UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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# Ação antimicrobiana e propriedades físico-químicas de diferentes pastas empregadas como medicação intracanal

# Antimicrobial activity and physicochemical properties of different pastes used as intracanal medication

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

Orientador: Prof. Dr. Marco Antonio Hungaro Duarte

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*“Ela acreditava em anjos e, porque acreditava, eles existiam.”*

***Clarice Lispector***

# RESUMO

## Ação antimicrobiana e propriedades físico-químicas de diferentes pastas empregadas como medicação intracanal

A medicação intracanal nas necropulpectomias tem como função principal corroborar com a ação antisséptica sobre micro-organismos que sobreviveram ao preparo biomecânico do sistema de canais radiculares. A associação de fármacos e outras substâncias à pasta de hidróxido de cálcio vem sendo sugerida, com intuito de aumentar a efetividade antisséptica da pasta. Pastas antibióticas também vêm sendo utilizadas pelo seu amplo espectro de ação. O objetivo deste estudo foi avaliar pH, liberação de íons cálcio, solubilidade e ação antimicrobiana sobre dentina infectada por biofilmes orais, das pastas: Hidróxido de Cálcio + Solução Salina (G1), Calen (G2), Calen PMCC (G3), Hidróxido de Cálcio + Clorexidina (G4), Hidróxido de Cálcio + Diantibiótica (G5) e Triantibiótica (G6). Materiais e Métodos: Todos os experimentos foram divididos em 6 grupos de acordo com as medicações em questão. Medição de pH e liberação de cálcio: dentes de acrílico tiveram seus canais preenchidos com as medicações intracanal (*N=10*) e suas coroas seladas. Estes dentes foram imersos em água deionizada e, após os períodos de 3, 24, 72 e 168 horas, foram trocados de frasco. Na água em que estavam imersos foi aferido o pH por meio de um peagâmetro e o cálcio liberado através de espectrofotômetro de absorção atômica. Teste de solubilidade: A solubilidade foi avaliada pela medição volumétrica das pastas inseridas em cavidades padronizadas em 60 dentes de acrílico *(N=10)*, antes e depois de submersas em água deionizada, usando imagens do Micro-CT. Ação antimicrobiana: Sobre blocos de dentes bovinos foi induzida a formação de biofilme *in vitro* de *Enterococcus faecalis e Enterococcus faecalis + Pseudomonas Aeruginosa*. Após a indução, as amostras foram tratadas com as pastas por 7 dias. A porcentagem de células vivas foi mensurada através do corante Live/Dead pelo microscópio confocal. (*N=20*) Resultados: G1, G2, G3 e G4 se comportaram de maneira semelhante nos testes de pH, cálcio e solubilidade. G5 e G6 obtiveram os maiores valores de solubilidade. Na ação antimicrobiana, G4 e G6 obtiveram os melhores resultados em ambos biofilmes. Conclusões: O veículo parece não interferir no pH, liberação de cálcio e solubilidade das pastas de hidróxido de cálcio analisadas. Nenhuma das pastas matou 100% das bactérias no biofilme. Mesmo em pH alto, 7 dias foi insuficiente para efetiva ação antimicrobiana das pastas G2 e G3 em biofilme. A associação do hidróxido de cálcio à pasta Diantibiótica não é favorável em relação à sua ação antimicrobiana. A pasta Triantibiótica foi a mais efetiva contra biofilmes, porém obteve a maior solubilidade.



