UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

BRUNO PIAZZA

Evaluation of the antimicrobial activity, quality of removal and influence on the bond strength of calcium hydroxide pastes associated with different substances

Avaliação da atividade antimicrobiana, qualidade da remoção e influência na resistência de união de pastas de hidróxido de cálcio associado a diferentes substâncias

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

Orientador: Prof. Dr. Rodrigo Ricci Vivan

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FOLHA DE APROVAÇÃO

BRUNO PIAZZA

DADOS CURRÍCULARES

17/07/1991	**Nascimento** **Botucatu – SP**
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“Se queres acordar toda a humanidade, então acorda-te a ti mesmo, se queres eliminar o sofrimento do mundo então elimina a escuridão e o negativismo em ti próprio.
Na verdade, a maior dadiva que podes dar ao mundo é aquela da tua própria autotransformação.”

Lao Tsé

RESUMO

Avaliação da atividade antimicrobiana, qualidade da remoção e influência na resistência de união de pastas de hidróxido de cálcio associado a diferentes substâncias

O hidróxido de cálcio é comumente utilizado usado em endodontia devido a suas propriedades físico-químicas. Entretanto novas associações à pasta de hidróxido de cálcio são propostas devido à modificação e resistência bacteriana adquirida. O presente trabalho tem como objetivos avaliar a associação da pasta de hidróxido de cálcio aos medicamentos: diclofenaco sódico, ibuprofeno e cloridrato ciprofloxacino avaliando a remoção destas medicações das paredes dentinárias, interferência na adesividade do cimento endodôntico e a difusão via túbulos dentinários sobre biofilme bacteriano extra radicular. O ensaio para a remoção das pastas utilizou 80 dentes bovinos posteriormente clivados no sentido longitudinal (Coroa-ápice) e remontados em mufla para a inserção das pastas. A remoção foi realizada sete dias após a inserção das medicações com auxílio de um ultrassom acoplado a um inserto Irrissonic além da remoção convencional através da combinação de instrumento de memória, seringa e cânula. As análises foram realizadas em microscopia eletrônica de varredura (MEV), aplicando scores para a avaliação da limpeza. Durante o ensaio mecânico de *push-out* 50 dentes bovinos foram divididos em grupos (n=10): Controle negativo (apenas instrumentado), controle positivo (hidróxido de cálcio + propilenoglicol), diclofenaco + hidróxido de cálcio + propilenoglicol, ibuprofeno + hidróxido de cálcio + propilenoglicol, cloridrato de ciprofloxacino + hidróxido de cálcio + propilenoglicol, após o preenchimento dos canais com as pastas os dentes foram armazenados por 7 dias até o momento da obturação. Os dentes então foram novamente armazenados por 7dias para a presa total do cimento e seccionados a 2 mm, 4 mm e 6 mm do forame e submetidos ao teste *push-out*. As falhas foram avaliadas através de microscopia eletrônica de varredura (MEV) e classificadas quanto ao seu tipo (adesiva, coesiva e mista). A realização do teste antimicrobiano foi realizada utilizando 26 dentes bovinos preparados e retirados dois blocos de dentina localizados nas faces mesial e distal de cada dente para crescimento e cultivo de biofilme de Enteroccocus faecalis. Após cultivo e amadurecimento os blocos foram reposicionados e subdivididos em dois grupos contendo 13 dentes e 26 blocos de dentina. Apenas um grupo receberá

ativação ultrassônica após inserção das respectivas pastas e ambos os grupos armazenados em estufa úmida a 36º graus Celsius durante 7 dias. As análises dos blocos de dentina foram realizadas com auxílio de um microscópio confocal de varredura a laser e corante Live and Dead para a obtenção de imagens das células bacterianas. Após o término dos ensaios, os dados foram tabulados e submetidos ao teste de D'agostino e Pearson para verificação da normalidade a qual se encontrou ausente e assim aplicados os testes de Kruskal-Wallis e para comparação múltipla o teste de Dunn.

Palavras chave: hidróxido de cálcio, ultrassom; push-out; microbiologia

ABSTRACT

Evaluation of the antimicrobial activity, quality of removal and influence on the bond strength of calcium hydroxide pastes associated with different substances

Calcium hydroxide is commonly used in endodontics because of its physic-chemical properties. However new associations to the calcium hydroxide paste are proposed due to the bacterial modification and resistance acquired. The aim of the present study was to evaluate the association of calcium hydroxide paste with diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride, evaluating the removal of these medications from dentin walls, interference with adhesion of endodontic cement and diffusion through dentinal tubules on bacterial biofilms extra radicular. The paste removal test used 80 bovine teeth later cleaved in the longitudinal direction (Crown-apex) and remounted in muffle for the insertion of the pastes. The removal was performed seven days after the insertion of the medications with the aid of an ultrasound coupled to an Irrissonic insert in addition to the conventional removal through the combination of memory instrument, syringe and cannula. The analyzes were performed in scanning electron microscopy (SEM), applying scores for cleaning evaluation. During the mechanical push-out test 50 bovine teeth were divided into groups (n = 10): Negative control (instrumented only), positive control (calcium hydroxide + propylene glycol), diclofenac + calcium hydroxide + propylene glycol, ibuprofen + calcium + propylene glycol, ciprofloxacin hydrochloride + calcium hydroxide + propylene glycol, after filling the channels with the pastes the teeth were stored for 7 days until the time of filling. The teeth were then stored again for 7 days for the total cement prey and sectioned at 2 mm, 4 mm and 6 mm from the foramen and subjected to the push-out test. The failures were evaluated by scanning electron microscopy (SEM) and classified according to their type (adhesive, cohesive and mixed). The antimicrobial test was performed using 26 prepared bovine teeth and removed two blocks of dentin located on the mesial and distal surfaces of each tooth for growth and biofilm culture of Enterococcus faecalis. After cultivation and maturation, the blocks were repositioned and subdivided into two groups containing 13 teeth and 26 blocks of dentin, where only one group received ultrasonic activation after insertion of the pulps and both groups stored in a humid oven at 36ºC for 7 days. The dentin blocks were analyzed using a confocal laser scanning microscope

and Live and Dead dye to obtain images of the bacterial cells. After completion of the tests, the data were tabulated and submitted to the D'agostino and Pearson test to verify normality, which was absent and thus the Kruskal-Wallis tests were applied and for a multiple comparison the Dunn test.

Keywords: calcium hydroxide, ultrasound; push-out; microbiology

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LIST OF ABREVIATIONS AND ACRONYMS

%	percentage
<	less than
>	greater than
#	tip diameter
º	degree
C	Celsius
nm	nanometers
mm	millimeter
ml	milliliter
n	number
NiTi	Nickel-Titanium
P	statistical significance
$Ca\ (OH)_2$	calcium Hydroxide
Ca_2	calcium
OH	Hydroxyl
NSAIDs	non-steroidal substances anti-inflammatory drugs
SG	sub group
pH	hydrogen potential
DNA	deoxyribonucleic acid.
ATCC	American type culture collection
BHI	Brain heart infusion
CFU	colony forming units
PUI	passive ultrasonic irrigation
SEM	scanning electron microscopy
EDTA	ethylenediaminetetraacetic acid
K	Kerr
NaOCl	Sodium hypochlorite
Mpa	Mega pascal

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

The aim of the biomechanical preparation is the total elimination of the microorganisms present in the root canal system (TAKAHASHI K, 1998; PETERS OA et al., 2001). However, this preparation exclusively promotes a small reduction of the microbiota due to the difficulty of cleaning in areas such as isthmus and anatomical complexities of the root canal system, besides the particularities of the different microbial groups (PAQUÉ F, GANAHL D, PETERS OA., 2009). Therefore, the indication of intracanal drug therapy becomes relevant for the elimination of bacteria still present, which promotes a significant increase in treatment success (TANOMARO FILHO M, LEONARDO MR, da SILVA LA, 2002).

There are several intracanal medications used in endodontics, among them Ca $(OH)_2$ (Calcium hydroxide) and its associations with different vehicles. Calcium hydroxide is characterized by being a white powder with alkaline pH around 12.8. Its obtainment is given by calcination of calcium carbonate and transformation into calcium oxide. After its hydration, we have as final product the formation of calcium hydroxide (ESTRELA C et al., 1995).

The calcium hydroxide has high power and wide spectrum against several species present in the endodontic microbiota (ATHANASSIADIS B, ABBOTT PV, WALSH LJ, 2007; SIQUEIRA, MAGALHAES, ROÇAS, 2007). Due to its presentation in powder, it becomes essential to use another substance that in association facilitates its insertion and promotes the release of hydroxyl and calcium ions, as well as to improve its microbial properties (SIQUEIRA & LOPES, 1999; LIMA et al. 2013). Dissociation into calcium and hydroxyl ions becomes indispensable in cases of bacterial pulp necrosis, where the action and presence of these highly oxidizing free radicals produce effective results. (ESTRELA C et al., 1998; SIQUEIRA & LOPES, 1999). Dissociation into calcium and hydroxyl ions becomes indispensable in cases of bacterial pulp necrosis, where the action and presence of these highly oxidizing free radicals produce effective results. (ESTRELA et al., 1998; SIQUEIRA & LOPES, 1999). The efficacy of Ca (OH) 2 is directly related to its highly elevated pH (12,8), the release of hydroxyl ions can alter the integrity of the cytoplasmic membrane through chemical injury to organic components and transport of nutrients and phospholipids or unsaturated fatty acids in the cytoplasmic membrane of bacterial components (ESTRELA et al., 1995).

Calcium ions in turn exhibit the ability to actively participate in osteocemental formation and mineralization in dentin tubules and other involved areas where mineralization occur (HOLLAND R et al., 1978; HOLLAND R et al., 1982; WAKABAYASHI H et al., 1995).

