte-derived macrophages*
mg	*Milligram*
mL	*Milliliter*
mm	*Millimeter*
nm	*Nanometer*
NO	*Nitric Oxide*
P	*Probability*
PBS	*Phosphate buffered saline*
P.aeruginosa	*Pseudomonas aeruginosa*
ºC	*Degree Celsius*
%	*Percent*
<	*Less-than*
≥	*Greater than or equal to*

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1 INTRODUCTION

1 INTRODUCTION

Calcium hydroxide (CH) is recommended as an intracanal medication for the treatment of apical periodontitis (SIQUEIRA & LOPES 1999). Its mechanism of antimicrobial action is related to the dissociation of calcium and hydroxyl ions, transforming the environment into an alkaline pH inhibiting the enzymatic activities that are essential for microbial survival, ie metabolism, growth and cell division (ESTRELA et al., 1995, SIQUEIRA & LOPES 1999). An important feature of CH is its ability to inactivate bacterial lipopolysaccharides (LPS) found on the outer membrane of gram-negative bacteria, as well as inactivate lipoteichoic acid (LTA) in gram-positive bacteria by attenuating the host response and reducing progress of apical periodontitis (BAIK et al., 2011).

The process of pulpal inflammation, necrosis, and tissue infection gradually progress to the apical region until the periradicular tissues are affected. There is a pattern of microbial colonization in the root canal system, resulting in apical periodontitis, which represents the most frequently diagnosed apical odontogenic disease in human teeth (SCHULZ et al., 2009), where the cause is a combination of strict and facultative anaerobic microbiota. (FUJI et al., 2009).

Some microorganisms are resistant to endodontic therapy and CH, resulting in a persistent infection (SIQUEIRA & LOPES 1999). Environmental changes, such as rising pH, can stimulate genetic cascades that modify the characteristics of the bacterial cell. The formation of biofilms also represents a bacterial adaptation that increases the resistance of microorganisms (SIQUEIRA & RÔÇAS 2008).

Enterococcus faecalis (*E. faecalis*) is one of the microorganisms often found in persistent infections and endodontic therapy failures (PINHEIRO et al., 2003). Studies show that *E. faecalis* can withstand adverse environmental conditions and has great resistance to direct exposure to CH dressing, which could determine the prevalence of this bacterium in persistent periapical lesions (EVANS et al., 2002, CHÁVEZ DE PAZ et al., 2003, MCHUGH et al., 2004, CHÁVEZ DE PAZ et al., 2007).

Pseudomonas aureginosa (P.aureginosa) is a gram-negative aerobic bacterium commonly found in hospital infection, resistant to antibiotics and antimicrobials due to the permeability of its lipopolysaccharide membrane (YOON et al., 2002). Its strength and ease in biofilm formation make it a model for gram-negative biofilms, being related to endodontic treatment failures (SIREN., 1997). *E. faecalis* and mixed biofilms (*E. faecalis and P.aureginosa*) are resistant to the usual treatment with CH (KLOTZ, RUTTEN, SMITH, BABCOCK, & CUNNINGHAM, 1993; ZANCAN et al., 2016). persistent root canal infection.

Contact of intracanal medication, either directly or indirectly, with periapical tissues can stimulate inflammatory cells, especially macrophages (BRACKETT et al., 2009; SOUSA, CALVALCANTI, MARQUES, 2009). These are also prevalent in endodontic infections and are the main cells of the immune system that destroy microorganisms, and are involved in the process of apical healing (LEONARDO, 1997; SOUSA, CALVALCANTI, MARQUES, 2009). Its main activity is phagocytosis, and during this process, they release a large amount of mediators that attract neighboring cells to the affected area to reconstruct it (CONSOLARO, 2009). They present great capacity for synthesis and secretion of intra and extracellular substances, among them intermediate nitrogen compounds, such as nitric oxide (QUEIROZ et al., 2005; CONSOLARO, 2009).

Nitric oxide (NO) is a reactive intermediate species of nitrogen oxide, synthesized by the activity of nitric oxide synthase, an enzyme present in macrophages (BOGDAN et al.,2015). Substances generated from the reaction of NO molecules with themselves or with other molecules, such as reactive oxygen species, act on various microorganisms, including *E. faecalis* (BAIK et al., 2008, PROLO et al., 2014, WEISS & SCHAIBLE 2015). When released in large quantities it can increase cellular metabolism and potentiate the inflammatory process, promoting the progression of periapical lesion (BAIK et al., 2011) and in small amounts, favors the foreign body isolation process (GUTIERREZ, 2006).

Among many ways to analyze the cytotoxicity of a material, NO dosing is being commonly used. (AZAR et al., 2000; SHIMAUCHI et al., 2001; TAKEICHI et al., 1998). This free radical gas participates in several pathological processes, such as

macrophage suppressing activity, inhibition of leukocyte adhesion in the endothelial wall, and cellular apoptosis (MONCADA; PALMER; HIGGS, 1991).

Considering the importance of the biological properties of intracanal medications, since for periapical tissue repair, they must not stimulate an exacerbated inflammatory response. Thus, a study evaluating the cytotoxicity action of the associations in the release of NO by macrophages becomes opportune.

In an attempt to increase the effectiveness of CH, substances with different antimicrobial agents and chemical characteristics have been used in combination with CH to enhance its antimicrobial action. Among them paramonchlorophenol, chlorhexidine, iodoform (ESTRELA et al., 2006; DELGADO et al., 2010; LIMA et al., 2013). More recent studies have shown that some non-steroidal anti-inflammatory drugs have antimicrobial action (FREITAS et al., 2017; CHOCKATTU et al., 2018). Silva et al., 2019 evaluated the cytotoxicity and biocompatibility of CH pastes associated with two types of NSAIDs, sodium diclofenac and ibuprofen in rat cell and subcutaneous culture concluded that the pastes were not cytotoxic and showed biocompatibility.

E. faecalis and mixed biofilms (*E. faecalis* and *P.aureginosa*) are resistant to the usual treatment with CH (KLOTZ, RUTTEN, SMITH, BABCOCK, & CUNNINGHAM, 1993; ZANCAN et al., 2016) persistent root canal infection.

The present study aimed to evaluate the effect of the association of NSAIDs and antibiotics with CH: diclofenac sodium, ibuprofen, amoxicillin and metronidazole, and their pH, calcium ion release, solubility, adhesion by *E. faecalis*, antimicrobial action against *E. faecalis* and *P. aueruginosa* mixed biofilm and *E. faecalis* biofilm and NO release by macrophages.

2 ARTICLES

2 ARTICLES

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

2.1 ARTICLE 1 - Title: Evaluation of the physical-chemical and antimicrobial properties of the NSAIDs and antibiotics association with calcium hydroxide paste

Abstract

Introduction: the aim of this study was to evaluate the effect of the association of NSAIDs and antibiotics with calcium hydroxide in relation to the antimicrobial activity of *E. faecalis* and *P. aueruginosa* mixed biofilm and the evaluation of physical-chemical properties such as pH, ion release. calcium and solubility. **Methods:** For the experiments the following combinations were prepared: G1: CH + Propylene glycol; G2: CH + 5% Sodium Diclofenac + Propylene Glycol; G3: CH + 5% Amoxycillin + Propylene Glycol; G4: CH + 5% Ibuprofen + Propylene Glycol; G5: CH + 5% Metronidazole + Propylene Glycol. For the antimicrobial analysis, the mixed biofilm of *E. Faecalis* and *P. Aueruginosa* in dentin blocks (n = 4) was induced. After the incubation period, the samples were randomly distributed and immersed in the experimental pastes for 7 days. Through live and dead dye and confocal microscope images were captured from the treated biofilm and analyzed in a bioImage program to measure the percentage of living cells. To evaluate the pH of the pastes, root canals of acrylic teeth were filled with pastes (n = 10 / group) and then the teeth were immersed in ultrapure water to measure the release of hydroxyl ions by means of a calibrated pH meter. Similarly, the release of calcium ions was measured by an atomic absorption spectrophotometer at 7, 15 and 30 days. To assess solubility, root canals of acrylic teeth were filled with pastes as previously mentioned (n = 10 / group) and digitized by computed microtomography before (initial) and after 7, 15 and 30 days of water immersion. ultrapure. Data were statistically compared ($P < 0.05$). **Results:** All groups had similar results regarding mass loss and antimicrobial action against mixed biofilm ($P > 0.05$). The highest release of hydroxyl ions was observed within 30 days for group G1 ($P < 0.05$). The release of calcium ions was higher in group G5 within 7 days ($P < 0.05$). **Conclusions:** The association of NSAIDs with calcium hydroxide did not interfere with pH, calcium ion release and solubility. There was a similar antimicrobial action with the combination with antibiotics for mixed biofilm.

Key Words: calcium hydroxide, sodium diclofenac, ibuprofen, biofilm.

Introduction

Microorganisms and their products are the main factors for the development of pulp and periapical diseases[1]. Endodontic treatment has its main objective the eradication or at least the reduction and control of these microorganisms[2].

Intracanal medication does a fundamental role in microbial control. Calcium hydroxide is the medication of choice worldwide due to the combination of its biological properties[3] and its antiseptic effects, which result from the release of hydroxyl and calcium ions[4]. The high pH of calcium hydroxide promotes microbial inhibition through an irreversible enzymatic reaction[5] and inactivates the lipopolysaccharides (LPS) contained on the wall of gram-negative bacteria.

Some microorganisms have been resistant to the alkaline pH of calcium hydroxide, such as Enterococcus faecalis, a bacterium commonly isolated in persistent root canal infections[6]. Moreover, the capacity of this microorganism to adhere, colonize and form biofilms favors species associations, increasing its resistance to antimicrobial substances[7]

P. aureginosa is a gram-negative bacterium commonly found in nosocomial infection, but it has also been linked to endodontic treatment failures. It is resistant to antibiotics and antimicrobials due to the permeability of its LPS composite membrane. Its strength and ease in biofilm formation make it an exemplar for gram-negative biofilms[8].

The association of several drugs has been suggested to potentiate their antimicrobial action of calcium hydroxide pastes. Among them are paramonchlorophenol, chlorhexidine, and iodoform[9,10,11]. More recent studies have shown that some non-steroidal anti-inflammatory drugs have antimicrobial action[12,13]. Satisfactory results were obtained with *E. faecalis* biofilm[12], but no studies are evaluating the effect of these associations on mixed biofilms.

Given the above, the objective of the present study was to analyze the effect of the association of NSAIDs and antibiotics with calcium hydroxide, namely: diclofenac sodium, ibuprofen, amoxicillin, and metronidazole and to evaluate their pH, calcium ion release, solubility, and antimicrobial action. against mixed biofilm of *E. faecalis* and *P. aueruginosa*. The null hypotheses tested were NSAIDs and antibiotics would not interfere with the antibiofilm action of calcium hydroxide paste and would not interfere with pH, calcium ion release and hydroxide paste solubility.