**Palavras-chave:** Hidróxido de Cálcio. Ação Antimicrobiana. Solubilidade.

# ABSTRACT

## Antimicrobial activity and physicochemical properties of different pastes used as intracanal medication

Introduction: The main function of intracanal dressing, in the treatment of teeth with pulpal necrosis, is the antimicrobial action against microorganisms which survived to the biomechanical preparation of root canal system. The combination of Calcium Hydroxide paste with other drugs has been suggested in order to increase the antiseptic capacity of the medication. The aim of this study was to evaluate the pH, calcium release, solubility and antimicrobial action on biofilms of the pastes: Calcium Hydroxide + Saline Solution (G1), Calen (G2), Calen CMCP (G3), Calcium Hydroxide + Chlorhexidine (G4), Double Antibiotic Paste (G5) and Triple Antibiotic Paste (G6). Material and methods: Measurement of pH and calcium release: acrylic teeth had their canal filled with the pastes in study (*N=10*) and the crown access sealed. Next, they were immersed in deionized water and after 3, 7, 15 and 30 days, removed from the flasks and put in a new flask. The pH and calcium ion of the water were measured by a pH meter and by an atomic absorption spectrophotometry, respectively. Solubility test: To evaluate the solubility, 60 acrylic teeth with standardized foramens (*N=10*) were filled with pastes and scanned at initial, 7, 15 and 30 days periods, before and after immersion in ultrapure water. The solubility of each specimen was the difference between the initial and final volume scanning. Antimicrobial activity: Biofilm in vitro of mono-specie (*Enterecoccus faecalis*) and dual-specie (*Enterococcus faecalis + Pseudomonas Aeruginosa*), were induced on blocks of bovine teeth. (*N=20*). Next, the samples were treated by the pastes for 7 days. The percentage of living cells were measured by using Live/Dead dye and confocal microscope. The data were statistically compared. Results: G1, G2, G3 and G4 did not present statistical difference to pH, calcium release and solubility values. G5 e G6 had the higher values of solubility. G4 e G6 presented the better action against biofilms. Conclusion:.The vehicle of paste seems not to interfere with pH, calcium release and solubility of calcium hydroxide pastes. None of the pastes killed 100% of the bacteria into the biofilm. Even with a high pH, 7 days may be an insufficient time for CH/P and CH/CMCP pastes to kill bacterial cells into the biofilm. Calcium hydroxide in addition to DAP, not favored its antimicrobial action. The TAP paste presented the bigger solubility and antimicrobial action.



**Key words:** Calcium Hydroxide. Antimicrobial action . Solubility.

# TABLE OF CONTENTS

# 1- Introduction

# 1 INTRODUCTION

Microorganisms and their products are the main etiologic factor of pulp and periradicular pathologies. A chronological evolution of caries diseases can lead an invasion by the pulp chamber of infectious agents. (1) When in main root canal, these microorganisms can invade areas of difficult access, such as dentinal tubules, apical surface, periodontal areas of root resorption, (2) anatomical complexities, apical deltas and isthmus. (3)

Besides, bacteria have the capacity to adhere and colonize dentinal walls, forming biofilms. This condition enhances the virulence and microbial resistance of them to the host immune system and antiseptics, making difficult the action of instruments and substances to kill them. (4) To complement these antimicrobial action in necro-pulpectomy, an intracanal dressing is required. (5)

Calcium hydroxide ($Ca(OH)_2$) paste has been widely used with this aim. It mechanisms of antimicrobial action is related to the ionic dissociation of $Ca^{2+}$ and $OH^-$ ions, that allow gradually a high pH, damaging essential enzymes for the survival of bacteria. But, it is ineffective to disinfect bacteria in form of biofilm (6) and *Enterococcus faecalis*, especially when they are within dentinal tubules. (7)

Therefore, the addition of antimicrobial vehicles on $Ca(OH)_2$ pastes have been suggested. They must complement it antiseptic capacity, physical and chemical properties. Camphorated paramonochlorophenol (CMCP) is the active vehicle widely used. It increases the spectrum of action of the $Ca(OH)_2$ paste, improving the ability of $Ca^{2+}$ and $OH^-$ ions to penetrate in dentinal tubules. (8) Studies have shown advantages with the association of the chlorhexidine instead of CMCP. The chlorhexidine presents substantiality, which favor a residual antimicrobial effects and antimicrobial activity against *Enterococcus faecalis* and *Candida albicans*. (9)

Used for it large spectrum of action against oral microorganisms and it ability to disinfect dentin. (7, 10), Triple Antibiotic Paste (TAP), a combination of metronidazole, ciprofloxacin and minocycline has been proposed as an intracanal dressing. Minocycline can cause discoloration of teeth, but the use of only metronidazole and ciprofloxacin called Double Antibiotic Paste (DAP) solve this problem.

The solubility of these pastes, the antimicrobial action and the ionic dissociation have been little studied. Based this fact, the objectives of present study were to evaluate the pH and calcium release of different intracanal dressings, the percentage of volumetric loss and the antimicrobial effect on mono specie (*Enterococcus faecalis*) and dual species (*Enterococcus faecalis + Pseudomonas Aeruginosa*) biofilms.