However, the use of calcium hydroxide medication has certain disadvantages, such as direct contact action, which makes it detrimental to microorganisms that have great capacity to enter and deepen in the dentinal tubules (SIQUEIRA & LOPEZ, 1999), as well as the long time required for its performance (SJOGREN U et al., 1997). Another relatively important factor to be observed is the resistance of the microorganisms to this medication, besides the characteristic of the pathogens and their virulence in transmitting the acquired resistance to future generations or between species (TROPE M, DELANO EO, ORSTAVIK D.,1999; ESTRELA et al., 1999; SIQUEIRA, MAGALHAES, ROÇAS, 2007).

Some studies have demonstrated the resistance of *Enterococcus faecalis* to calcium hydroxide. TROPE M, DELANO EO, ORSTAVIK D, (1999), ESTRELA et al., (1999) and SIQUEIRA JF JR, MAGALHÃES KM, RÔÇAS IN, (2007) reported finding resistance of the pathogen at high pH levels resulting from the release of hydroxyl ions in aqueous medium. The resistance reported in this literature to $Ca(OH)_2$ can be explained by the presence of a proton pump present in its cytoplasm (EVANS et al., 2002). This defense mechanism acts when negatively charged hydroxyl ions enter the bacterial cytoplasm by raising its pH, so the proton pump is activated, and it directs positively charged potassium molecules into the cell acidifying its cytoplasm and preventing the action and occurrence of enzymatic inhibition (EVANS et al., 2002; ESTRELA, et al., 1995). SUKAWAT & SRISUAN (2002) verified the resistance of *Enterococcus faecalis* to three formulations of calcium hydroxide pastes. Their results demonstrated ineffectiveness in eliminating the pathogen when the powder was mixed with sodium chloride or chlorhexidine. LIN YH et al., (2003) analyzed associations of other substances with calcium hydroxide and chlorhexidine and could conclude that this powder only associated with the vehicle without other substances was not effective in the elimination of E*nterococcus faecalis.*

Another relevant factor in this species is its virulence factors. *Enterococcus. faecalis* produces cytolysins with activity on human, sheep and horse erythrocytes, aggregation substances responsible for the agglutination of microorganisms and facilitating the exchange between plasmids. E. faecalis strains still produce pheromones, peptides capable of amplifying the transfer of plasmid DNA by strains in a conjugative process promoting increased resistance in the bacterial line and by amplifying the inflammatory response during the infectious process (KAYAOGLU G, ØRSTAVIK D, 2004). Another important component is lipoteichoic acid, besides the adhesin, important factors of virulence inducers of tumor necrosis factor (TNF), aggressively modulating the immune response in persistent lesions (KAYAOGLU G, ØRSTAVIK D, 2004).

These previously reported factors increase resistance to medications used during treatment beyond painful symptomatology to patients. This is one of the few microorganisms that has shown in vitro to resist the antibacterial effect of $Ca(OH)_2$ (WEIGER R et al., 1995, EVANS et al., 2002).

Due to the resistance presented by this microorganism and its presence in persistent lesions, the literature emphasizes the association of other substances to improve the effectiveness of calcium hydroxide, as proposed by DOTTO et al., 2006, MANZUR A et al., 2007 and MOHAMADI Z & ABBOT PV in 2009.

MIDENA RZ et al. (2015) Verified the association of *Casearia sylvestris* and chlorhexidine in association with calcium hydroxide pastes in the tissue inflammatory response in subcutaneous rats and antimicrobial tests with *Enterococcus faecalis*. It was possible to conclude that both associations were effective in reducing microorganisms, but chlorhexidine had a greater inflammatory response in the subcutaneous tests.

VALERA et al., (2016) verified the association of calcium hydroxide with chlorhexidine and zinziber, acting on *Candida albicans*, *Enterococcus faecalis* and Escherichia coli and their toxins. It was verified that the association of these substances with calcium hydroxide effectively reduced the microorganisms, but it was not possible to eliminate them completely and their toxins.

Some associations have shown promise in eliminating the pathogens found in endodontic treatment. The work done by de FREITAS et al. (2017) associated to calcium hydroxide in different pastes substances such as diclofenac sodium, ibuprofen and hydrochloride ciprofloxacin, in order to verify its pH and antimicrobial activity. It can be concluded that the associations did not interfere in the pH of the pastes and the 3 associations showed a significant difference in relation to the pure paste.

In addition to the associations with calcium hydroxide to potentiate its effect, devices can be used to improve its action. Since calcium hydroxide acts through direct contact, the use of ultrasonic equipment for agitation of the pastes is recommended. The use of ultrasound when used for irrigation solutions promotes a great deal of agitation, promoting the contact of the irrigating solution in areas of anatomical difficulties and dentinal tubules resulting in a greater cleaning (WISEMAN et al., 2011).

Similarly, ultrasonic agitation may favor the penetration of toothpaste into dentinal tubules and areas of anatomical complexity. Studies have been carried out to confirm this theory, including that of DUARTE MAH et al. (2012), who performed agitation of the calcium hydroxide paste on teeth with simulated external resorptions, verifying a higher presence of hydroxyl ions in the groups which were agitated with ultrasound, demonstrating the permeability of the paste through the dentinal tubules when shaken with ultrasound.

In addition to the study by Duarte, the study by ARIAS MP et al. (2016) verified the effect of ultrasonic agitation on penetration and disinfection in teeth infected with E. faecalis. The results showed better penetration of the paste with ultrasonic agitation, besides the greater antimicrobial activity of this drug.

Without doubt, the use of the calcium hydroxide paste is essential for successful endodontic treatment. However, after its use, the residues from the calcium hydroxide paste inside the root canal can cause interference in the penetration of the endodontic sealer in the dentinal tubules (CALT S & SERPER A, 1999) and reduction of the adhesion of the sealing materials (ERDEMIR et al., 2004) and also the possibility of reacting with the cement used and interfering with its physic-chemical properties (HOSOYA N et al., 2004).

In 2008, BARBIZAM JV et al. Verified the adhesiveness of Ephiphany sealer after filling the canal with calcium hydroxide slurries. It can be concluded after the push-out test that all the slurries used decreased cement tack, even if the adhesion values were acceptable.

Amin SA, Seyam RS and El-Samman MA, in 2012 evaluated the adhesion of the iRoot SP, MTA Fillapex and AH plus sealer after filling the channel with calcium hydroxide paste. It can be concluded after the push out test and statistical analysis that the presence of calcium hydroxide affected the adhesiveness of iRoot SP and MTA Fillapex cements, not interfering with the adhesiveness of AH Plus.

GUIOTTI et al., (2014), verified the adhesiveness of MTA fillapex, Sealapex and AH plus sealer after the presence of intracanal medication with calcium hydroxide. It can be concluded that the presence of the calcium hydroxide in the walls of the canal decreased the adhesiveness of all the sealers.

Several papers in the literature show that the use of ultrasound can promote better removal of $Ca(OH)_2$ residues present in the root canal. YÜCEL et al. (2013) compared new irrigation systems over conventional methods in the removal of calcium hydroxide pastes. It can be verified that the ultrasonic agitation presented superior results in relation to the other methods.

ÇAPAR IM et al. (2014) verified the removal of the calcium hydroxide paste with different irrigation methods and concluded that the ultrasonic agitation presented better results than the other groups.

ZORZIN J et al., (2017) evaluated the amount of calcium hydroxide removed using different volumes of irrigating solution in addition to activation methods. It can be concluded that none of the methods completely removed, but the ultrasonic agitation was more effective than the other methods.

The evidences demonstrated before the association of several substances with the $Ca(OH)_2$ as well as auxiliary methods for the diffusion of the medications through the dentin structure, so their removal are essential for high level endodontics. The lack of data regarding the efficacy of novel $Ca(OH)_2$ pastes associated with diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride and the application of ultrasonic agitation are a gap within the literature. In view of these doubts, the execution of the present study becomes relevant.

2 ARTICLES

Article 1 According to the Dental Press Endodontics guide lines

Evaluation of the antimicrobial action on extra-root biofilms using calcium hydroxide associated with different drugs. In Vitro Study.

Abstract

Introduction: The objective of the present study was to evaluate the in vitro antibiofilm activity of calcium hydroxide associated with different non-steroidal substances anti-inflammatory drugs (NSAIDs) and antibiotics in extra-root biofilms. **Methods:** Twenty-nine teeth were prepared in a special way to accommodate Enterococcus faecalis biofilms extra-root and receive treatment with their respective pastes. The groups analyzed were as follows (n = 24 blocks). Group 1: Ultrasonic activation and Group 2: no ultrasonic activation. Then the groups were again divided into 4 subgroups containing 3 teeth and 6 dentin blocks per medication: SG1: Calcium hydroxide + propylene glycol (control), SG2: Calcium hydroxide + propylene glycol + ibuprofen, SG3: Calcium hydroxide + propylene glycol + diclofenac sodium and SG4: Calcium hydroxide + propylene glycol + ciprofloxacin hydrochloride. After 7 days all specimens were examined under confocal laser scanning microscopy at 40x magnification to verify the efficacy of the drugs in the elimination of the extra-root biofilms. The images were analyzed by a Las X and the values obtained were tabulated for later statistical analysis. Data were subjected to statistical analysis at a significance level of 5%. **Results:** The results regarding the percentage of live cells are shown a lower percentage of viable cells with the use of the conventional calcium hydroxide paste, followed by the paste containing ibuprofen, cipro and diclofenac respectively. The group in which the medications were agitated showed lower values in the microbial elimination. However, none of the pastes among them besides the presence or absence of ultrasonic agitation was statistically significant. **Conclusion:** The combination of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride have a low antimicrobial activity in relation to extra-root biofilms, and the association of these drugs with ultrasonic agitation suggests that the drug used is limited and limit its action potential.

Key words: intracanal medication, calcium hydroxide, ibuprofen, diclofenac, ciprofloxacin hydrochloride

Introduction

The biomechanical preparation reduces the microbial component significantly, however the presence of anatomical amphractuosities does not allow effective elimination of the microorganisms, providing adequate antisepsis (*Byström & A,Sundquist* G., 1981; El Karim I. et al. 2007). To obtain the expected success, the use of different methods and medications between the sessions are indicated to complement the residual microbial elimination after the chemical-mechanical preparation (Bystrom A. et al. 1985; Siqueira JF Jr. et al. 2007).