Materials and methods

Five pastes were evaluated in the experiments. For the preparation of the pastes calcium hydroxide (CH) was combined by mass, in the proportion of 5% of the total paste weight:

1. G1: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + propylene glycol;
2. G2: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de diclofenac sodium + propylene glycol;
3. G3: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de amoxicillin + propylene glycol;
4. G4: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de ibuprofen + propylene glycol;
5. G5: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de metronidazole + propylene glycol.

Experiment 1: Antimicrobial Test and Microscopic Analysis

Biofilm Growth

This test was performed using mixed biofilm obtained with the *Enterococcus faecalis* strain (American Type Culture Collection) ATCC 29212, and *P. aueruginosa* ATCC 10145. For this 15 µL of each bacteria were placed in 3mL of BHI bovine brain and heart broth. (Oxoid, Basingstoke, UK), separately, and then stored in a greenhouse at 37 ° C for overnight growth. After overnight growth, the bacterial density was adjusted to 10^9 cells / mL *E. faecalis* ATCC (29212) and 10^7 cells / mL *P. aeruginosa* ATCC (10145) by an atomic absorption spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density of 1 to 600 nm according to MacFarland Standard Scale 0.5.

Dentin Surface Infection

Dentin blocks were obtained from bovine central incisors with a 4.0 mm diameter trephine under abundant irrigation and subsequently autoclaved. After adjusting the microbial density the dentin surfaces were infected. For each dentin block, 100 µl

E.faecalis + 100 µl *P. aueruginosa* + 1500 µl BHI were used and placed in each well of 24-well culture plates according to the methodology of van der Waal et al. The boards were placed in an oven at 37ºC for 4 days and the BHI changed every 2 days.

Antimicrobial Test for *E. faecalis* Biofilm and *E. faecalis* + *P. Aeruginosa* Biofilm

After the incubation period, the infected samples were washed with 1mL of distilled water to remove bacteria not adhered to the biofilm. Then, the samples were randomly distributed into 6 groups (n = 5), according to the experimental pastes plus the positive control (no treatment). For the contact test dentin samples were immersed in the experimental pastes and incubated at 37ºC for 7 days.

Microbiological Analysis

Biofilm viability was evaluated using the SYTO 9 and propidium iodide technique (Live / Dead BacLight Viability Kit; Molecular Probes, Eugene, OR). After the time established in contact with the pastes, the blocks were washed with saline and stained in a dark environment with 15 µl dyes for 15 minutes. Then they have been washed again and analyzed directly by confocal laser scanning microscopy (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Four images of random areas were obtained from each sample with a magnification of 40X. In total, there were 5 samples per group, 20 images for each medication.

Quantification of total biovolume and percentage of (dead cells) found after antimicrobial treatment was performed using the bioImage_L software (www.bioImageL.com).

Experiments 2 and 3: pH of the Pastes and Calcium Ion Release

For experiments 2 and 3 maxillary central incisor teeth of standardized foramen acrylic resin at 400 nm were filled with the previously described pastes (n = 10). Then the teeth were sealed with temporary restorative coltosol (Vigodent, Rio de Janeiro, Rio de Janeiro, Brazil) and individually immersed in a plastic container containing 10 mL of ultrapure water. In the periods of 7, 15 and 30 days, the teeth were removed and immersed in a new plastic container containing 10mL of ultrapure water. The

release of hydroxyl and calcium ions from the water contained in these containers was measured using a pH meter (Model 371; Micronal, São Paulo, SP, Brazil) calibrated and standardized with buffer solutions 4, 7 and 12 and Atomic absorption spectrophotometer (AA6800; Schimadzu, Tokyo, Japan) equipped with a calcium-ion-specific hollow cathode lamp, respectively. This methodology was based on the method of Duarte et al. (2009)[14].

Experiment 4: Micro–computed Tomographic Volumetric Solubility

For solubility analysis, root canals of 50 acrylic teeth were filled with the experimental pastes (n = 10) and immediately after that, the samples were scanned with a microtomography (SkyScan 1174v2; SkyScan, Kontich, Belgium). Using as parameters voxel size of 19.70 mm, 0.5_ rotation steps, and a 360_ rotation. Each scan consisted of 373 TIFF images with 1024_1304 pixels. Subsequently, the samples were individually immersed in plastic vials containing 10 mL of water and stored at 37 ° C. On the intervals of 7, 15 and 30 days, the acrylic teeth were removed from the container, and new scans were performed using the same parameters. already described. The obtained images were reconstructed and the volume (mm3) of the pastes was measured with the CTan software (CTan v1.11.10.0, Sky-Scan). Solubility values for each specimen were calculated by subtracting the final volume from the initial volume. The result obtained from this calculation represented the total volume lost during immersion in that time interval. The solubility percentage was calculated by dividing the total volume lost by the initial volume and multiplying the result by 100.

Statistical Analysis

Shapiro - Wilk test was performed to verify data distribution. In all analyses, the absence of normality was observed. A comparison between groups was performed using the Kruskal Wallis test with Dunn post-hoc. The significance level was set at 5%.

Results

Table 1 shows the percentages of live cells of the different biofilms after contact with the experimental pastes. There was a statistical difference only to the negative control ($P < 0.05$). The group that most reduced viable microorganisms was G3 (Calcium hydroxide + 5% amoxicillin) ($P < 0.05$)

Table 2 shows the values obtained for the release of calcium Ca2 + and hydroxyl OH - ions (experiments 2 and 3). Group G5 (CH + 5% metronidazole) had the highest release of hydroxyl ions at 7 and 15 days ($P < 0.05$). In the 30 days, the G1 group (CH) had the highest release ($P < 0.05$). Group G5 (CH + 5% metronidazole) was the group with the highest calcium ion release in all periods ($P < 0.05$).

Table 3 shows the percentage of solubility of pastes at time intervals 7, 15 and 30 days. There was no statistically significant difference ($P > 0.05$) between the groups tested.

TABLE 1 : Median (Med) and Minimum and Maximum (Min–Max) Values of the Percentage of Live Cells of Biofilm after Contact with the Experimental Medicaments for a week

Group	CH	CHD	CHA	CHI	CHM	Controle negativo
Med (Min-Max)	28,41 (1,348 – 75,11)[b]	35,60 (7,905 – 77,51)[b]	9,345 (0,1833 – 91,31)[b]	24,86 (1,015 – 64,73)[b]	16,12 (0,3437 – 37,74)[b]	96,59 (49,78 – 99,74)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in columns indicate statistically significant intergroup differences in the same time period.

TABLE 2: Medians (Med) and minimum and Maximum (Min- Max) Values for pH and Calcium Release (mg/L) of the pastes in different Studied Periods

	7 days		**15 days**		**30 days**	
	Med (Min- Max)		Med (Min- Max)		Med (Min- Max)	
Group	pH	Ca^{2+}Realese	pH	Ca^{2+}Realese	pH	Ca^{2+}Realese
CH	7,460 (7,210 - 11,63)[ab]	115,0 (76,14 – 167,9)[a]	7,090 (6,850 - 10,27)[a]	29,96 (15,01- 58,15)[a]	10,74 (7,790 - 11,07)[a]	23,75 (6,650 – 38,81)[a]
CHD	7,690 (7,580 - 11,65)[a]	159,0 (83,96 – 206,3)[abc]	10,13 (7,750 - 10,90)[b]	43,06 (15,55 – 85,85)[abcd]	10,00 (7,940 - 11,21)[a]	17,64 (4,300 – 38,23)[a]
CHA	7,890 (7,660 - 8,180)[abc]	166,6 (122,8 – 205,2)[abc]	9,830 (7,900 - 10,75)[ab]	61,70 (43,10 – 127,1)[bcd]	9,930 (8,070 - 11,04)[a]	28,02 (7,460 – 95,03)[a]
CHI	8,260 (7,970 - 11,62)[bc]	189,8 (134,9 – 215,3)[bc]	10,20 (8,280 - 11,06)[cb]	61,88 (33,71 – 108,4)[d]	9,990 (7,770 - 11,20)[a]	26,38 (4,580 – 64,37)[a]
CHM	8,620 (7,970 - 11,72)[c]	193,8 (138,9 – 269,3)[c]	10,42 (8,000 - 10,77)[db]	73,05 (41,89 – 141,6)[d]	9,140 (7,880 - 11,48)[a]	60,56 (1,830 – 86,74)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in columns indicate statistically significant intergroup differences in the same time period.

TABLE 3. Median (Med) and Minimum and Maximum (Min–Max) Values of the Paste Volume Initially and after 7, 15, and 30 Days of Immersion in 10 mL Deionized Water, and the Percentage of Lost Volume Comparing the 7, 15, and 30 Day Period Volumes with the Initial Volume

	Initial	**7 days**		**15 days**		**30 days**	
	Med (Min-Max**)**	Med (Min- Max)		Med (Min- Max)		Med (Min- Max)	
Group	Volume	Volume	% of lost volume (7 days _ Initial)	Volume	% of lost volume (15 days _ Initial)	Volume	% of lost volume (30 days _ Initial)
CH	2.85(2.19-8.57) [a]	2.09(1.16-6.69) [a]	17.36 (4.55-59.28)[a]	2.18(1.25-7.52) [a]	13.90(9.10-56.20)[a]	1.93(1.16-5.39) [a]	23.17(11.93-59.04)[a]
CHD	2.48(1.93-3.97) [a]	2.20(0.20-3.88) [a]	14.73 (-32.72-90.98)[a]	2.11(1.38-3.47) [a]	25.06(-33.42-44.43)[a]	1.76(0.84-2.90) [a]	41.03(-37.47-60.83)[a]
CHA	2.97(0,00-3.63) [a]	2.91(2.17-6.95) [a]	18.36 (-15.34 -32.06)[a]	2.64(1.94-6.68) [a]	28.17(-12.47-38.84)[a]	2.47(1.62-6.02) [a]	37.24(2.54-45.24)[a]
CHI	2.48(1.93-3.97) [a]	2.20(0.20-3.88) [a]	13.27 (-80.84-26.28)[a]	2.11(1.38-3.47) [a]	9.09(-69.92-28.43)[a]	1.76(0.84-2.90) [a]	20.08(-38.38-62.40)[a]
CHM	3.00(1.62-9.14) [a]	2.76(1.13-8.57) [a]	13.73 (-0.10-29.87)[a]	2.34(1.24-8.12) [a]	17.50(5.10-36.38)[a]	1.87(1.13-7.51) [a]	21.00(9.76-52.54)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different letters in columns indicate statistically significant intergroup differences in the same time period.