# 2- Article

# 2 ARTICLE

The article presented in this dissertation was written according to instructions and guidelines for article submission presented in Journal of Endodontics.

## 2.1 Article 1

Title: Antimicrobial activity and physicochemical properties of different pastes used as intracanal medication

**Introduction:** The aim of this study was to evaluate the pH, calcium release, solubility and antimicrobial action on biofilms of the pastes: Calcium Hydroxide + Saline Solution (G1), Calen (G2), Calen CMCP (G3), Calcium Hydroxide + Chlorhexidine (G4), Double Antibiotic Paste + calcium hydroxide (G5) and Triple Antibiotic Paste (G6).

**Methods:** Acrylic teeth (*N=10*) with root canal filled with the intracanal dressings were immersed in ultrapure water for measurement of pH level and calcium ion release (atomic absorption spectrophotometer) at 3, 7, 15 and 30 days. For the solubility, acrylic teeth (*N=10*) with root canal filled with the pastes were scanned by a Micro-CT, before (initial) and after (7, 15 and 30 days) immersion in ultrapure water. The solubility of each specimen was the difference between the initial and final volume scanning. In the antimicrobial analysis, biofilm in vitro of mono-specie (*Enterecoccus faecalis*) and dual-species (*Enterococcus faecalis + Pseudomonas Aeruginosa*), were induced on dentin blocks. (*N=20*) After, they were treated by the pastes for 7 days. The percentage of living cells were measured by using Live/Dead dye and confocal microscope. The data were statistically compared ($P<0.05$).

**Results:** G1, G2, G3 and G4 did not present statistical significant differences to pH, calcium release and solubility values. G5 e G6 presented the higher values of solubility. G4 e G6 favored the better antimicrobial action.

**Conclusion:** The vehicle did not interfere in the pH, calcium release and solubility of calcium hydroxide pastes. None of the pastes killed 100% of the bacteria into the biofilm. Calcium hydroxide in addition to the Double Antibiotic paste did not favor it antimicrobial action. The Triple Antibiotic paste presented the biggest solubility and antimicrobial action.

**Key words:** Intracanal dressing, pH, calcium release, solubility, antimicrobial action.

## Introduction

The microbial colonization of the root canal spaces and their products, as a consequence of caries or traumatism, are etiologic factors of pulp and periapical diseases. These bacteria may be located in isthmuses, dentinal tubules and ramifications which difficult the action of instruments and substances to kill them. Besides that, microorganisms capacity to adhere, colonize and form biofilms, favors the associations between microbial species which increases the resistance of bacteria to antimicrobials. (1)

Therefore, oftentimes root canal cleaning and shaping effectively reduces microbiota but not enough for it antisepsis, so the use of an intracanal dressing is necessary. Calcium hydroxide pastes has been widely used as an intracanal dressing. It antimicrobial action is related with the ionic dissociation of $Ca^{2+}$ and $OH^-$ ions, which gradually allow a high pH that interfere with the enzymatic system located on the cytoplasmic membrane of the bacteria. (2)

The vehicle of the paste determines the velocity of ionic dissociation, solubility and diffusibility. Some vehicles provides a faster ionic dissociation of the paste, favoring a faster on alkalinity of dentin, apical and periapical tissues, however may encourage a greater to causticity of the paste. (3) Inert vehicles associated with $Ca(OH)_2$ may be ineffective to kill the *Enterococcus Faecalis* which is a bacterium commonly isolated in persistent root canal infections. This microorganism presents resistance to the alkaline pH, especially when in form of biofilm. (4)

To increase the disinfectant action of the calcium hydroxide paste, the association of antimicrobial vehicles has been suggested. Camphorated paramonochlorophenol (CPCM) is the most used, expanding the spectrum of action of $Ca(OH)_2$ and improving its ability to penetrate in dentin. (5) Studies have shown advantages with the association of the chlorhexidine instead of CPCM. The chlorhexidine presents substantiality, which favor a residual antimicrobial effects. (6)

In cases of failures in endodontics, the employment of local application of antibiotics has been suggested. (7, 8) The combination of metronidazole, ciprofloxacin and minocycline in triple antibiotic paste (TAP) is benefic because of polymicrobial action in root canal infections, been able to eradicate anaerobic, gram-positive and gram-negative microorganisms. (7) Minocycline can cause discoloration of teeth, so the use of only metronidazole and ciprofloxacin called double antibiotic paste (DAP) have been suggested (9). However, the solubility of these pastes, the antimicrobial action and the ionic dissociation have been little studied.