Calcium Hydroxide (powder) is certainly the most commonly used intracanal medication due to its biological effects as well as its antimicrobial properties (Desai S & Chandler N. 2009). The Ca $(OH)_2$ powder in a carrier has been widely used for this purpose. As a slow-acting antiseptic, its antimicrobial and biological actions occur by ionic dissociation of Ca_2 + and OH ions. The dissociation of these particles allows the diffusion of hydroxyl and calcium ions from the walls of the root canal through the dentinal tubules and areas of anatomical difficulty (Gomes BP et al. 2002; Estrela C et al. 1998; Estrela et al. 1999).

The biological and antimicrobial effects are directly related to alkalinity as well as the release of calcium ions (Estrela C et al., 1998). The high alkalinity related to pH increase leads to enzymatic inversion reactions promoting bacterial inhibition (Siqueira JF & Lopes HP. 1999). The action of ions after dissociation will act on tissue mineralization (Mizuno M & Banzai Y. 1999), this occurs due to the binding to carbon dioxide used as a bacterial substrate for cellular respiration being transformed into calcium carbonate as a major factor in the mineralization, besides the expression of factors of the fibronectin gene (Seux et al. 1991; Estrela et al. 1998). Another important capacity of calcium hydroxide is the inactivation of Lipopolysaccharides, a highly aggressive virulence factor presents in the outer membrane of gram-negative bacteria and an important role in the development of the disease (Tanomaru JM. et al. 2003).

However, the presence of persistent infections may occur due to microorganisms remaining in endodontic procedures, as well as the presence of extra-radicular infections (Siqueira JF & Roças IN. 2008; Tronstad L. et al. 1990). Some factors may increase microbial resistance, increasing pH to the bacterial environment may induce genetic modifications with changes in bacterial cell characteristics. In addition, the formation of biofilms drastically increases the resistance of microorganisms to the use and application of drugs (Siqueira JF, Roças IN., 2008).

Several bacterial species are involved in endodontic failure, among them the enterococcus faecalis commonly found in persistent infections (Pinheiro ET. et al. 2003; Nakajo K. et al. 2006; Ferreira FB. et al. 2007), this microorganism demonstrates resistance to intracanal medication therapy using calcium hydroxide (Dastidar SG. et al. 2000, Nakajo K. et al. 2006; Ferreira FB. et al. 2007). For this reason, the use of other antiseptic agents in association with calcium hydroxide were proposed to improve the action spectrum in the elimination of the microorganisms present intra and extra radicular

Different studies propose the addition of other substances to (AINES and Antibiotics) calcium hydroxide to improve its activity antimicrobial action (Delgado RJ. et al. 2010; Lima RA. et al. 2013). Anti-inflammatory drugs have been shown to be a potential drug associated with calcium hydroxide exhibiting excellent antimicrobial elimination (Dastidar SG. et al. 2000). Highly bactericidal action against gram positive microorganisms and gram-negative bacteria, inhibiting bacterial DNA synthesis was found when the use of diclofenac sodium was employed (Dastidar SG. et al., 2000).

Studies by Salem-Milani A. et al. (2013) also demonstrate that the antimicrobial activity exhibited in radial diffusion studies also demonstrate excellent results when used diclofenac sodium, ibuprofen, amoxicillin and gentamicin against E. faecalis ATCC 29212 (American Type Culture Collection, Rockville, MD). When compared to the results exhibited by the calcium hydroxide paste, a higher bacterial elimination was observed with the use of NSAIDs and the use of antibiotics. de Freitas et al. 2017 carried out the association of medicinal products with calcium hydroxide such as diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride and obtained excellent results in association with the medicines used, not interfering in the pH of the pastes as well as effective microbial elimination.

The calcium hydroxide pastes associated with diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride were only tested in direct contact with the microorganisms. However, the evaluation of the elimination of microorganisms at a distance, as well as the presence of extra-radicular biofilms eliminated by the dissociation of the pastes used through the dentin tubules has not yet been evaluated. This work aims to verify the elimination of simulated extra - apical biofilm through the application of intracanal calcium hydroxide pastes and their dissociation through the dentinal tubules. The null hypothesis tested was, the addition of NSAIDs and ciprofloxacin would eliminate the enterococcus faecalis extra root antibiofilm differently from the conventional paste of calcium hydroxide.

Material and methods

Preparation of Bovine Dentin Specimens

Twenty-nine teeth were prepared in a special way to accommodate Enterococcus faecalis biofilms. Dentin tubes were made approximately 10 mm long. The most apical portion was eventually sealed with epoxy resin to prevent intracanal medication from escaping. After this step two sections were performed on the buccal and lingual surfaces, removing two blocks of dentin from each specimen. The blocks were removed from the buccal and lingual surfaces, measuring 5 mm in length by 1.5 mm in thickness, all blocks were polished in a polishing machine to allow the tubules to be exposed and to plan the convexity area of the buccal and lingual surfaces in order to facilitate the reading in confocal microscopy of laser scanning (Figure 1). The specimen cleaning protocol was the same as that adopted by Freitas et al., (2017) in which it consisted, and the dentin segments were treated with 1% sodium hypochlorite for 30 minutes and 17% EDTA for 5 minutes to remove organic waste and possible presence of the smear layer. To verify the cleaning method, 3 blocks were observed by scanning electron microscopy. The blocks were sterilized in autoclave at 121ºC.

Contamination of the Dentin

For induction of the biofilm on the dentin blocks, the methodology recommended by Guerreiro-Tanomaru et al. 2013 was adopted. A standard strain of *Enterococcus. faecalis* ATCC (American Type Culture Collection, Manassas, VA) 4083 was used for biofilm formation. After confirmation of strain purity by Gram staining and colony morphology, and biochemical identification, microorganism was reactivated in 4 ml of sterile BHI broth and kept in an oven at 37 ° C 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. The cell density was 1.5 x 108 colony forming units per ml (CFU / ml). In two 24-well cell culture plates were placed the 52 dentin blocks with one of the surfaces marked with pencil and facing down. The blocks were then submerged with 3.6 ml of sterile BHI broth added with 0.4 ml of the standardized bacterial inoculum. The plates were placed in a bacteriological oven at 37 ° C for 21 days. For there to be no nutrient deficiency for the bacterial cells, the BHI culture medium of each specimen was totally exchanged every 48 hours without the addition of new microorganisms.

At the end of the biofilm formation period, all blocks were removed from culture broths, rinsed 3 times with sterile buffered saline to remove planktonic cells, aspirated dried with a sterile needle and repositioned again on the buccal and lingual surfaces where were removed with the biofilm area facing the root canal walls and stabilized with Top Dam (FGM produtos odontológicos Ltda, Joinville, Brazil) and polymerized for 20 seconds for the implementation of intracanal medication insertion protocols.

Antibiofilm Activity Test

After replacement of the blocks, the teeth were divided into 2 groups (n = 24 blocks). Group 1: Ultrasonic activation and Group 2: no ultrasonic activation. Then the groups were again divided into 4 subgroups containing 3 teeth and 6 dentin blocks per medication: SG1: Calcium hydroxide + propylene glycol (control), SG2: Calcium hydroxide + propylene glycol + ibuprofen, SG3: Calcium hydroxide + propylene glycol + diclofenac sodium and SG4: Calcium hydroxide + propylene glycol + ciprofloxacin hydrochloride. All the slides were inserted with the help of a gentle spiral. After the filling of the channels with the folders of their respective groups, the first group with an Irrisonic insert (Helse dental tecnology, Brazil, São Simão) was coupled to an EMS model P100 (SEM Switzerland, Nyon) in the power of 20%. The agitation was carried out in the mesio-distal direction so that the paste could penetrate and diffuse through dentinal tubules reaching the target site on the face of the dentin block, located the fixed biofilm. The specimens will then be stored in a humid oven at 37 ° C for seven days.

Confocal Microscopic Analysis

The specimens were placed into Petri dishes and stained with 50 ml Live/Dead BacLight Bacterial Viability L7012 solution (Molecular Probes,Inc, Eugene, OR) trickled over the dentin. After the application of dyes, the Petri dishes were closed and wrapped in tinfoil to allow dye diffusion into the specimens, in the absence of light, at a temperature of 37ºC for 20 minutes according to the manufacturer’s guidelines. To prepare thedye Live/Dead BacLight, 1.5 ml component A and 1.5 ml component B were added to 0.97 ml 0.85% saline solution. The marker colored the viable cells in green and the cells with membrane damage in red.

All specimens were examined under confocal laser scanning microscopy (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany) at 40xmagnification. Six specimens were photographed in each group, and 3 images were taken per specimen, totaling 18 pictures per group. The images were analyzed by a Las X (Leica TCS-SPE; Leica Microsystems

GmbH, Mannheim, Germany) and the values obtained were tabulated for later statistical analysis.

Statistical analysis

After obtaining the data and tabulation, the data will be submitted to statistical analysis, and the D'agostino and Pearson test will be applied to verify normality. In case of normality, the values will be submitted to the ANOVA test followed by the Tukey test. If normality is not found, the Kruskal-Wallis tests will be applied and for a multiple comparison the Dunn test.

Results

The results regarding the percentage of live cells are shown in table 1. The results showed a lower percentage of viable cells with the use of the conventional calcium hydroxide paste, followed by the paste containing ibuprofen, cipro and diclofenac respectively. The group in which the medications were agitated showed lower values in the microbial elimination. However, none of the pastes among them besides the presence or absence of ultrasonic agitation was statistically significant.

Discussion

The null hypothesis for this work was not accepted. It was expected that the addition of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride to calcium hydroxide would also promote significant bacterial elimination as well as direct contact. However, the microbial elimination in extra-root biofilms was discrete.

The methodology used to test the antimicrobial action of pastes and substances based on calcium hydroxide is the same and grounded within the scientific literature using confocal laser scanning microscopy and live / dead dye aid for cell labeling (Ordinola – Zapata et al. 2012. However, the methodology used for the simulation of extra-radicular microorganisms is absent in the literature, even in in vitro articles are not found or methodologies like is used. This further justifies the necessity and accomplishment of studies with this objective in the search of drugs that can eliminate them without the need of periapical surgeries and fill in the gap within the literature.