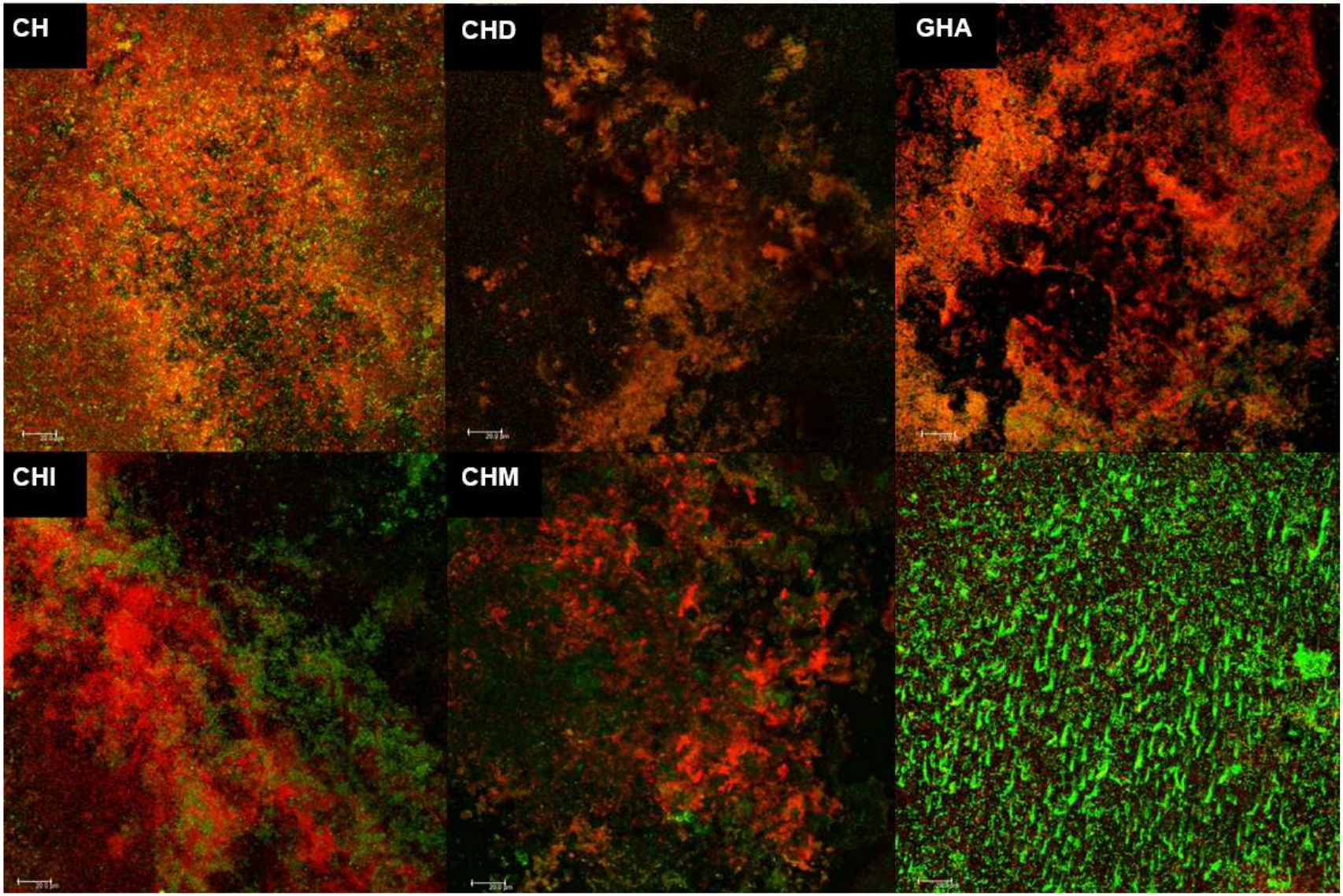

FIGURE 1. Confocal laser scanning microscopy of biofilms treated. Viable cells are indicated in green, and not viable cells are indicated in red. Each picture represents an area of 275 x 275 µm

Discussion

Several medications have been suggested in association with CH to enhance its biological properties and reduce the microbial load inside the root canals[15,16]. In this study, some associations of NSAID and antibiotics with calcium hydroxide were tested. The null hypothesis was partially rejected once there was a statistical difference in antimicrobial action, pH and calcium ion release between the tested medications to the control. The association of NSAIDS and antibiotics with calcium hydroxide did not affect the solubility of the studied pastes.

Studies have shown that some anti-inflammatory drugs have antimicrobial action[17,18,19] and the association of these anti-inflammatory drugs with calcium hydroxide has been shown effective against *E. faecalis* bacteria biofilms without changing the pH of the paste[12, 13].

The polymicrobial nature of root canal infections[20] makes the duo-species biofilm test important, better represents the clinical situation of an endodontic infection, the organization of multispecies biofilm favors the bacterial capacity for material exchange, making them more resistant and capable of leading the interspecific spread of antibiotic-resistant genes[21].

The results showed no differences in cell viability between the associations and the pure paste. Although NSAIDS have antimicrobial activity against gram-positive cocci, their activity against gram-negative species is lower due to the extrusion of these components by multidrug resistance efflux pumps[22]. Also, *P. Aeruginosa* can undergo phenomena variations that make it more resilient according to its environment[23].

E. faecalis, a bacterium commonly found in cases of endodontic failures[24,25,26], has the ability to invade dentinal tubules and remains viable within them[27,28,29], besides presenting resistance to various antimicrobial substances, surviving in alkaline environment[30,6] and not be totally eliminated from the root canal[31].

P. aeruginosa, is a gram-negative, enteric, bacillus, aerobic, bacterium that can survive under anaerobic conditions[32], has motility due to the presence of flagella and is related to failure in endodontic treatment[8]. It is predominant in hospital infections, being resistant to antibiotics and antiseptics due to a permeability barrier offered by its external membrane composed by lipopolysaccharide - LPS and easy to exchange

genetic material. As a producer of notorious extracellular polymeric substance, it is commonly chosen as a gram-negative bacterial model in biofilm studies[33].

To assess the pH and calcium ion release, standardized foraminal widening acrylic teeth with a diameter of 400 nm [14,34] were used. This foraminal enlargement represents a real clinical situation since it has been recommended in cases of necropulpectomy [35,36] for a bacterial reduction in the canal system, especially in the apical third of the root.

In this study, pH values ranging from 7.4 to 8.6 were observed in the first period of 7 days to 9.1 to 10.4 in the last period, which may favor the antimicrobial action of pastes. A high pH of CH pastes promotes microbial inhibition through an irreversible enzymatic reaction and can stimulate genetic cascades that modify bacterial cell characteristics.

To evaluate the solubility of pastes, the methodology proposed by Cavenago, 2014[37] was used. Computed tomography sought to find the volumetric loss of the paste, subtracting from the total value of the initial volume of the tooth filled with the paste the final volume after each period.

The vehicle associated with CH has a direct influence on pH, calcium ion release and solubility. Propylene glycol was the vehicle of choice used in all associations with CH justifying the study results where there was no difference in solubility in the different groups. The powder/liquid ratio was standardized so that no paste became more fluid, which could result in a higher pH higher calcium release and solubility and consequently higher cytotoxicity in periapical tissues[38].

References

1. Siqueira JF Jr, Lopes HP. Bacteria on the apical root surfaces of untreated teeth with periradicular lesions: a scanning electron microscopy study. Int Endod J 2001;34:216-20.
2. Siqueira JF Jr, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod 2008;34:1291-1301.
3. Estrela C, Holland R. Calcium hydroxide: study based on scientific evidences. J Appl Oral Sci 2003;11:269-82.
4. Siqueira JF Jr, de Uzeda M. Influence of different vehicles on the antibacterial effects of calcium hydroxide. J Endod 1998;24:663-5.
5. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32:361-9.
6. Nakajo K, Komori R, Ishikawa S, Ueno T, et al. Resistance to acidic and alkaline environments in the endodontic pathogen Enterococcus faecalis. Oral Microbiol Immunol. 2006;21:283-8.
7. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MA, et al. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod 2013;39:346-50.
8. Siren EK, Haapasalo MP, Ranta K, et al. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. Int Endod J 1997;30:91–5.
9. Estrela C, Estrela CR, Hollanda AC, et al. Influence of iodoform on antimicrobial potential of calcium hydroxide. J Appl Oral Sci 2006;14:33-7.
10. Delgado RJ, Gasparoto TH, Sipert CR, et al. Antimicrobial effects of calcium hydroxide and chlorhexidine on Enterococcus faecalis. J Endod 2010;36:1389–93.
11. Lima RA, Carvalho CB, Ribeiro TR, et al. Antimicrobial efficacy of chlorhexidine and calcium hydroxide/camphorated paramonochlorophenol on infected primary molars: a split-mouth randomized clinical trial. Quintessence Int 2013;44:113–22.
12. de Freitas RP, Greatti VR, Alcalde MP, et al. Effect of the Association of Nonsteroidal. Anti-inflammatory and Antibiotic Drugs on Antibiofilm Activity and pH of Calcium Hydroxide Pastes. J Endod 2017;43:131-134.

13. Chockattu SJ, Deepak BS, Goud KM. Comparison of anti-bacterial efficiency of ibuprofen, diclofenac, and calcium hydroxide against Enterococcus faecalis in an endodontic model: An in vitro study. J Conserv Dent 2018;21:80-84.

14. Duarte MA, Midena RZ, Zeferino MA, et al. Evaluation of pH and calcium ion release of calcium hydroxide pastes containing different substances. J Endod 2009; 35: 1274-7.

15. Estrela C, Bammann LL, Pimenta FC, Pécora JD. Control of microorganisms in vitro by calcium hydroxide pastes. Int Endod J 2001;34:341-5.

16. Pereira TC, da Silva Munhoz Vasconcelos LR, Graeff MSZ, et al. Intratubular decontamination ability and physicochemical properties of calcium hydroxide pastes. Clin Oral Investig 2018.

17. Dastidar SG, Ganguly K, Chaudhuri K, et al. The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. Int J Antimicrob Agents 2000;14:249-51.

18. Dutta NK, Annadurai S, Mazumdar K, et al. Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. Int J Antimicrob Agents 2007;30:242-9.

19. Salem-Milani A, Balaei-Gajan E, Rahimi S, et al. Antibacterial Effect of Diclofenac Sodium on Enterococcus faecalis. J Dent (Tehran) 2013;10:16-22.