Several reports of failure during endodontic therapy are related to the absence of elimination of enterococcus faecalis (Sundqvist G et al. 1998; Hancock HH 3rd et al. 2001; Rôças IN et al. 2004. This microorganism presents a high resistance threshold against several drugs used during the intracanal medication step. Calcium hydroxide is undoubtedly the most used intracanal medication during endodontic treatment, however this microorganism presents high resistance to this drug due to the presence of a proton pump present in its cytoplasm (Evans M, et al., 2002). Another factor that is relevant to the failure of endodontic therapy is the ability of this microorganism to penetrate deeply into dentinal tubules (Chivatxaranukul P et al. 2008; Ran S et al. 2015), as well as present in cases of extra root biofilms demonstrated by Sunde PT et al. 2002.

In view of this problem, medicinal associations with calcium hydroxide are proposed. de Freitas et al., 2017 performed an association of NSAIDs and antibiotic to calcium hydroxide demonstrating no changes in the pH of the pastes as well as an increase in the elimination efficiency of enterococcus faecalis. However, the diffusion of the ions through the dentin and the possible elimination of microorganisms present on the extra radicular surface were not tested.

The results found after conducting this research showed inefficiency of all pastes in the elimination of the microorganisms present in the simulated extra radicular surface. This result suggests that the extension of the dentinal tubules as well as the presence of inorganic apatite hydroxide and other components present in the dentin may be acting on the neutralization of acids and bases (Wang JD, Hume WR, 1998) formed with the combination of calcium, NSAIDs and antibiotics decreasing their microbial potential

However, despite the absence of a significant statistical difference, higher elimination values of microbial agents could be observed when ultrasonic agitation was not used. Studies in the literature claim that ultrasonic action on the drug may cause degradation of the active molecule in question with loss of therapeutic activity as well as undesirable effects caused by the formation of new compounds generated as a result of degradation (Riesz and Kondo, 1992).

Furthermore, the combination of the calcium hydroxide associated with the vehicle together with the physic-chemical effects of the ultrasound promotes an increase in the release of hydroxyl ions (Duarte et al, 2007). However, it is a combination based on the results obtained, suggesting diminishing the antimicrobial effects of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride associated with calcium hydroxide. Studies have reported that the increase of pH caused by the increase of hydroxyl ions promotes the degradation of these active principles, rendering them ineffective (Ballesteros et al., 2003, Jeong et al., 2010a; Luo et al., 2012).

Because it is a first experiment, in addition to the limitations of the present study, more research should be done to better understand and understand the action and association of these drugs associated with calcium hydroxide.

Conclusion

The authors were able to conclude that the combination of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride have a low antimicrobial activity in relation to extra radicular biofilms, and the association of these drugs with ultrasonic agitation suggests that the drug used is limited and limit its action potential.

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Table 1 - Percentage of live cells

	CH	CH+IBU	CH+DIC	CH+CIPRO	Control (no paste)
with/U	26,45(21,96-38,66)^{Aa}	42,03(8,062-51,62)^{Aa}	55,74(23,20-62,94)^{Aa}	53,28(22,16-67,58)^{Aa}	53,92(48,36-77,00)^{Aa}
without/U	24,19(21,30-30,58)^{Aa}	43,44(6,717-45,21)^{Aa}	31,02(14,07-55,09)^{Aa}	22,19(9,043-88,16)^{Aa}	

Different Lowercase identifiers show statistical difference at the same line in relation to associated medication ($p<0,05$). Different uppercase at the same column show difference about presence or absence of ultrasound.

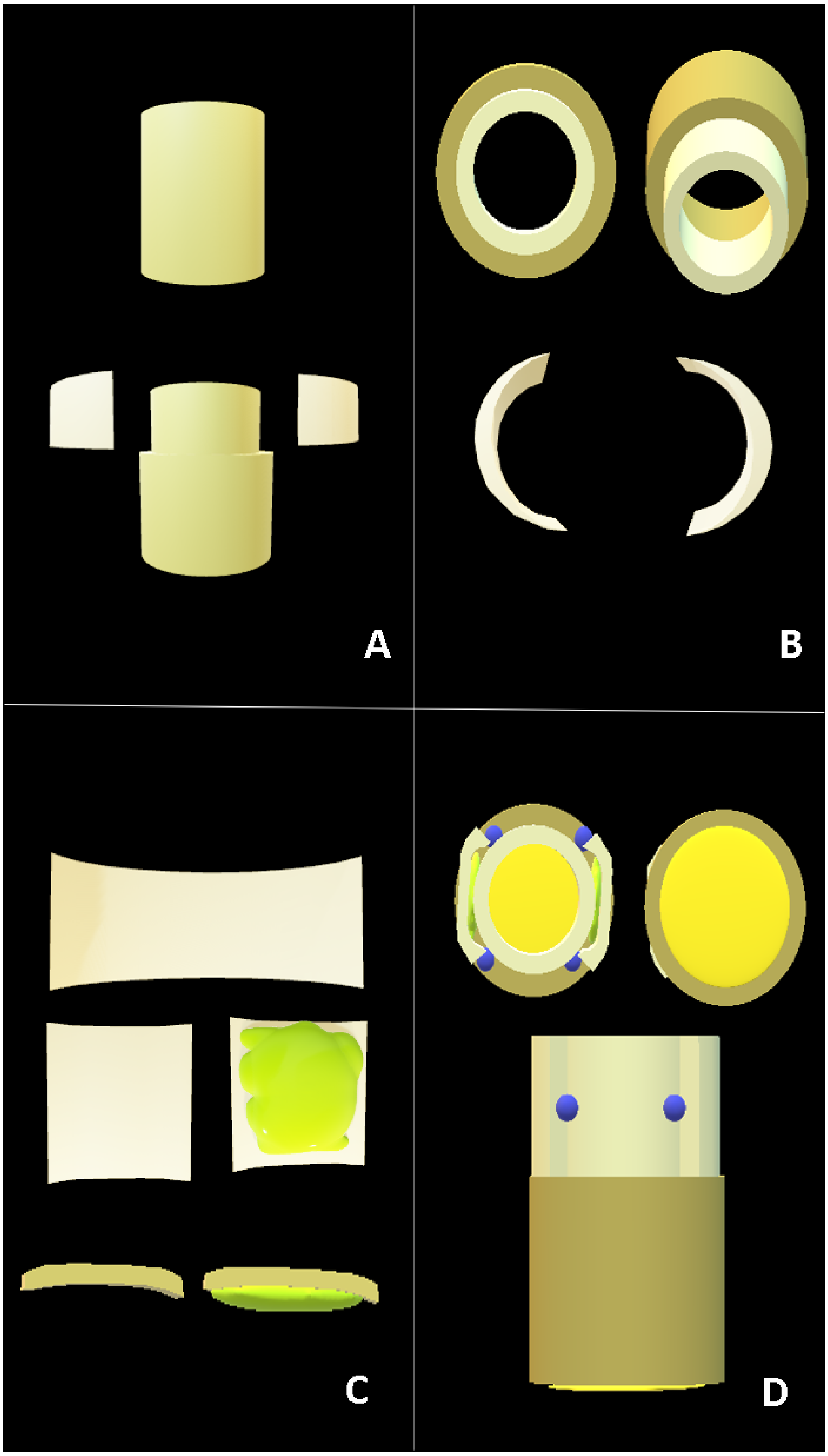

Figure 1: Three-dimensional model demonstrating methodology of simulation of extra-root biofilms. Three-dimensional model demonstrating methodology of simulation of extra-root biofilms. In A we can visualize the side face of a dentin tube obtained from the cervical portion of a bovine tooth before and after obtaining the dentin blocks removed on the mesial and distal surfaces. In B we can visualize an image of the more cervical portion, being possible to also visualize the entrance of root canal besides the blocks removed from the proximal faces. In C, we can visualize the dentin blocks after removal of the proximal surfaces and after polishing and contamination with bacterial biofilms. In D we can visualize the reassembly of the dentin tube and the sealing of the more apical portion with araldite