20. Rôças IN, Siqueira JF Jr, Aboim MC, et al. Denaturing gradient gel telectrophoresis analysis of bacterial communities associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;98:741-9

21. Jhajharia K, Parolia A, Shetty KV, et al. Biofilm in endodontics: A review. J Int Soc Prev Community Dent. 2015;5:1-12.

22. Laudy AE. Non-antibiotics, Efflux Pumps and Drug Resistance of Gram-negative Rods. Pol J Microbiol. 2018 30;67:129-135.

23. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature. 2002 18;416:740-3.

24. Peciuliene V, Reynaud AH, Balciuniene I, et al. Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. Int Endod J. 2001;34:429-34.

25. Pinheiro ET, Gomes BP, Ferraz CC, et al. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J. 2003;36:1-11.
26. Stuart CH, Schwartz SA, Beeson TJ, et al.Enterococcus faecalis: its rolein root canal treatment failure and current concepts in retreatment.J Endod 2006;32:93-8.
27. Siqueira JF Jr, De Uzeda M, Fonseca ME. A scanning electron microscopic evaluation of in vitro dentinal tubules penetration by selected anaerobic bacteria. J Endod 1996;22:308-10.
28. Love RM. Enterococcus faecalis--a mechanism for its role in endodontic failure. Int Endod J 2001;34:399-405.
29. Chivatxaranukul P, Dashper SG, Messer HH. Dentinal tubule invasion and adherence by Enterococcus faecalis. Int Endod J 2008;41:873-82.
30. Evans M, Davies JK, Sundqvist G, et al. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2002;35:221–8.
31. Guerreiro-Tanomaru JM, Morgental RD, Flumignan DL, et al. Evaluation of pH, available chlorine content, and antibacterial activity of endodontic irrigants and their combinations against Enterococcus faecalis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112:132-5.
32. Yoon SS, Hennigan RF, Hilliard GM, et al. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 2002;3:593–603.
33. Bazire A, Diab F, Jebbar M, et al. Influence of high salinity on biofilm formation and benzoate assimilation by Pseudomonas aeruginosa. J Ind Microbiol Biotechnol. 2007;34:5-8.
34. Zancan RF, Vivan RR, Milanda Lopes MR, et al . Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. J Endod 2016;42:1822-1828.
35. Cruz Junior JA, Coelho MS, Kato AS, et al. The Effect of Foraminal Enlargement of Necrotic Teeth with the Reciproc System on Postoperative Pain: A Prospective and Randomized Clinical Trial. J Endod 2016;42:8-11.
36. Rodrigues RCV, Zandi H, Kristoffersen AK, et al. Influence of the Apical Preparation Size and the Irrigant Type on Bacterial Reduction in Root Canal-treated Teeth with Apical Periodontitis. J Endod 2017;43:1058-1063.

37. Cavenago BC, Pereira TC, Duarte MA, et al. Influence of powder-to-water ratio on radiopacity, setting time, pH, calcium ion release and a micro-CT volumetric solubility of White mineral trioxide aggregate. Int Endod J 2014;47:120-6.
38. Rehman K, Saunders WP, Foye RH, et al. Calcium ion diffusion from calcium hydroxide-containing materials in endodontically-treated teeth: an in vitro study. Int Endod J 1996;29:271–9.

2.2 ARTICLE 2 - Title: Antimicrobial evaluation, adhesion and nitric oxide production of the association of NSAIDs and antibiotics with calcium hydroxide paste.

Abstract

This study aimed to evaluate the effect of the association of NSAIDs and antibiotics with calcium hydroxide to E. faecalis antimicrobial capacity, adhesion and the production of nitric oxide (NO) from stressed macrophages with the same associations. Methodology: They were divided into five groups: G1: calcium hydroxide + propylene glycol; G2: calcium hydroxide + 5% sodium diclofenac + propylene glycol; G3: Calcium hydroxide + 5% Amoxicillin + Propylene Glycol G4: Calcium hydroxide + 5% Ibuprofen + Propylene Glycol; G5: Calcium hydroxide + 5% Metronidazole + Propylene Glycol. For antimicrobial tests, bovine dentin blocks were obtained and biofilm induction was performed. After the incubation period, the specimens were divided between groups (n = 5) and immersed in the experimental pastes and incubated at 37ºC for 7 days. For the adhesion test, dentin blocks were obtained and placed in sterile petri dishes and then the provided pastes were placed on each dentin block and placed in a greenhouse at 37ºC for 7 days. The specimens were then contaminated using Enterococcus faecalis bacteria (ATCC 29212), where each inoculum + dentin block was added to each well of a 24-well plate and kept in an oven at 37ºC for one hour. Through live and dead dye and confocal microscope images were captured and analyzed using a bioImage program to measure the percentage of viable and non-viable cells. For the expression of NO, the pastes were inserted into the plates containing the macrophages adhered for 24 hours in an oven at 37 ° C, and then the NO production analysis was performed by Greiss reaction. Data were statistically compared ($P < 0.05$). Results: The highest antimicrobial action was observed in group G5 followed by G4, as well as for adhesion of M.O and release of nitric oxide. All groups were statistically different from the positive control in all tests ($P < 0.05$). Conclusions: The association of NSAIDs and antibiotics contributed to the antimicrobial action of calcium hydroxide paste. The use of metronidazole increased Enterococcus faecalis bacterial cell adhesion and the production of NO by macrophages.

Keywords: calcium hydroxide, sodium diclofenac, ibuprofen, biofilm.

Introduction

Apical periodontitis can be considered an untreated caries sequelae or present when endodontic treatment fails or is not effective to remedy the existing infection. Endodontic infection is composed of different types of bacteria and the predominance in cases where treatment was not effective is strict and facultative anaerobic bacteria (Fuji et al. 2009).

E. faecalis, constantly related to secondary and persistent infections (Pinheiro et al. 2003), is a facultative, gram-positive and fermentative anaerobic bacterium. It has virulence factors related to aggregation and adhesion to the dentin substrate (Pinheiro et al. 2013), being able to invade dentinal tubules and remain viable even at alkaline pH (Love et al. 2001; Nakajo et al. 2006).

When endodontic treatment fails and infection persists, cells of our immune system are activated, which are polymorphonuclear leukocytes, macrophages, and lymphocytes. The main cells involved are macrophages, which are in high concentration in the apical region. Macrophages are key actors in immune defense and pathological processes, especially host response to intracellular bacteria (Riccuci et al. 2006; Weiss & Schaible, 2015).

Macrophages produce reactive species to eliminate internalized microorganisms. NO is a reactive intermediate species of nitrogen oxide, synthesized by the activity of nitric oxide synthase, an enzyme present in macrophages. NO is released when macrophages come into contact with a foreign particle in an attempt to perform phagocytosis. When released in large quantities it can increase cellular metabolism and potentiate the inflammatory process; and in small quantities, it favors the process of isolation of the foreign body, cytodifferentiation fibroblasts and exacerbating protein and collagen synthesis, which characterizes post-obturation repair with formation of fibrous capsule or mineralized tissue (Gutierrez et al. 2006).

Calcium hydroxide makes the alkaline environment unsuitable for microorganisms to survive and multiply but is not capable of eliminating *E. faecalis* if not for direct contact due to its defense mechanisms (Nakajo et al. 2006). Several drugs have been suggested in association with calcium hydroxide to potentiate its antimicrobial action. Among them paramonochlorophenol, chlorhexidine, iodoform (Estrela et al. 2006, Delgado et al. 2010, Lima et a. 2013). More recent studies have shown that some nonsteroidal anti-inflammatory drugs, when combined with calcium

hydroxide, have antimicrobial action (Freitas et al. 2017, Chockattu et al. 2018). Some associations of anti-inflammatories with calcium hydroxide showed satisfactory results when compared to *E. faecalis biofilm* (Freitas et al. 2017).

However, the effect of some associations on these biofilms, as well as on *E. faecalis* adhesion in case of recontamination and on NO production by macrophages is still unknown.

Therefore, the objectives of this work were to evaluate the effect of pure calcium hydroxide paste associated with antibiotics and anti-inflammatory agents on biofilm and the adhesion of *E. faecalis* to dentin, and on the expression of NO in macrophages.

Materials and methods

Five pastes were evaluated in the experiments. For the preparation of the pastes calcium hydroxide (CH) was combined by mass, in the proportion of 5% of the total paste weight:

G1: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + propylene glycol;
G2: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de diclofenac sodium + propylene glycol;
G3: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de amoxicillin + propylene glycol;
G4: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de ibuprofen + propylene glycol;
G5: CH (Biodinâmica ®, Ibiporã, PR, Brazil) + 5% de metronidazole + propylene glycol.

Experiment 1: Antimicrobial Test

Preparation of specimens

Twenty-five dentin blocks were obtained from bovine central incisors with a 4.0 mm diameter trephine under abundant irrigation. After cutting, the blocks were sanded using a polishing machine. The dentin blocks were treated with 1% sodium hypochlorite for 30 minutes and 17% EDTA for 5 minutes to remove organic residues and smear layer. The blocks were then sterilized with an autoclave at 121 ° C.

Biofilm Formation

To perform the biofilm induction has used the methodology recommended by Guerreiro-Tanomaru et al, 2013. For biofilm formation a standard strain of *E. faecalis* was used ATCC 29212 (American Type Culture Collection) was used. After confirming the purity of the strain, performed by Gram staining and colony morphology, and biochemical identification, the microorganism was reactivated in 4 mL of sterile BHI broth and kept in an oven at 37 oC for 12 hours. After this period the optical density of the medium was measured and adjusted in a spectrophotometer (Model 600 Plus, Femto, São Paulo, SP, Brazil) with a wavelength of 600 nm. Cell density was 1.5 x 10 8 colony-forming units per mL (CFU / mL). In two 24-well cell culture plates, dentin blocks were placed with one of the pencil-marked surfaces facing downwards. Then the blocks were submerged with 3.6 mL of sterile BHI broth added with 0.4 mL of the standard bacterial inoculum. The plates were placed in a bacteriological oven at 37 oC for 21 days. In order to avoid nutrient deficiency for bacterial cells, the BHI culture medium of each specimen was fully changed every 48 h without the addition of new microorganisms.

Antimicrobial Test

After the incubation period, the infected samples were washed with 1 mL of distilled water to remove bacteria not adhered to the biofilm. Then they were randomly assigned to 5 groups according to the experimentais folders plus the positive control (no treatment) (n = 5). For the contact test dentin samples were immersed in the experimental pastes and incubated at 37ºC for 7 days.