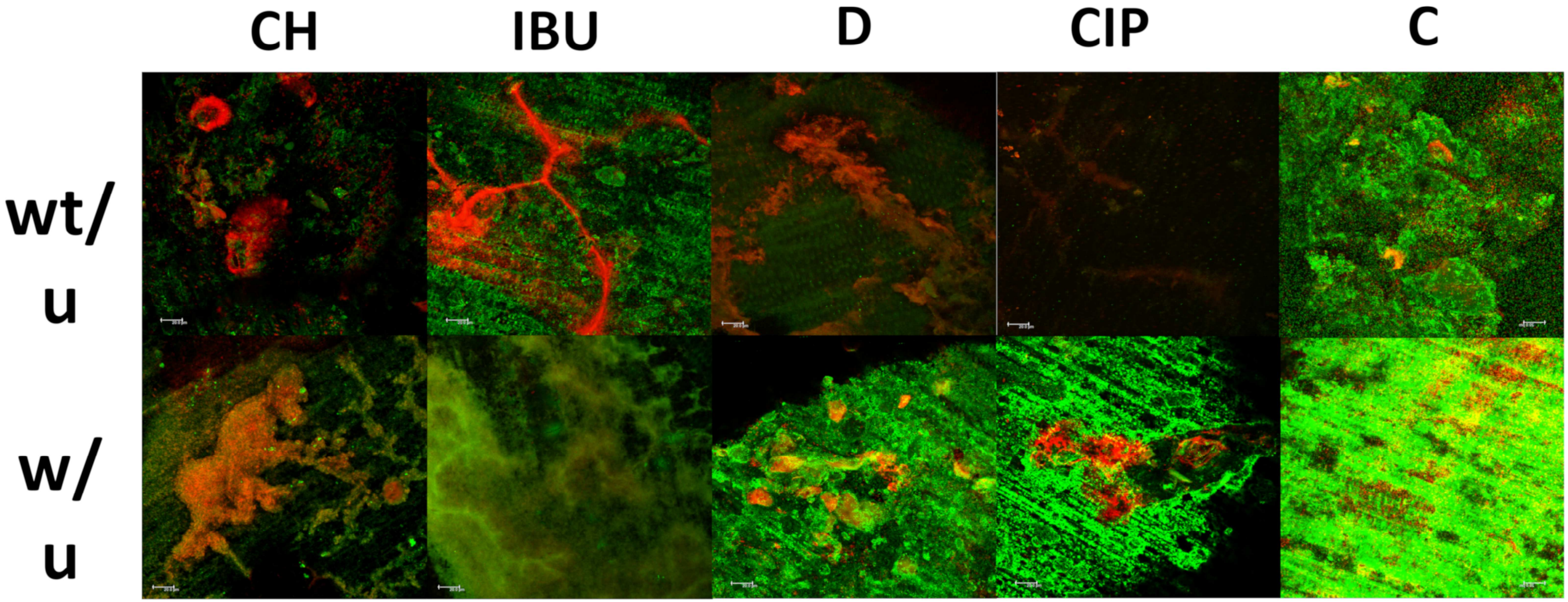

Figure 2. Confocal laser scanning microscopy of extra root biofilms treated with (A- B) calcium hydroxide + polyethylene glycol, (C-D) calcium hydroxide + polyethylene glycol+ Ibuprofen, (E-F) calcium hydroxide + polyethylene glycol + Sodium diclofenac, and (G-H) calcium hydroxide + propylene glycol + ciprofloxacin hydrochloride (I-J) the control group. Figures in column W/U (with ultrasonic) receive ultrasonic agitation, figures in WT/U (without ultrasonic agitation) no receive ultrasonic agitation treatment. Live cells are indicated in green, and dead cells are indicated in red. Each Picture represents an area of 275 x275 mm.

Article 2 According to the Journal of Endodontics guide lines.

Evaluation of calcium hydroxide paste removal associated with different drugs and its interference in the adhesiveness of a resinous sealer.

Abstract

Introduction: The objective of this study was to evaluate the effects of intracanal calcium hydroxide medication (Ca $(OH)_2$) associated with diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride during its removal and the effects on the adhesiveness of epoxy resin Sealer Plus cement. **Material and Methods:** 130 bovine teeth, instrumented and standardized with master apical file K80# were selected. For the removal test, 80 teeth were selected and divided into 2 groups: Master apical file and irrigation with cannula and syringe and Passive Ultrasonic Irrigation (PUI) , these two groups were further subdivided according to the medication to be received in: positive control ($Ca(OH)_2$ + propylene glycol), diclofenac sodium ($Ca(OH)_2$ + propylene glycol + diclofenac sodium 5%) ibuprofen ($Ca(OH)_2$ + propylene glycol + ibuprofen 5 %) and ciprofloxacin ($Ca(OH)_2$ + propylene glycol + ciprofloxacin 5%).and analyzed in Scanning Electron Microscopy. (SEM). For the push-out test 50 teeth divided into 5 groups were used: negative control (Only instrumentation) positive control ($Ca(OH)_2$ + propylene glycol), diclofenac sodium ($Ca(OH)_2$ + propylene glycol + diclofenac sodium 5%) ibuprofen ($Ca(OH)_2$ + propylene glycol + ibuprofen 5 %) and ciprofloxacin ($Ca(OH)_2$ + propylene glycol + ciprofloxacin 5%).After the push-out test, the obtained failures were classified and the data of all tests submitted to statistical analysis. **Results:** The results revealed no statistical difference between the intracanal medications regardless of the cleaning method used. The residues of the medications Diclofenac and ibuprofen influenced the adhesiveness of Sealer Plus presenting a statistically significant difference between them. The drug ibuprofen showed lower values of adhesiveness at the apical level when compared to the positive and negative control groups **Conclusion:** It was concluded that the association of the medicines with Ca $(OH)_2$ did not hamper the removal of the pastes independent of the associated drug, however none of the removal protocols was effective at complete removal. The remaining residues from the pastes containing diclofenac sodium and ibuprofen, influence in the adhesiveness of Sealer.

Key words: Intracanal medication, Calcium hydroxide, Ultrasound, push-out.

Introduction

Ca $(OH)_2$ is certainly the most widely used medication as a complement to the chemical-mechanical preparation and has excellent antimicrobial properties and biocompatibility. (1,2). To achieve these properties, it is important to obtain a compact and homogeneous filling in the total extension of the root canal system (3). However, resistant infections are related to microorganisms resistant to the steps of biomechanical preparation and intracanal medication (4). Some species of bacteria present in endodontic failures show resistance to Ca $(OH)_2$ medication (5). In relation to this problem the association of other antiseptic substances is proposed. Nonsteroidal drugs and antibiotics have proven antimicrobial activity when associated with Ca $(OH)_2$ (6).

Regarding to conventional Ca $(OH)_2$ pastes it must be completely removed before the obturation step. Among the techniques for the removal of Ca $(OH)_2$. The most used is a combination with the master apical file and syringe and cannula irrigation containing sodium hypochlorite and EDTA17% (7). Other methods such as rotary instruments, Self-adjusting file, EndoVac and EndoActivator are more efficient in the removal of the Ca $(OH)_2$ paste (8-10). Some devices such as an insert coupled to ultrasonic equipment are a satisfactory option to improve the efficiency of the irrigation in addition to offering excellent cost-benefit (11-13).

However, several studies have demonstrated that´s not possible to remove the Ca $(OH)_2$ completely from de root canal system (14,-16). The remnant Ca $(OH)_2$ blocks the penetration of sealers in dentinal tubules, could interfere with dentin adhesion, decreasing the dentin bond strength, increases the micro apical leakage and might interact with zinc oxide eugenol sealers (17-20).

The gold standard sealers are methacrylate-based resin materials propitiating a proper sealing of the root canal system. AH Plus is the most used resin sealer in several mechanical tests of push-out (21). Recently a new resin sealer has been commercialized, Sealer Plus presents characteristics like AH Plus (22). However, some studies have shown that the residual presence of Ca $(OH)_2$ can affect the bond strength of this group of sealers (23).

Recently new drugs such as nonsteroidal anti-inflammatory and antibiotics have been added Ca $(OH)_2$ pastes aiming at a better elimination of the microorganisms present in the root canal system. Medications such as diclofenac sodium, ibuprofen and ciprofloxacin demonstrated excellent antimicrobial properties when associated with Ca $(OH)_2$. Freitas et al., 2017 demonstrated excellent properties of the associations of these medications with Ca $(OH)_2$. The hypothesis theorized in this work would be that the addition of these drugs may facilitate or hinder the removal of Ca $(OH)_2$ pastes from the dentin walls as well as improve or affect the bond strength of Sealer Plus cement.

Material and methods

Specimens preparation

All specimens (130 Teeth) were instrumented by step-back technique with type K files of the second and third series, and the apical stops were standardized with the file size 80#. During instrumentation, irrigation with cannula and syringe with 5 ml of 2.5% sodium hypochlorite, 17% of EDTA irrigation for 3 minutes and final irrigation with saline solution.

Paste removal test

After the preparation of the specimens, 80 teeth were cleaved with the aid of a diamond disk and a lecron. Cavities were then made to delimit the areas of scanning SEM. After this step the teeth were reassembled and inserted in addition silicone. In this experiment 2 groups were evaluated being divided into conventional removal (master apical file + syringe and cannula) and Passive Ultrasonic Irrigation (PUI). The 2 groups were still sub divided into 6 groups (n=10): positive control (Ca $(OH)_2$ + propylene glycol), diclofenac sodium (Ca $(OH)_2$ + propylene glycol + diclofenac sodium 5%) ibuprofen (Ca $(OH)_2$ + propylene glycol + ibuprofen 5 %) and ciprofloxacin (Ca $(OH)_2$ + propylene glycol + ciprofloxacin 5%). The pastes were inserted with the assistance of a Lentullo instrument coupled to a VDW silver (VDW GmbH, Munich, Germany) at 300 rpm and 1 newton of torque, the ultrasonic agitation was performed using an EMS (Electro Medical Systems S.A., Nyon, Sweden) ultrasound and irrisonic ultrasonic insert in 20% power and stored for 7 days in a humid oven at 36º Celsius. The conventional protocol was performed with the initial removal of medication with memory instrument (80#k) and irrigation with 10 mL of 2.5% NaOCl, 17% EDTA for 3 minutes terminated with an irrigation of 10 mL of saline solution. The ultrasonic group was performed with 3 agitations of 20 seconds of 2.5% NaOCl, 1 saline agitation for 20 seconds, 3 agitations with 17% EDTA for 20 seconds and 1 final saline agitation for another 20 seconds. Then the specimens were disassembled and taken to SEM for obtaining 300x magnification images of cervical, middle and apical thirds. With the obtaining of the images scores of 0 to 3 were attributed to the classification of root canal cleansing, where the score 0: represents absence of $Ca(OH)_2$, score 1: small amount of $Ca(OH)_2$ (covering ≤ 20% of the surface), score 2: moderate presence of $Ca(OH)_2$ (covering 20 to 60% of the surface) and score 3: large amount of $Ca(OH)_2$ (covering> 60% of the surface).

Push-out Test

The push-out test used 50 teeth and divided them after instrumentation into 5 groups (n=10): Only instrumentation and none medication (negative control), positive control (Ca $(OH)_2$ + propylene glycol), diclofenac sodium (Ca $(OH)_2$ + propylene glycol + diclofenac sodium 5%) ibuprofen (Ca $(OH)_2$ + propylene glycol + ibuprofen 5 %) and ciprofloxacin (Ca $(OH)_2$ + propylene glycol + ciprofloxacin 5%).. The teeth were filled with their respective intracanal medications as well as performing ultrasonic agitation of the pastes used in each group. The specimens were then stored in a humid oven at 36 degrees Celsius for 7 days. The intracanal medications was removed with 3 agitations of 20 seconds of 2.5% NaOCl, 1 saline agitation for 20 seconds, 3 agitations with 17% EDTA for 20 seconds and 1 final saline agitation for another 20 seconds, after this period the specimens were filled only with Sealer Plus endodontic sealer and again stored in an oven for 7 days for the sealer setting time. The teeth were sectioned into slices approximately 2 mm from the apex and divided into apical, middle and cervical thirds and polished to plan their apical and cervical surfaces. The measures of the slices as height, diameter of the channel area was obtained for later application in formula to obtain the values referring to the displacement force in Mpa. The push-out test itself was performed in an INSTRON universal test machine and the values obtained in this test were added to the other values by means of the formula $SL = \pi(R + r)\sqrt{h^2 + (R + r)^2}$ to obtain to displacement force (Mpa) and the evaluation of the type of failure (adhesive, cohesive and mixed) was carried out by scanning electron microscopy in a 50x magnification.