Microbiological analysis

The viability of the biofilm was evaluated using the SYTO 9 propidium iodide technique (Live / Dead BacLight Viability Kit; Molecular Probes, Eugene, OR). After the time established in contact with the pastes, the blocks were washed with saline and stained in a dark environment with 15 µL of dye for 15 minutes. Then they have washed again and observed directly by confocal laser scanning microscopy (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Four random area images

were obtained for each sample with a 40X lens. In total there were 5 samples per group, 20 images for each medication.

The bioImage_L software (www.bioImageL.com) was used to quantify the total biovolume and the percentage of viable and unviable cells found after antimicrobial treatment.

Experiment 2: Adhesion

Obtaining specimens

Bovine dentin blocks were obtained in the same way as described in experiment 1.

Then, the dentin blocks were placed in sterile petri dishes and the pastes were placed on them, which were then oven baked at 37ºC for 7 days.

Specimen Contamination

After the 7-day period in which the specimens were kept in the oven at 37ºC, the specimens were contaminated using *Enterococcus faecalis* bacteria (ATCC 29212). To this end, 15µl of bacterial suspension was added to the 3ml brain and heart infusion broth (BHI) (Difco; Diagnostics BD, Sparks, MD) for overnight growth. (24 hours period). In a spectrophotometer then, the bacterial density was adjusted to 1x107UFC / mL.

For contamination, in each well of a 24-well plate were added: 975µl BHI + 25µl inoculum + dentin block and kept in a 37 ° C oven for one hour.

Adhesion Analysis

At this time, to remove microorganisms not adhered to the surface of the block, the specimens were washed thoroughly with 2 mL phosphate-buffered saline (PBS).

The specimens were then stained with the Live / Dead kit for 15 minutes in a dark environment and immediately analyzed using a TCS-SPE inverted confocal

microscope (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). To measure the results, the biovolume was analyzed through the bioImage_L program.

Experiment 3: Nitric oxide release by macrophages

This study was approved by the Research Ethics Committee of the Bauru School of Dentistry, University of São Paulo. (CEP / FOB-USP) under CAAE: 66228717.4.0000.5417. After signing the informed consent form, as provided for in Resolution 196/96 of the National Health Council, blood collection was performed in healthy volunteer patients.

3.1 Obtainment and culture of macrophages from human peripheral blood monocytes

Thirty mL of brachial vein blood was collected from 3 healthy individuals by a calibrated technician with the aid of vacuum glass tubes (BD Vacutainer), containing sodium heparin as an anticoagulant. For the differentiation of mononuclear cells, the Histopaque 1083 (Sigma-Aldrich) gradient was added to the biological material diluted with PBS and centrifuged for 25 minutes at 400 rcf at room temperature (21ºC). Afterward, 3 layers formed with different concentration gradients, in which the interface was collected between the plasma (above) and the red series (below). The obtained cells were washed in RPMI 1640 (Invitrogen) and adjusted to a concentration of 1x10 6 monocytes per ml of culture medium. These were then plated into 24-well culture plates (Orange Scientific, Belgium) and incubated at 37 ° C in a humidified atmosphere (5% CO2 / air) for 2 hours so that monocytes could adhere to the plate. Afterward, the plates were again washed twice with RPMI 1640 so that non-adherent cells, including lymphocytes, were then removed. Then 1 mL RPMI 1640 containing 10% fetal bovine serum (FCS) and 1% penicillin was added to wells containing adherent monocytes. Next, 24-well plates (Orange Scientific, Belgium) were incubated for 7 days (time required for monocytes to differentiate into macrophages) at 37 ° C in a humidified atmosphere (5% CO2 / air) and every 48 hours. hours, 500 µl of culture medium from each well was renewed.

3.2 Preparation of Pastes

The medications were individually prepared according to the proportions previously established in this work, and mixed in RPMI 1640 medium, reaching a concentration of 0.1 g / mL (100%). From then on, we obtained a second dilution in RPMI 1640 at a concentration of 0.1%. The samples were incubated in an oven at 37ºC for 24 h. The positive control group is composed of RPMI 1640 only. For all groups, the experiment was performed in triplicate.

3.3 Release of Nitric Oxide by Macrophages

After the incubation period of the pastes (topic 3.2), they were inserted into the plates containing the attached macrophages (topic 3.1), and this exposure occurred for 24 hours in a 37 ° C greenhouse in a humidified atmosphere (5% CO2 / air). Next, the analysis of NO production was performed by Greiss reaction. One hundred µL of the culture supernatant from each well was mixed with 1% sulfanilamide in 2.5% phosphoric acid solution (100 µL) for 10 minutes and then 100 µL of 1% N- (1-naphthyl) ethylenediamine in 2.5% phosphoric acid added at the same time. For evaluation of NO production the 490 nm absorption was used in a microplate reader (Synergy 2, BioTek, VT, USA) using sodium nitrite standard curve with RPMI 1640 as a comparison.

Statistical analysis

Shapiro - Wilk test was performed to verify data normality, in all analyzes absence of normality, was observed. A comparison between groups was performed using the Kruskal-Wallis test and the Dunn posthoc test. The significance level was set at 5%.

Results

Table 1 shows the percentages of viable biofilm cells after contact with the experimental pastes. All associations significantly reduced the number of viable bacterial cells relative to the positive control and the pure CH group. (P> 0.05)

Table 2 shows the percentages of cells adhered to the specimen (biovolume) (experiments 1) in the adhesion test. The highest cell adhesion was observed in group G1 (CH) followed by group G5 (CH+ 5% metronidazole). The group that least adhered to cells was the control group (where there was no intracanal medication), which was significantly different from the other groups studied (P <0.05).

Nitric oxide production induced by different combinations of CH medications is presented in **Table 3**. Nitric oxide production was observed in all associations; group G5 (CH + 5% metronidazole) had the highest production of all groups, being statistically larger than the negative control (P> 0.05).

TABLE 1 Median (Med) and Minimum and Maximum (Min–Max) Values of the Percentage of Live Cells after Contact with the Experimental Medicaments for a Week

Groups	CH	CHD	CHA	CHI	CHM	Negative Control
% of live bacteria	61,93(24,96-99,09)[a]	11,28 (0,33 – 87,33)[b]	8,535 (0,02 –76,39)[b]	4,542(0,27–63,46)[b]	3,246 (0,401–50,72)[b]	90,92 (52,71 – 99,74)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different letters in columns indicate statistically significant intergroup differences in the same time period

TABLE 2 Median (Med) and Minimum and Maximum (Min - Max) Biovolume Values of Adhered Bacterial Cells After one hour of contact with the dentin discs previously reated with the experimental drugs

Groups	CH	CHD	CHA	CHI	CHM	Negative Control
Med (Min-Max)	20105 (2489 – 59800)[bd]	5724 (276,5 - 152477)[f]	4203 (736,5 – 101,371)[cf]	4616 (743,8 - 21162)[ef]	11454 (200,1 - 13780)[bf]	1290 (397,3 - 17785)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different letters in columns indicate statistically significant intergroup differences in the same time period

TABLE 3 Median (Med) and Minimum and Maximum (Min – Max) Values of nitric oxide release by macrophages stressed with experimental pastes

Groups	CH	CHD	CHA	CHI	CHM	Negative Control
Med (Min-Max)	32,45(32,00-36,09)[ab]	210,2 (183,8-253,4)[ab]	37,45 (37,00-37,91)[ab]	658,8 (655,6-660,2)[ab]	867,8 (867,8-868,3)[b]	14,00(10,82-14,45)[a]

Kruskal-Wallis with a Dunn post hoc P value <.05. Different letters in columns indicate statistically significant intergroup differences in the same time period

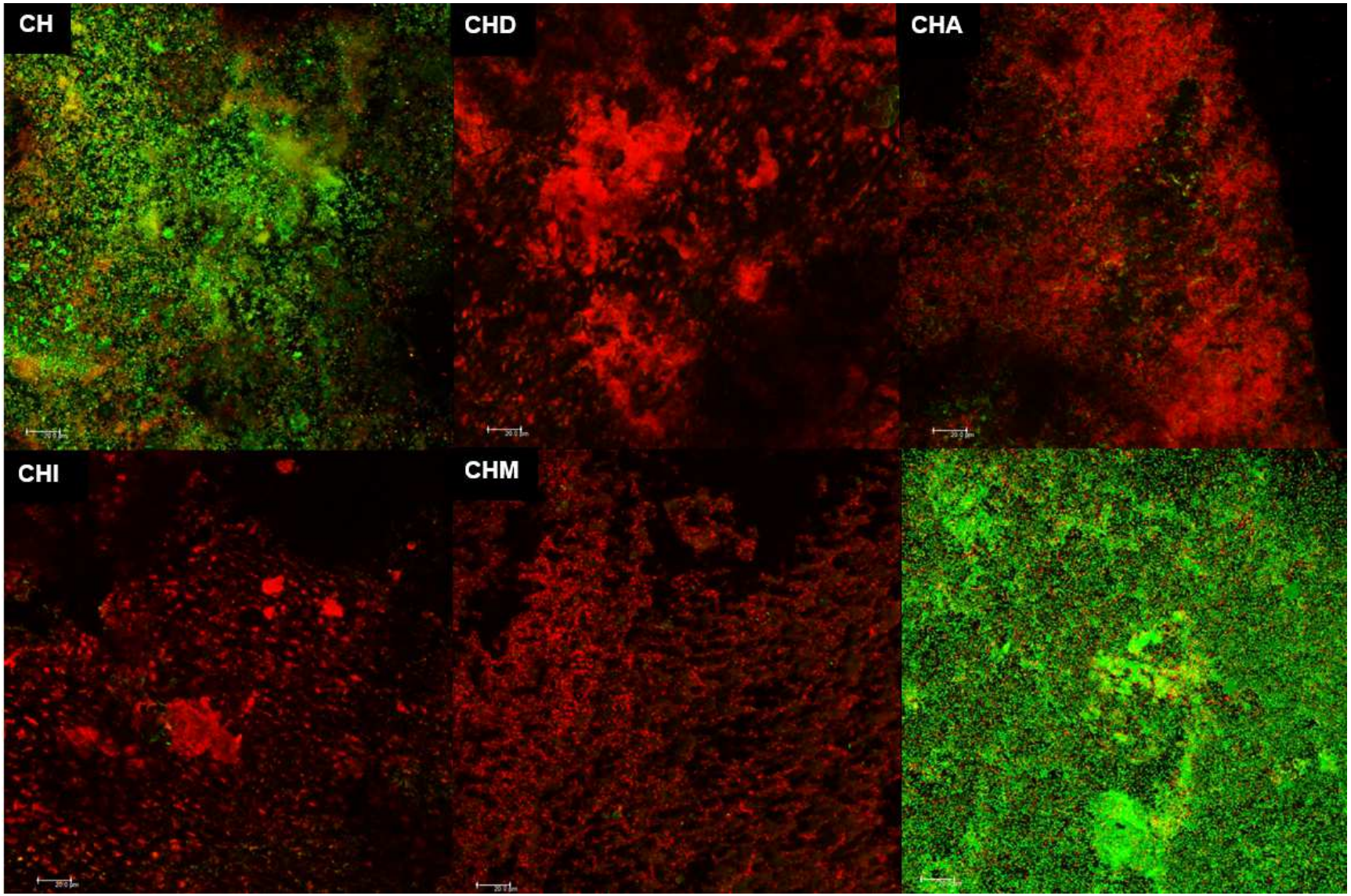

FIGURE 1. Confocal laser scanning microscopy of biofilms treated. Viable cells are indicated in green, and not viable cells are indicated in red. Each picture represents an area of 275 x 275 µm

Discussion

The null hypothesis was rejected, as there was a statistical difference between the tested medications regarding antimicrobial action, adhesion of microorganisms to dentin and NO release between the studied paste associations. The use of intracanal medication has as its main purpose to promote antiseptic action, reducing the microbial load in the canal (Siqueira et al., 2008).