Statistical Analysis

The data were tabulated in excel and submitted to statistical analysis and D'agostino and Pearson to verify normality. Checking the absence of normality were applied the Kruskal-Wallis tests and for multiple comparison the Dunn test. For the intra - group comparison, the

Friedman statistical test was applied. The test of Chi- squares was used to verify statistical difference between failures after push-out test.

Results

The results regarding the removal of the same intracanal medication showed no statistical difference when compared to the removal method ($p>0,05$). The comparison between the different intracanal medications with and without ultrasonic agitation did not show a statistically significant difference ($p>0,05$). Regarding the apical, middle and cervical levels, there were no statistically significant differences ($p> 0.05$). However, none of the protocols used totally removed the used drug pastes.

Regarding the mechanical push-out test, a significant statistical difference between the diclofenac-containing group and the ibuprofen-containing group could be noted at all levels analyzed ($p <0.05$), significant statistical difference was also noted between negative control and diclofenac containing group at the apical third ($p < 0.05$). The positive control and negative control groups showed statistically significant difference when compared to the group containing ibuprofen at the apical level ($P <0.05$). Finally, the other groups did not present a statistically significant difference between them.

In relation to failures obtained after the mechanical test of push-out there was a predominance of adhesive failures in all thirds analyzed without significant statistical difference ($p> 0.05$).

Discussion

The use of sealant materials with good adhesion to the dentin walls, maintenance of the absence of oral and periapical fluids, as well as the structural maintenance of the root tissue during static, functional and operative conditions (25-27), are properties that are fundamental to the success of endodontic therapy.

For these requirements to be achieved, after the use of $Ca(OH)_2$ during the intracanal medication step, the complete removal of the paste must be performed, since its residues will affect the adhesion strength between the dentin and the endodontic sealer. However, new drug formulations are being proposed against bacterial resistance, Freitas et al., (2017) proposed the association of three drugs individually associated with $Ca(OH)_2$: diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride, obtaining excellent results for bacterial elimination. In hypothesis, these drugs when associated with Ca $(OH)_2$ would facilitate or aggravate the removal of the new pastes and the residues after their removal, would influence the adhesiveness to the Sealer Plus resin cement.

Clinically these are essential and decisive factors for the success of endodontic treatment, since numerous studies have shown that up to the present moment it is not possible to carry out the complete removal of the Ca $(OH)_2$ pastes(14-16) These remaining residues can still interact physiochemically with the sealing material, thus increasing apical infiltration and even compromising the entire treatment (17,28-30).

There are innumerable technical proposals for the removal of intracanal medication (31-33), the most commonly used technique is the apical master file associated with copious irrigation (34). This combination is easily employed but should be used with caution not to wear the channel walls because the residue of the intracanal medication will remain mixed with the smear layer created by the coating of the canal walls and prevent its removal (35).

Another method widely used and presenting excellent results for the removal of Ca $(OH)_2$ is the use of ultrasonic equipment (36-38). (The good results found in the literature are influenced by thermal, mechanical, chemical, reflexive and shock wave generation, these effects are called biological effects of ultrasound. In addition to its clinical availability, ultrasonic irrigation also promotes greater elimination of organic tissue, planktonic bacteria and dentin remains of the root canal when compared to conventional irrigation (32), which makes it commonly used.

For the verification of the residual amount of Ca $(OH)_2$ in the dentin walls, one of the most used methods is performed through the application of scans after analysis in SEM. This is a methodological evidence in detail the surface and morphology of the walls of the root canal as well radiolucent materials (39). Several magnifications are proposed in the literature by different authors. Thus, this work used a magnification of 300x as well as Yücel et al., 2013 (40) to be able to perform the visualization as much as possible of the dentinal tubules in relation to the third one to be analyzed, thus avoiding the induction of the blind evaluators in relation to the cleaning of the walls.

To correlate the effect of residues from intracanal medication used and the adhesiveness of endodontic sealers in In vitro tests, the push-out test is the most widely used and widely accepted (41,42). This test is easily applicable and allows an adequate analysis regarding the bond strength of the endodontic sealers to the channel walls (43). However, the non-uniform distribution of gutta-percha shear and deformation in response to the application of compressive force during the test is disadvantageous. To minimize this problem, this study filled the channels only with endodontic sealer, to ensure total contact of the material with the walls of the root canal (44,45). In this experimental study, the comparison between the modified pastes and the conventional Ca $(OH)_2$ paste between them by their removal was not statistically significant. This condition suggests that there is no interference to the addition of medications to Ca $(OH)_2$ between pastes and dentin, being irrelevant to its removal and discarding some of the hypotheses considered. The comparison between the removal techniques used did not demonstrate a significant statistical difference between conventional irrigation and the use of passive ultrasonic irrigation. This work agrees with the findings found by Kourti E, Pantelidou O., 2017 (46) and there was no statistical difference between the use of the conventional irrigation associated with the master apical file when compared to PUI. However, several studies have demonstrated a better efficacy of removal with the use of ultrasound as well as the findings by Khaleel HY. et al., 2013 (47), Kirar DS. et al., 2017 (48). This may have occurred because of several factors so many may affect the removal efficiency of Ca $(OH)_2$, including the position of the syringe needle; the anatomical type as straight roots, amount of irrigant and concentration besides the application of instruments (10,36,49-51). Yet none of the protocols effectively removed Ca $(OH)_2$ independent of the associated medication.

Regarding the remaining residues and their interference on the epoxy resin sealer, the push-out test revealed that the drug diclofenac sodic demonstrated to improve the adhesiveness of the endodontic sealer in relation to the other drugs and the control groups, however the values were not statistically significant, except when computed to the ibuprofen group and compared at cervical level to the negative control group. It is suggested that residues from these pastes may have influenced the adhesiveness of the sealant cement or even altering the dentin structurally favoring or impairing adhesion.

In relation to the cervical third the anatomy of the dentinal tubules is directly related to the adhesiveness of the sealer as well as the presence of collagen fibers present. A possible hypothesis for this occurred is that the dressing with Ca $(OH)_2$ in addition of the drug had no effect negative in adhesion strength, because the cervical portion presents dentinal tubules and collagen fibers in these regions more homogeneous and well distributed than in other root thirds (45,52).

The failures occurred after the push-out test in the majority were of the adhesive type. This probably occurred due to the large amount of intracanal medication remaining in the walls of the root canal, these data corroborate with the researches of Ackay M. et al., 2014

(53) and Gokturk H. et al., 2016 (54) reporting the predominance of adhesive failures in groups with or without Ca $(OH)_2$ used.

Therefore, new studies should be carried out to evaluate the effects of these new pastes and their removal protocols, as well as to study the mechanisms that promote or difficult adhesion of endodontic Sealers to root dentin.

Conclusion

It can be concluded that the combination of medications such as diclofenac sodium, ibuprofen and ciprofloxacin hydrochloride individually associated with Ca $(OH)_2$ did not prove difficult to remove after methods of removal as did the conventional Ca $(OH)_2$. None of the cleaning methods completely removed the intracanal medications used. However, the remaining residues can affect the adhesiveness of Sealer Plus

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Table 1. - Medium±min/max of clean dentin walls.

	Ca (OH)2	**US Ca(OH)2**	**Cip**	**US Cip**	**Ds**	**US Ds**	**Ibu**	**US Ibu**
Apical	1,000 (0,0 -2,000) Aab	0,5000 (0,0 – 1,000) Aa	2,000 (1,000 – 3,000) Ab	1,000 (0,0 – 3,000) Aab	1,000 (0,0 – 3,000) Aab	1,000 (0,0 – 1,000) Aab	1,000 (0,0 – 1,000) Aab	1,000 (0,0 – 2,000) Aab
Medium	1,000 (0,0 -2,000) Aab	0,5000 (0,0 – 2,000) Aab	2,000 (0,0 -3,000) Aa	1,000 (0,0 – 2,000) Aab	1,000 (0,0 – 3,000) Aab	1,000 (0,0 – 1,000) Aab	1,000 (0,0 – 1,000) Aab	1,000 (0,0 – 1,000) Ab
Cervical	1,000 (1,000 – 2,000) Aa	1,000 (0,0 – 2,000) Aa	2,500 (0,0 – 3,000) Aa	1,000 (1,000 -1000) Aa	1,000 (1,000 – 3,000) Aa	1,000 (1,000 – 1,000) Aa	1,000(0,0 – 1,000) Aa	1,000 (1,000 – 1,000) Aa

Lowercase identifiers show statistical difference at the same level in relation to the removal technique and associated medication. Upper case identifiers show statistical difference between levels ($P<0.05$).

Table 2. Medium ± min/max of the push-out test.

	Diclofenac	Ibuprofen	Ciprofloxacin	Positive Control	Negative Control
Apical	14,75 (9,390 – 23,08) a	5,450 (1,670 – 8,310) b	10,74 (6,810 – 17,27) ab	10,35 (6,680 – 18,41) a	11,65 (4,440 – 22,50) a
Medium	12,02 (6,540 – 26,64) a	6,865 (2,500 – 12,07) b	9,300 (7,600 – 17,28) ab	9,065 (3,960 – 15,92) ab	9,425 (4,820 – 20,50) ab
Cervical	12,35 (9,010 – 28,55) a	7,855 (2,640 – 10,65) b	8,575 (6,680 – 15,89) ab	9,530 (5,170 – 17,61) ab	8,230 (5,610 – 14) b

Lowercase identifiers show statistical difference at the same level in relation to associated medication ($p<0{,}05$).

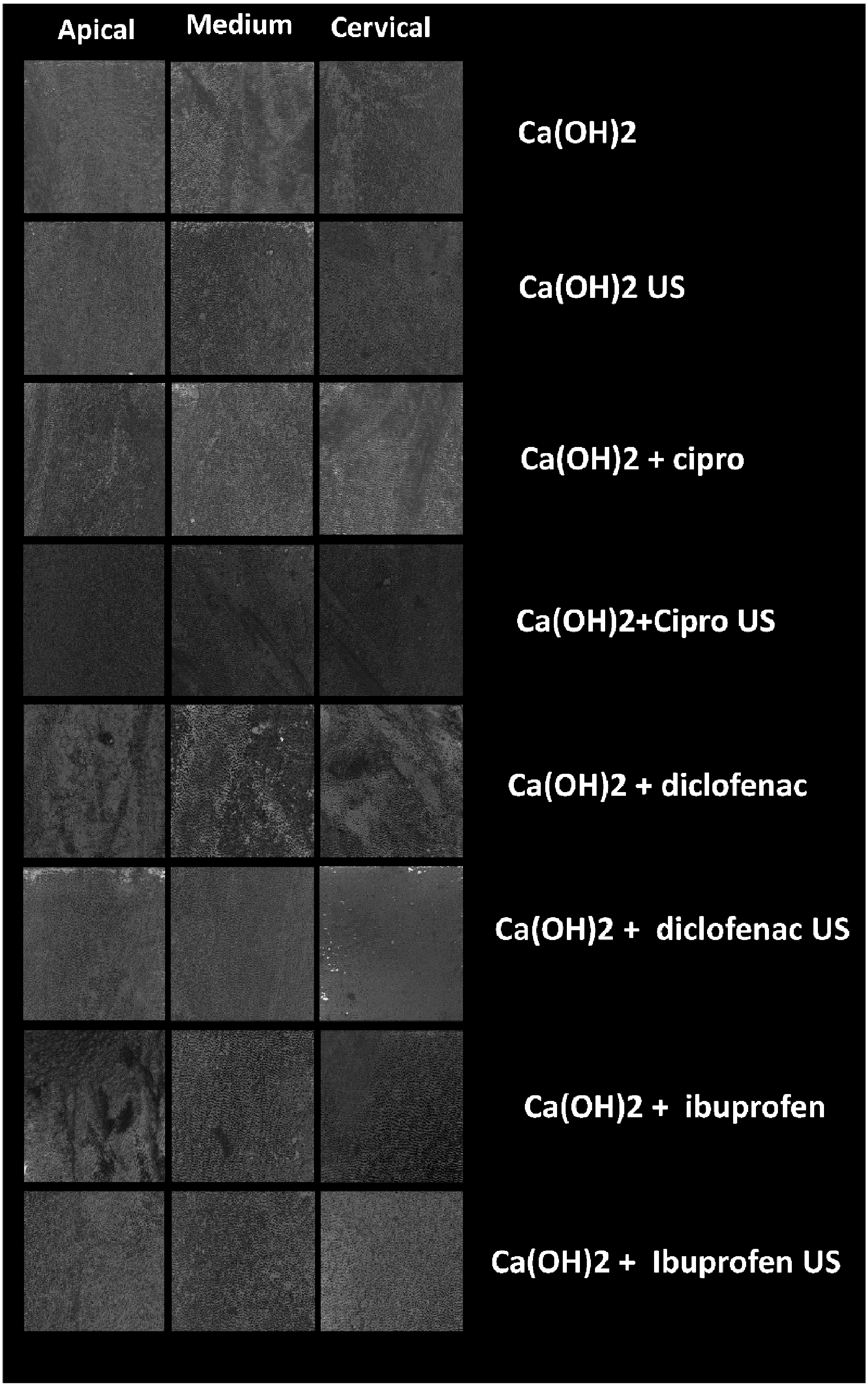

Figure 1. SEM images obtained for cleaning after immediate removal of calcium hydroxide pastes associated with different medications.

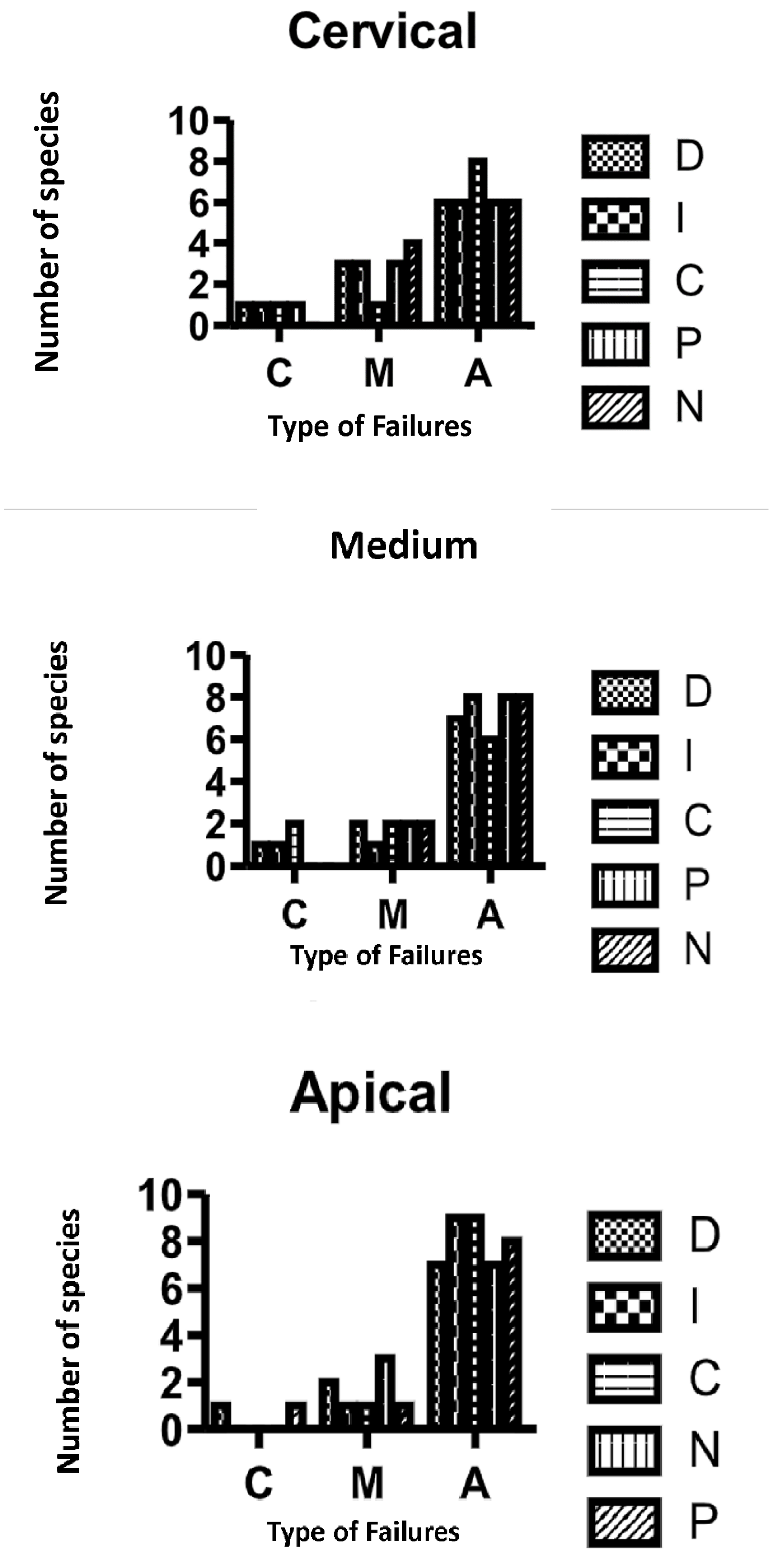

Figure 2: Graphs evidencing the number of failures, in relation to the type obtained after mechanical push-out test

3 Discussion

3 DISCUSSION

The use of different intracanal medications is proposed to complement bacterial elimination effectively. However, several reports of failure during endodontic therapy are related to the absence of elimination of enterococcus faecalis or others microorganism (SUNDQVIST G et al. 1998; HANCOCK HH 3RD et al. 2001; ROÇAS IN et al. 2004. This microorganism presents a high resistance threshold against several drugs used during the intracanal medication step. Calcium hydroxide is undoubtedly the most used intracanal medication during endodontic treatment, however enterococcus faecalis presents high resistance to this drug due to the presence of a proton pump present in its cytoplasm (EVANS M, et al., 2002). Another factor that is relevant to the failure of endodontic therapy is the ability of this microorganism to penetrate deeply into dentinal tubules (CHIVATXARANUKUL P et al. 2008; RAN S et al. 2015), as well as present in cases of extra root biofilms demonstrated by SUNDE PT et al. 2002.

In view of this problem, medicinal associations with calcium hydroxide are proposed. de Freitas et al., 2017 performed an association of NSAIDs and antibiotic to calcium hydroxide demonstrating no changes in the pH of the pastes as well as an increase in the elimination efficiency of enterococcus faecalis. However, the diffusion of the ions through the dentin and the possible elimination of microorganisms present on the extra radicular surface were not tested.

After the use and use of intra-root medications the use of sealant materials with good adhesion to the dentin walls, maintenance of the absence of oral and periapical fluids, as well as the structural maintenance of the root tissue during static, functional and operative conditions are properties that are fundamental to the success of endodontic therapy (TAGGER M. et al.,2002; CHEN H et al.,2008; HUFFMAN BP et al.,2009).

For these requirements to be achieved, after the use of $Ca\ (OH)_2$ during the intracanal medication step, the complete removal of the paste must be performed, since its residues will affect the adhesion strength between the dentin and the endodontic sealer. However, new drugs formulations are being proposed against bacterial resistance, FREITAS et al., (2017) as previously mentioned and obtained excellent results in bacterial elimination. In hypothesis, these drugs when associated with Ca $(OH)_2$ would facilitate or aggravate the removal of the new pastes and the residues after their removal, would influence the adhesiveness to the Sealer Plus resin cement.