CH is a nonspecific antimicrobial and is the most common substance used as an intracanal medication, it is also known to induce mineralized tissue formation (Mizuno & Banzai 2008) and inactivate lipopolysaccharides found in the outer membrane of gram-negative bacteria (Tanomaru et al 2003).

Enterococcus faecalis is one of the microorganisms present in persistent endodontic infection and is known to withstand adverse environmental conditions and to have high resistance to direct exposure to CH dressing (Evans et al 2002, Chávez de Paz et al 2007).

In the present study, it was proportionally associated with anti-inflammatory CH such as diclofenac sodium and ibuprofen and the antibiotics amoxicillin and metronidazole. In the antimicrobial action against biofilm, the results showed that the groups that most reduced the number of viable cells was G5 (CH + 5% metronidazole) followed by G4 (CH + 5% ibuprofen), but all Antibiotic / NSAID combinations were effective in reducing bacterial viability in the *E. faecalis* biofilm. The concentrations of medications used in association with CH were based on a study by Freitas et al., 2017.

Medicines do not have a single specific function; chemicals that have moderate to powerful antimicrobial properties are called "non-antibiotics". The mechanism of action of antimicrobial activity of anti-inflammatory drugs such as diclofenac and ibuprofen is unclear. Studies have proposed inhibiting bacterial DNA synthesis (Dastidar et al 2000) or compromising membrane activity Dutta et al 2007, Dutta & Mazumdar2007 as possible underlying mechanisms.

The use of intracanal antibiotics such as triantibiotic modified triantibiotic and diantibiotic paste is suggested in the literature mainly for pulp revascularization, to cover a broader spectrum of action against the various types of microbial species that inhabit the canal (Akgun et al.2009). Although both benefit from the antimicrobial action of CH, the indiscriminate use of antibiotics creates a selection, and as a result,

bacterial species are becoming increasingly resistant (Reynolds et al. 2009). In addition to the problem of bacterial resistance, antibiotics induce tissue cytotoxicity (Windley et al. 2005). However, an alternative that has not been studied is the interaction of the antimicrobial action of antibiotics with the biological benefits of CH.

During sample preparation, the teeth were irrigated with sodium hypochlorite and EDTA to remove residual remains and smear layer, as it is used during the mechanical chemical preparation of the root canal. These irrigants alter dentin composition (Tartari et al. 2016) and exposure of *E. faecalis* to collagen and EDTA also increases its resistance to CH disinfection (Kayaoglu et al. 2009; George and Kishen 2008). Intracanal medications may also alter dentin composition by denaturing its collagen content (Whitbeck et al. 2011) and influencing the adhesion of microorganisms to the surface.

Another factor that may contribute to the adhesion of E.faecalis to dentin is its virulence factors related to aggregation and adhesion to the dentin substrate as collagen (Pinheiro et al. 2013, Love et al 2001), namely: ace (protein-ligand) collagen), wing and wing 373 (aggregating substance), cylA (hemolysin activator), esp (protein surface) and gelE (gelatinase) (Barbosa-Ribeiro et al. 2016).

In the adhesion experiment, the groups containing medication favored a significantly higher bacterial cell adhesion than the negative control. Kayaoglu et al (2004), studying *E. Faecalis* adhesion under alkaline pH conditions observed that the increase in pH up to 8.5 that occurs with alkaline drugs such as CH increases *E. faecalis* binding capacity to collagen. in vitro.

The group with pure CH paste followed by the CH group plus 5% metronidazole favored the adhesion of *E. faecalis* cells. In contrast, it was the groups that left the largest number of unviable bacterial cells in 1h that remained in contact.

Metronidazole has a broad spectrum of bactericidal action against strict anaerobes; but is not able to eliminate all facultative bacteria (Hoshino1992, Er et al 2007, Quirynen2002, Sato et al 199 3, Kim et al. 2010), so there is a need for other drugs to be used together to disinfect root dentin.

Macrophages are prevalent in endodontic infections and are the main immune cells that destroy microorganisms. NO is a reactive intermediate species of nitrogen oxide, synthesized by the activity of nitric oxide synthase, an enzyme present in macrophages. Substances generated from the reaction of NO molecules with themselves or with other molecules, such as reactive oxygen species, act on various

microorganisms, including *E. faecalis* (Baik et al. 2008, Prolo, Alvarez, Radi, 2014, Weiss, Schaible. 2015).

The release of nitric oxide by macrophages that were in contact with the paste was higher in group G5, the other groups had no statistically significant difference with the negative control. NO is important in defense against microorganisms, but it is also a chemical mediator of inflammation related to tissue damage when released in large quantities.

CH is a nonspecific antimicrobial and is the most common substance used as an intracanal medication, just as anti-inflammatory drugs cannot cause bacterial resistance, which can happen when antibiotics are used indiscriminately. Anti-inflammatory drugs have been shown to be effective in antimicrobial action in association with CH. It is believed that this association may contribute beyond the antimicrobial effect to the analgesic and anti-inflammatory effect of the endodontic postoperative period (Moskow et al, 1984; Chance et al, 1987; Ehrmann; Messer & Adams 2003).

References

1. Akgun OM, Altun C, Guven G (2009) Use of triple antibiotic paste as a disinfectant for a traumatized immature tooth with a periapical lesion: a case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. **108**, e62–e65.

2. Baik JE, Ryu YH, Han JY, Im J, Kum KY, Yun CH, Lee K, Han SH (2008) Lipoteichoic acid partially contributes to the inflammatory responses to Enterococcus faecalis. Journal of Endodontics **34**, 975-82.

3. Barbosa-Ribeiro M, De-Jesus-Soares A, Zaia AA, Ferraz CC, Almeida JF, Gomes BP (2016) Antimicrobial Susceptibility and Characterization of Virulence Genes of Enterococcus faecalis Isolates from Teeth with Failure of the Endodontic Treatment. Journal of Endodontics **42**,1022-8.

4. Chance K, Lin L, Shovlin FE, Skribner J (1987) Clinical trial of intracanal corticosteroid in root canal therapy. Journal of Endodontics **13**, 466-8.

5. Chávez de Paz LE, Bergenholtz G, Dahlén G, Svensäter G (2007) Response to alkaline stress by root canal bacteria in biofilms. International Endodontic Journal **40**, 344-55.

6. Chockattu SJ, Deepak BS, Goud KM (2018) Comparison of anti-bacterial efficiency of ibuprofen, diclofenac, and calcium hydroxide against Enterococcus faecalis in an endodontic model: An in vitro study. Journal Conservative Dentistry **21**, 80-84.

7. Dastidar SG, Ganguly K, Chaudhuri K, Chakrabarty AN (2000) The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. International Journal Antimicrobial Agents **14**, 249-51.

8. Delgado RJ, Gasparoto TH, Sipert CR, Pinheiro CR, Moraes IG, Garcia RB, Bramante CM, Campanelli AP, Bernardineli N (2010) Antimicrobial effects of calcium hydroxide and chlorhexidine on Enterococcus faecalis. Journal of Endodontics **36**,1389-93.

9. Dutta NK, Annadurai S, Mazumdar K, Dastidar SG, Kristiansen JE, Molnar J,Martins M, Amaral L (2007) Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. International Journal Antimicrobial Agents **30**, 242-9.

10. Dutta NK, Mazumdar K, Dastidar SG, Park JH (2007) Activity of diclofenac used alone and in combination with streptomycin against Mycobacterium tuberculosis in mice. International Journal Antimicrobial Agents **30**, 336-40.

11. Ehrmann EH, Messer HH, Adams GG (2003) The relationship of intracanal medicaments to postoperative pain in endodontics. International Endodontic Journal **36,** 868-75.

12. Er K, Kuştarci A, Ozan U, Taşdemir T (2007) Nonsurgical endodontic treatment of dens invaginatus in a mandibular premolar with large periradicular lesion: a case report. Journal of Endodontics **33**, 322–324.

13. Estrela C, Estrela CR, Hollanda AC, Decurcio Dde A, Pécora JD (2006) Influence of iodoform on antimicrobial potential of calcium hydroxide. Journal of Applied Oral Science **14**, 33-7.

14. Evans M, Davies JK, Sundqvint G, Figdor D (2002) Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. International Endodontic Journal **35**, 221-8.

15. de Freitas RP, Greatti VR, Alcalde MP, Cavenago BC, Vivan RR, Duarte MA,Weckwerth AC, Weckwerth PH (2017) Effect of the Association of Nonsteroidal. Anti-inflammatory and Antibiotic Drugs on Antibiofilm Activity and pH of Calcium Hydroxide Pastes. Journal of Endodontics **43**, 131-134.

16. Fujii R, Saito Y, Tokura Y, Nakagawa KI, Okuda K, Ishihara K (2009) Characterization of bacterial flora in persistence apical periodontitis lesions. Oral *Microbiology Immunology* **24**, 502-5.

17. George S, Kishen A (2008) Influence of photosensitizer solvent on the mechanisms of photoactivated killing of Enterococcus faecalis. Photochem Photobiol **84**, 734-40.

18. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M (2013) Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. Journal of Endodontics **39**, 346-50.

19. Gutierrez JCR. Avaliação de citotoxicidade de materiais obturadores de canais radiculares: influência na liberação de fator de necrose tumoral-á, interferon-ɤ e óxido nítrico em cultura de células murinas. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da Unesp, 2006.

20. Hoshino E, Ando N, Sato M, Kota K (1992) Bacterial invasion of non-exposed dental pulp. International Endodontic Journal **25**, 2-5.