Clinically these are essential and decisive factors for the success of endodontic treatment, since numerous studies have shown that up to the present moment it is not possible to carry out the complete removal of the Ca $(OH)_2$ pastes (KUGA MC et al.,2012; de FARIA-JÚNIOR NB et al. RÖDIG T, VOGEI S, Zapf A, HÜLSMANN M, 2010). These remaining residues can still interact physiochemically with the sealing material, thus increasing apical infiltration and even compromising the entire treatment (17,28-30 (KONTAKIOTIS EG, WU MK, WESSELINK PR., 1997; MARGELOS J et al., 1997; RICUCCI D & LANGELAND K.,1997).

Several methods are proposed to verify the bacterial elimination, as well as the residues left by the medications used and their interactions with the sealing cement. The methodology used to test the antimicrobial action of pastes and substances based on calcium hydroxide is the same and grounded within the scientific literature using confocal laser scanning microscopy and live / dead dye aid for cell labeling (ORDINOLA – ZAPATA et al. 2012). However, the methodology used for the simulation of extra-radicular microorganisms is absent in the literature, even in in vitro articles are not found or methodologies like is used Regarding the methodology applied to the elimination of root extra biofilms, it is a new methodology without possible comparisons in the literature, making it relevant to new studies.

For the verification of the residual amount of Ca $(OH)_2$ in the dentin walls, one of the most used methods is performed through the application of scans after analysis in SEM. This is a methodological evidence in detail the surface and morphology of the walls of the root canal as well radiolucent materials (VAN ELDIK DA et al., 2004). Several magnifications are proposed in the literature by different authors. Thus, this work used a magnification of 300x as well as YÜCEL et al., 2013 to be able to perform the visualization as much as possible of the dentinal tubules in relation to the third one to be analyzed, thus avoiding the induction of the blind evaluators in relation to the cleaning of the walls.

To correlate the effect of residues from intracanal medication used and the adhesiveness of endodontic sealers in In vitro tests, the push-out test is the most widely used and widely accepted (PANITVISAI P & MESSER HH, 1995; da CUNHA

LF et al., 2010). This test is easily applicable and allows an adequate analysis regarding the bond strength of the endodontic sealers to the channel walls WILLIAMS C et al., 2006. However, the non-uniform distribution of gutta-percha shear and deformation in response to the application of compressive force during the test is disadvantageous. To minimize this problem, this study filled the channels only with endodontic sealer, to ensure total contact of the material with the walls of the root canal (ERSAHAN S & AYDIN C, 2010; SOUSA-NETO MD et al., 2005).

Regarding the expected results, the null hypothesis for this work was not accepted. It was expected that the addition of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride to calcium hydroxide would also promote significant bacterial elimination as well as direct contact. However, the microbial elimination in extra-root biofilms was discrete. In addition, the pastes were removable as was the conventional calcium hydroxide paste. Although it is not possible to completely remove all the paste, but residues left by the calcium hydroxide + diclofenac sodium paste but increased the retention in relation to used sealer.

The results found after conducting this research showed inefficiency of all pastes in the elimination of the microorganisms present in the simulated extra radicular surface. This result suggests that the extension of the dentinal tubules as well as the presence of inorganic apatite hydroxide and other components present in the dentin may be acting on the neutralization of acids and bases (WANG JD & HUM WR, 1998) formed with the combination of calcium, NSAIDs and antibiotics decreasing their microbial potential

However, another intriguing factor found, despite the absence of a significant statistical difference, higher elimination values of microbial agents could be observed when ultrasonic agitation was not used. Studies in the literature claim that ultrasonic action on the drug may cause degradation of the active molecule in question with loss of therapeutic activity as well as undesirable effects caused by the formation of new compounds generated as a result of degradation (RIESZ P & KONDO T, 1992).

Furthermore, the combination of the calcium hydroxide associated with the vehicle together with the physic-chemical effects of the ultrasound promotes an increase in the release of hydroxyl ions (DUARTE MAHet al, 2007). However, it is a combination based on the results obtained, suggesting diminishing the antimicrobial effects of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride associated with calcium hydroxide. Studies have reported that the increase of pH caused by the increase of hydroxyl ions promotes the degradation of these active principles, rendering them ineffective (BALLESTEROS et al., 2003, JEONG J et al., 2010a; LUO X et al., 2012).

In relation to the removal of the intracanal medications and the residues of the interacting with the sealer. This experimental study, the comparison between the modified pastes and the conventional Ca $(OH)_2$ paste between them by their removal was not statistically significant. This condition suggests that there is no interference to the addition of medications to Ca $(OH)_2$ between pastes and dentin, being irrelevant to its removal and discarding some of the hypotheses considered. The comparison between the removal techniques used did not demonstrate a significant statistical difference between conventional irrigation and the use of passive ultrasonic irrigation. This work agrees with the findings found by KOURTI E & PANTELIDOU O., 2017 and there was no statistical difference between the use of the conventional irrigation associated with the master apical file when compared to PUI. However, several studies have demonstrated a better efficacy of removal with the use of ultrasound as well as the findings by KHALEEL HY. et al., 2013, KIRAR DS. et al., 2017. This may have occurred because of several factors so many may affect the removal efficiency of Ca $(OH)_2$, including the position of the syringe needle; the anatomical type as straight roots, amount of irrigant and concentration besides the application of instruments (WIGLER R et al., 2017; ÇAPAR ID et al., 2014; WISEMAN A et al., 2011; MA JZ et al., 2015; ALTURAIKI S et al., 2015). Yet none of the protocols effectively removed Ca $(OH)_2$ independent of the associated medication.

Regarding the remaining residues and their interference on the epoxy resin sealer, the push-out test revealed that the drug diclofenac sodic demonstrated to improve the adhesiveness of the endodontic sealer in relation to the other drugs and the control groups, however the values were not statistically significant, except when computed to the ibuprofen group and compared at cervical level to the negative control group. It is suggested that residues from these pastes may have influenced the adhesiveness of the sealant cement or even altering the dentin structurally favoring or impairing adhesion.

In relation to the cervical third the anatomy of the dentinal tubules is directly related to the adhesiveness of the sealer as well as the presence of collagen fibers present. A possible hypothesis for this occurred is that the dressing with Ca $(OH)_2$ in addition of the drug had no effect negative in adhesion strength, because the cervical portion presents dentinal tubules and collagen fibers in these regions more homogeneous and well distributed than in other root thirds (AKCAY M. et al., 2014; KOURTI E. & PANTELIDOU O., 2017).

The failures occurred after the push-out test in the majority were of the adhesive type. This probably occurred due to the large amount of intracanal medication remaining in the walls of the root canal, these data corroborate with the researches of ACKAY M. et al., 2014 and GOKTURK H. et al., 2016 reporting the predominance of adhesive failures in groups with or without $Ca(OH)_2$ used.

Despite the difficulties encountered and the limitations of the present study, new studies should be carried out to understand the interactions and mechanisms between the association of calcium hydroxide and the medicaments used as well as the physical and chemical effects that the ultrasound can exert on them.

4 CONCLUSION

4 CONCLUSIONS

The authors were able to conclude that the combination of ibuprofen, diclofenac sodium and ciprofloxacin hydrochloride have a low antimicrobial activity in relation to extra radicular biofilms, and the association of these drugs with ultrasonic agitation suggests that the drug used is limited and limit its action potential. It may also be concluded that combination of medications individually associated with $Ca(OH)_2$ did not prove difficult to remove after methods of removal as did the conventional $Ca(OH)_2$. None of the cleaning methods completely removed the intracanal medications used. However, the remaining residues can affect the adhesiveness of Sealer Plus

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Bariri, 02 de Setembro de 2017

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Assinatura

FOLHA DE ROSTO

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1. Projeto de Pesquisa:
Avaliação da atividade antimicrobiana, qualidade da remoção e influência na resistência de união de pastas de hidróxido de cálcio associado a diferentes substâncias

2. Área de Conhecimento: Endodontia

PESQUISADOR RESPONSÁVEL

3. Nome: Rodrigo Ricci Vivan		4. Cargo/Função: Professor/Orientador
5. CPF: 300.818.338-73	6. Endereço: Endereço: Alameda Dr. Octávio Pinheiro Brisolla, 9-75 - Jardim Brasil, Bauru - SP, 17012-901	
7. Nacionalidade: Brasileiro	8. Telefone: (014)98156-4945	9. E-mail: rodrigo.vivan@fob.usp.br

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PESQUISADOR EXECUTOR

10. Nome: Bruno Piazza		11. Cargo/Função: Aluno/Doutorando
12. CPF:413.371.608-65	13. Endereço: Praça Coronel Raphael de Moura Campos, 92-a	
14. Nacionalidade: Brasileiro	15. Telefone: (14)99671-7797	16. E-mail: bpiazzabtu@gmail.com

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17. Universidade de São Paulo	18. CNPJ: 63025530/0029-05	19. Unidade: Faculdade de Odontologia de Bauru
20. Telefone: (14) 3235-8000	21. Endereço: Alameda Dr. Octávio Pinheiro Brisolla, 9-75 - Jardim Brasil, Bauru - SP, 17012-901	

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Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Dentística, Materiais dentários e Endodontia

Bauru, 18 de Julho de 2018

Senhora Presidente,

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Atenciosamente,

Prof. Dr. Rodrigo Ricci Vivan	Prof. Dr. Marco Antônio Húngaro Duarte
Pesquisador Responsável	Chefe do Departamento de Endodontia

Profª Drª Ana Paula Campanelli
Presidente da Comissão de Ética no Uso de Animais

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Número do artigo: 267
Título: **Avaliação da ação antimicrobiana sobre biofilmes extra radiculares utilizando hidróxido de cálcio associado a diferentes medicamentos. Estudo In Vitro.**

Seu nome e e-mail foi cadastrado como autor do artigo cuja submissão (ou correção) da versão do artigo sob número 1 foi finalizada.

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