21. Kayaoglu G, Erten H, Bodrumlu E, Ørstavik D (2009) The resistance of collagen-associated, planktonic cells of Enterococcus faecalis to calcium hydroxide. Journal of Endodontics **35**, 46-9.

22. Kayaoglu G, Ørstavik D (2004) Virulence factors of Enterococcus faecalis:relationship to endodontic disease. Critical Reviews in Oral Biology & Medicine **15**, 308-20.

23. Kim JH, Kim Y, Shin SJ, Park JW, Jung IY (2010) Tooth discoloration of immature permanent incisor associated with triple antibiotic therapy: a case report. Journal of Endodontics **36**, 1086-91.

24. Lima RK, Guerreiro-Tanomaru JM, Faria-Júnior NB, Tanomaru-Filho M (2012) Effectiveness of calcium hydroxide-based intracanal medicaments against Enterococcus faecalis. International Endodontic Journal **45**, 311-6.

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

26. Mizuno M, Banzai Y (2008) Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin. International Endodontic Journal **41**, 933-8.

27. Moskow A, Morse DR, Krasner P, Furst ML (1984) Intracanal use of a corticosteroid solution as an endodontic anodyne. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology **58**, 600-4.

28. Nakajo K, Komori R, Ishikawa S, Ueno T, Suzuki Y, Iwami Y, Takahashi N (2006) Resistance to acidic and alkaline environments in the endodontic pathogen Enterococcus faecalis. Oral Microbiology Immunology **21**, 283-8.

29. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ (2003) Microorganisms from canals of root-filled teeth with periapical lesions. International Endodontic Journal. **36**, 1-11.

30. Pinheiro ET, Penas PP, Endo M, Gomes BP, Mayer MP (2012) Capsule locus polymorphism among distinct lineages of Enterococcus faecalis isolated from canals of root-filled teeth with periapical lesions. Journal of Endodontics **38**, 58-61.

31. Prolo C, Alvarez MN, Radi R (2014) Peroxynitrite, a potent macrophage-derived oxidizing cytotoxin to combat invading pathogens. Biofactors **40**, 215-25.

32. Quirynen M, Teughels W, De Soete M, van Steenberghe D (2002) Topical antiseptics and antibiotics in the initial therapy of chronic adult periodontitis: microbiological aspects. Periodontology 2000 **28**, 72-90.

33. Reynolds K, Johnson JD, Cohenca N (2009) Pulp revascularization of necrotic bilateral bicuspids using a modified novel technique to eliminate potential coronal discolouration: a case report. International Endodontic Journal **42,** 84–92.

34. Ricucci D, Pascon EA, Ford TR, Langeland K (2006) Epithelium and bacteria in periapical lesions. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology **101**, 239-49.

35. Sato T, Hoshino E, Uematsu H, Noda T (1993) In vitro antimicrobial susceptibility to combinations of drugs on bacteria from carious and endodontic lesions of human deciduous teeth. Oral Microbiology Immunology **8**, 172-6.

36. Siqueira JF, Roças IN (2008) Clinical implications and microbiology of bacterial persistence after treatment procedures. Journal of Endodontics **34**,1291–301.

37. Schulz M, von Arx T, Altermatt HJ, Bosshardt D (2009) Histology of periapical lesions obtained during apical surgery. Journal of Endodontics **35**, 634-42.

38. Tanomaru JM, Leonardo MR, Tanomaru Filho M, Bonetti Filho I, Silva LA (2003) Effect of different irrigation solutions and calcium hydroxide on bacterial LPS. International Endodontic Journal **36**, 733-9.

39. Tartari T, Wichnieski C, Bachmann L, Jafelicci M Jr, Silva RM, Letra A, van der Hoeven R, Duarte MAH, Bramante CM (2018) Effect of the combination of several irrigants on dentine surface properties, adsorption of chlorhexidine and adhesionof microorganisms to dentine. International Endodontic Journal **51**, 1420-1433.

40. Weiss G, Schaible EU (2015) Macrophage defense mechanisms against intracellular bacteria. Immunological Reviews **264**,182-203.

41. Whitbeck ER, Quinn GD, Quinn JB (2011) Effect of Calcium Hydroxide on the Fracture Resistance of Dentin. Journal of Research of the National Institute of Standards and Technology **116**, 743-9.

42. Windley W, Teixeira F, Levin L, Sigurdsson A, Trope M (2005) Disinfection of immature teeth with a triple antibiotic paste. Journal of Endodontics **31**, 439-43.

3 DISCUSSION

3 DISCUSSION

Calcium Hydroxide (CH) is an antimicrobial substance most commonly used in endodontics as an intracanal medication with the main purpose of controlling microorganisms present in the endodontic infection. It has several advantages such as induction of mineralized tissue (MIZUNO & BANZAI 2008), antimicrobial activity (SIQUEIRA & LOPES 1999) and inactivation of lipopolysaccharides present in the membrane of gram-negative bacteria (TANOMARU et al., 2003). Its mechanism of action is through the release of calcium and hydroxyl ions making the environment alkaline and unsuitable for microbial survival. However, some bacteria resist treatment causing maintenance of the endodontic infection.

When endodontic treatment fails and endodontic infection persists, bacteria that have been resistant to treatment remain in the root canal system and apical periodontitis is formed. Failure - related bacteria in endodontic treatment include *E. faecalis*, a facultative, gram-positive and fermentative anaerobic bacterium that can invade dentinal tubules and remain viable even at alkaline pH (LOVE et al., 2001; NAKAJO et al., 2006). It has virulence factors related to aggregation and adhesion to the dentin substrate (PINHEIRO et al., 2013).

In order to potentiate the antimicrobial action of CH, several combinations of drugs such as chlorhexidine, camphorated paramonochlorophenol, furacinated paramonoclofophenol, antibiotics, and anti-inflammatory drugs have been suggested in the literature (ESTRELA et al., 2006; DELGADO et al., 2010; LIMA et al., 2013; ZANCAN et al., 2016; FREITAS et al., 2017). In the present study, it was proportionally associated with anti-inflammatory calcium hydroxide such as diclofenac sodium and ibuprofen and the antibiotics amoxicillin and metronidazole.

CH is a strong base, poorly soluble in water, its pH is around 12.6 (SIQUEIRA et al., 1999). It acts directly and indirectly on the enzymatic activity of bacteria by inhibiting their growth. Thus there may be a reversible (temporary) enzymatic inactivation when the bacterium is placed at pH above or below optimal for its functioning and its irreversibility may be observed under extreme pH conditions for

long periods promoting the total loss of its ability. biological (ESTRELA & HOLLAND 2003).

The combinations tested did not change the pH of HC, metronidazole, and ibuprofen favored alkaline pH in the first 2 periods (7 and 15 days). Similarly with the release of calcium ions. The solubility of the folders was not changed by the associations.

Initial adhesion of microorganisms to surfaces is the first step in biofilm formation and may result in persistence or chronic infections (BACA et al. 2011). The adhesion activity of *E. faecalis* is enhanced by calcium hydroxide. Cultivated at high pH, it increases the binding capacity of *E. faecalis* collagen (KAYAOGLU et al., 2004; KAYAOGLU, ERTEN, & ORSTAVIK, 2005). The results of this study corroborate the work since there was greater adhesion of bacterial cells in the groups that contained intracanal medication, statistically different from the negative control.

The bacteria in the root canal are organized in a matrix that protects them, the biofilm. The extracellular matrix of biofilm leads to better protection against environmental changes, the inability of antibiotics to reach bacteria within the biofilm (CHÁVEZ DE PAZ, 2007). Due to bacterial interactions and variations in oxygen pressure within the root canal, a restricted group of species can grow and survive (SUNDQVIST, 1994). A test was performed with two types of biofilm, one of monospecies with *E. faecalis* bacteria and mixed biofilm where two types of bacteria were used, *E. faecalis* and *P. aureginosa*.

In this study, intracanal medication was placed in direct contact with the biofilm, which probably does not happen in the root canal system due to its great anatomical variability (MONSARRAT et al., 2016), which has been a limitation of the methodology. Even so, CH is ineffective in eliminating *E. faecalis*, which is linked to cases of endodontic treatment failure due to its resistance to alkaline media, particularly when finding as biofilm (ZANCAN et al., 2016).

In the *E. faecalis* biofilm antimicrobial test, the group that most reduced bacterial cells was the G5 group (HC + 5% metronidazole), but not statistically different from the other associations. In the mixed biofilm antimicrobial test (*E. faecalis* and *P. Aeruginosa*) the G3 group (HC + 5% amoxicillin) reduced the most

viable bacterial cells, followed by the G5 group (HC + 5% metronidazole). The two different groups of control.

Combinations of antibiotics such as tri-antibiotic paste (minocycline + ciprofloxacin + metronidazole) and di-antibiotic (ciprofloxacin + metronidazole) are used in the root canal to cover a broader spectrum of action, showing efficacy against microorganisms associated with endodontic treatment failure, such as *E. faecalis* (STUART et al., 2006; Yang et al., 2013). Taneja & Kumari (2012) concluded that triantibiotic paste can be used as an alternative medication when CH does not eliminate symptoms in persistent infections.

Metronidazole is a nitroimidazole compound, widely used for its broad spectrum and strong antibacterial activity against anaerobic cocci as well as Gram-negative and Gram-positive bacilli. Its action occurs through permeate the bacterial cell membrane, reaching the nucleus and binding to DNA, disrupting its helical structure, causing cell death, but does not show activity against aerobes (ROCHE & YOSHIMORI., 1997), nor with facultative anaerobes (WALTIMO et al., 2003), so the association of this antibiotic with another substance is necessary. Besides, the use of metronidazole has been advocated because of its low induction of bacterial resistance (SLOTS & TING 2002).

The use of antibiotics as an intracanal medication is not recommended because it causes the selection of resistant bacteria (REYNOLDS et al., 2009) and induces tissue cytotoxicity (WINDLEY et al., 2005).

The groups that were associated with anti-inflammatory drugs also made it impossible to statistically different bacteria from the positive control and without statistical differences from the groups associated with antibiotics. Another important clinical factor in the association of anti-inflammatory drugs with CH paste would be a topical action in controlling inflammation, which could reduce the risk of pain in patients following endodontic intervention (MOSKOW et al., 1984; CHANCE et al., 1987; EHRMANN et al., 2003).

Considering that the pastes are in direct contact with the apical and periapical tissues an important factor to be studied is the biocompatibilities of the pastes. The release of nitric oxide is one of the means to evaluate biocompatibility

since the production of this gas molecule is induced by various inflammatory stimuli (KORHONEN et al., 2005) and its release through macrophages, the main cells involved. in the process of apical repair.

The group that most released nitric oxide was G5 (HC + 5% Metronidazole), which was statistically different from the negative control. The other groups also released, and the group of anti-inflammatory drugs that released the most was G4 (HC + 5% Ibuprofen). SILVA et al., 2019 tested the cytotoxicity and biocompatibility of HC associated with amoxicillin, ibuprofen and diclofenac sodium in rat subcutaneous and cell culture, the authors observed that the associations were not cytotoxic and showed biocompatibility.

4 CONCLUSIONS

4 CONCLUSIONS

- All combinations of medications with CH paste did not interfere in the physicochemical properties studied compared to paste without additions;

- In antimicrobial activity, all anti-inflammatory drugs significantly reduced the number of viable bacteria compared to control and pure paste.

- On adhesion, specimens in which the CH paste associated with metronidazole was placed increased bacterial cells adhered within one hour of contact. This group was also the one that maintained the highest pH for up to 15 days compared to the others.

- NO production by stressed macrophages was similar in the anti-inflammatory groups and lower for diclofenac sodium.

REFERENCES

REFERENCES

Azar NG, Heidari M, Bahrami ZS, Shokri F. In vitro cytotoxicity of a new epoxy resin root canal sealer. J Endod 2000; 26:462-5.

Baca P, Junco P, Arias-Moliz MT, González-Rodríguez MP, Ferrer-Luque CM. Residual and antimicrobial activity of final irrigation protocols on Enterococcus faecalis biofilm in dentin. J Endod 2011;37(3):363-6.

Baik JE, Jang KS, Kang SS, et al. Calcium hydroxide inactivates lipoteichoic acid from Enterococcus faecalis through deacylation of the lipid moiety. J Endod 2011; 37:191–196.

Baik JE, Ryu YH, Han JY, Im J, Kum KY, Yun CH, Lee K, Han SH. Lipoteichoicacid partially contributes to the inflammatory responses to Enterococcus faecalis. J Endod 2008;34(8):975-82.

Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol 2015;36:161–178.

Brackett MG, Marshall A, Lockwood PE, Lewis JB, Messer RLW, Bouillaguet S, et al. Inflammatory suppression by endodontic sealers after aging 12 weeks in vitro. J Biomed Mater Res B Appl Biomater 2009; 91:839-44.

Chance et al , 1987; Chance K, Lin L, Shovlin FE, Skribner J. Clinical trial of intracanal corticosteroid in root canal therapy. J Endod 1987;13(9):466-8.

Chávez De Paz LE, Dahlén G, Molander A, Möller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J 2003;36(7):500-8.

Chávez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod 2007;33: 652–662.

Chockattu SJ, Deepak BS, Goud KM. Comparison of anti-bacterial efficiency of ibuprofen, diclofenac, and calcium hydroxide against Enterococcus faecalis in an endodontic model: An in vitro study. J Conserv Dent 2018; 21:80-84.

Consolaro A. Inflamação e reparo: um sílabo para a compreensão clínica e implicações terapêuticas. Maringá: Dental Press; 2009. 352p.

de Freitas RP, Greatti VR, Alcalde MP, et al. Effect of the Association of Nonsteroidal. Anti-inflammatory and Antibiotic Drugs on Antibiofilm Activity and pH of Calcium Hydroxide Pastes. J Endod 2017; 43:131-134.

Delgado RJ, Gasparoto TH, Sipert CR, et al. Antimicrobial effects of calcium hydroxide and chlorhexidine on Enterococcus faecalis. J Endod 2010; 36:1389–93.

Ehrmann EH, Messer HH, Adams GG. The relationship of intracanal medicaments to postoperative pain in endodontics. Int Endod J 2003; 36(12):868-75.

Estrela C, Estrela CR, Hollanda AC, et al. Influence of iodoform on antimicrobial potential of calcium hydroxide. J Appl Oral Sci 2006; 14:33-7.

Estrela C, Holland R. Calcium hydroxide: study based on scientific evidences. J Appl Oral Sci 2003; 11:269-82.

Estrela C, Sydney GB, Bammann LL, Felippe Júnior O. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Braz Dent J 1995;6(2):85-90.

Evans M, Davies JK, Sundqvist G, et al. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2002; 35:221–8.

Fujii R, Saito Y, Tokura Y, Nakagawa KI, Okuda K, Ishihara K. Characterization of bacterial flora in persistence apical periodontitis lesions. Oral Microbiol Immunol 2009; 24:502-5.

Gutierrez JCR. Avaliação de citotoxicidade de materiais obturadores de canais radiculares: influência na liberação de fator de necrose tumoral-á, interferon-ɤ e óxido nítrico em cultura de células murinas. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da Unesp, 2006.

Kayaoglu G, Erten H, Ørstavik D. Growth at high pH increases Enterococcus faecalis adhesion to collagen. Int Endod J 2005; 38(6):389-96.

Kayaoglu G, Ørstavik D. Virulence factors of Enterococcus faecalis:relationship to endodontic disease. Crit Rev Oral Biol Med 2004; 15(5):308-20.

Klotz SA, Rutten MJ, Smith RL, Babcock SR, Cunningham MD. Adherence of Candida albicans to immobilized extracellular matrix proteins is mediated by calcium-dependent surface glycoproteins. Microb Pathog 1993; 14(2):133-47.

Korhonen R, Lahti A, Kankaanranta H, et al. Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy 2005; 4:471–479.

Lima RA, Carvalho CB, Ribeiro TR, et al. Antimicrobial efficacy of chlorhexidine and calcium hydroxide/camphorated paramonochlorophenol on infected primary molars: a split-mouth randomized clinical trial. Quintessence Int 2013; 44:113–22.

Love RM. Enterococcus faecalis--a mechanism for its role in endodontic failure. Int Endod J 2001; 34:399-405.

McHugh CP, Zhang P, Michalek S, et al. pH required to kill Enterococcus faecalis in vitro. J Endod 2004; 30:218–219.

Mizuno M, Banzai Y. Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin. Int Endod J 2008; 41(11):933-8.

Moncada S, Palmer RM, Higgs EA. Nitric oxide, physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109-42.

Monsarrat P, Arcaute B, Peters OA, Maury E, Telmon N, Georgelin-Gurgel M, Maret D. Interrelationships in the Variability of Root Canal Anatomy among the Permanent Teeth: A Full-Mouth Approach by Cone-Beam CT. PLoS One 2016 20;11(10):e0165329.

Moskow A, Morse DR, Krasner P, Furst ML. Intracanal use of a corticosteroid solution as an endodontic anodyne. Oral Surg Oral Med Oral Pathol 1984; 58(5):600-4.

Nakajo K, Komori R, Ishikawa S, Ueno T, et al. Resistance to acidic and alkaline environments in the endodontic pathogen Enterococcus faecalis. Oral Microbiol Immunol. 2006; 21:283-8.

Pinheiro ET, Gomes BP, Ferraz CC, et al. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J. 2003; 36:1-11.

Pinheiro ET, Penas PP, Endo M, Gomes BP, Mayer MP. Capsule locus polymorphism among distinct lineages of Enterococcus faecalis isolated from canals of root-filled teeth with periapical lesions. J Endod 2012; 38:58-61.

Prolo C, Alvarez MN, Radi R. Peroxynitrite, a potent macrophage-derived oxidizing cytotoxin to combat invading pathogens. Biofactors 2014; 40:215-25.

Queiroz CES, Soares JA, Leonardo RT, Carlos IZ, Dinelli W. Evaluation of cytotoxicity of two endodontic cements in a macrophage culture. J Appl Oral Sci 2005; 13:237-42.

Reynolds et al. 2009 Reynolds K, Johnson JD, Cohenca N. Pulp revascularization of necrotic bilateral bicuspids using a modified novel technique to eliminate potential coronal discolouration: a case report. Int Endod J 2009; 42:84–92.

Rôças IN, Siqueira JF Jr. Root canal microbiota of teeth with chronic apical periodontitis. J Clin Microbiol 2008; 46(11):3599-606.

Roche Y, Yoshimori RN. In-vitro activity of spiramycin and metronidazole alone or in combination against clinical isolates from odontogenic abscesses. J Antimicrob Chemother 1997; 40(3):353-7.

Schulz M, von Arx T, Altermatt HJ, Bosshardt D. Histology of periapical lesions obtained during apical surgery. J Endod 2009; 35(5):634-42.

Shimauchi H, Takayama S, Narikawa-kiji M, Shimabukuro Y, Okada H. Production of interleukin-8 and nitric oxide in human periapical lesions. J Endod 2001; 27: 749-52.

Silva GF, Cesário F, Garcia AMR, Weckwerth PH, Duarte MAH, de Oliveira RC, Vivan RR. Effect of association of non-steroidal anti-inflammatory and antibiotic agents with calcium hydroxide pastes on their cytotoxicity and biocompatibility. Clin Oral Investig. 2019; 28.

Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999; 32:361-9.

Siqueira JF Jr, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod 2008; 34(11):1291-1301.e3.

Siren EK, Haapasalo MP, Ranta K, et al. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. Int Endod J 1997; 30:91–5.

Slots J, Ting M. Systemic antibiotics in the treatment of periodontal disease. Periodontol 2002; 28:106-76.

Sousa LR, Cavalcanti BN, Marques MM. Effect of laser phototherapy on the release of TNF-á and MMP-1 by endodontic sealer-stimulated macrophages. Photomed Laser Surg 2009; 27:37-42.

Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surg Oral Med Oral Pathol 1994; 78(4):522-30.

Takeichi O, Saito I, Hayashi M, Tsurumachi T, Saito T. Production of human-inducible nitric oxide synthase in radicular cysts. J Endod. 1998; 24:157-60.

Taneja K, Kumari M. Use of triple antibiotic paste in the treatment of large periradicular lesions. J Investig Clin Dent 2012; 3:72–76.

Tanomaru JM, Leonardo MR, Tanomaru Filho M, et al. Effect of different irrigation solutions and calcium hydroxide on bacterial LPS. Int Endod J 2003; 36: 733–9.

Waltimo TM, Sen BH, Meurman JH, Ørstavik D, Haapasalo MP. Yeasts in apical periodontitis. Crit Rev Oral Biol Med 2003; 14(2):128-37.

Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev 2015; 264:182-203.

Windley W, Teixeira F, Levin L, et al. Disinfection of immature teeth with a triple antibiotic paste. J Endod 2005; 31:439–443.

Yang J, Zhao Y, Qin M, et al. Pulp revascularization of immature dens invaginatus with periapical periodontitis. J Endod. 2013; 39:288–292.

Yoon SS, Hennigan RF, Hilliard GM, et al. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 2002; 3:593–603.

Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB,Duarte MA. Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. J Endod 2016; 42(12):1822-1828.