Based this fact, the objectives of present study were to evaluate the pH and calcium release of different intracanal dressings, the percentage of volumetric loss and the antimicrobial effect on mono species (*Enterococcus faecalis*) and dual species (*Enterococcus faecalis + Pseudomonas Aeruginosa*) biofilms. The null hypothesis test was that the pastes presents similarity in the pH, calcium release, solubility and antimicrobial action.

## Methods

### *Intracanal Dressings*

Six intracanal dressings were evaluate:

- G1 (CH/S) - $Ca(OH)_2$ ((Merck & Co., NJ)/Saline (1 gr/ 0,5 mL);
- G2 (CH/P) - $Ca(OH)_2$/ polyethilene glycol (Calen, S.S. WHITE Artigos Dentários Ltd, Rio de Janeiro, Brasil);
- G3 (CH/CMPC) - $Ca(OH)_2$/ polyethilene glycol/ CMPC (Calen PMCC, S.S. WHITE Artigos Dentários Ltd, Rio de Janeiro, Brasil);
- G4 (CH/CHX) - $Ca(OH)_2$ ((Merck & Co., NJ)/ 2% chlorhexidine gluconate/ propylene glycol (1 gr/0,3 mL/0,2mL);
- G5 (CH/DAP) - Double paste (metronidazole, ciprofloxacin) /$Ca(OH)^2$ ((Merck & Co., NJ) /Saline (500 mg of each antibiotic/500 mg $Ca(OH)^2$ /1mL);
- G6 (TAP) - Triple Paste (metronidazole, ciprofloxacin, minocycline)/Saline (500 mg of each antibiotic/ 1 mL).

### *Experiment 1 and 2: pH and calcium ion release*

For the pH and calcium release tests, 60 artificial maxillary central incisors confectioned of resin acrylic with an artificial foramen standardized to a diameter of 400 µm were filled according to the 6 pastes in analyze ($N$= 10), according to the methodology of Duarte. (10) Each teeth was immersed individually in a plastic bottle containing 10 mL of ultrapure water and after 7, 15 and 30 days, the teeth were moved to a new plastic bottle with an equal volume of new ultrapure water. The pH and calcium release of the solutions were analyzed with a pH meter (model 371; Micronal, São Paulo, SP, Brazil) and with an atomic

absorption spectrophotometer (AA6800; Schimadzu, Tokyo, Japan) equipped with a calcium-ion-specific holllow cathode lamp, respectively. This methodology is based on Duarte

***Experiment 3: micro-CT volumetric solubility***

For the solubility analysis, sixty acrylic teeth with the same standardization of experiment 1 and 2 have the canals filled with the experimental pastes ( $N$= 10). Immediately after, the samples were scanned using an X -ray desktop microfocus CT scanner (SkyScan 1174v2; SkyScan, Kontich, Belgium). The parameter used in the image capture were a voxel size of 19.70 µm, 0.5º rotation step, with a 360º rotation. Each scan consisted of 373.tif images with 1024 x 1304 pixels. Next, the samples were individually immersed in plastic bottles containing 10 ml of deionized water, and stored at 37 ° C for 168 hours. So, acrylic teeth were removed from the bottles and a second scanning was made, using the same parameters of the first stage. Reconstruction of the images obtained of scanning were made and the volume ($mm^3$) of the pastes was get using CTan software (CTan v1.11.10.0, SkyScan). The solubility of each specimen was the difference between the initial and final volume scanning, having the total of volume lost during immersion. The percentage of solubility was calculate by the division of the volume lost by the volume total. The scanning was performed at periods of 0, 7, 15 and 30 days.

***Experiment 4 and 5: Antimicrobial Test and Microscopical Analysis***

***Sample preparation***

Dentin blocks were obtained from bovine central incisors with fully developed roots using trephine drills of 4.0-mm diameter under abundant irrigation. The incisors were positioned laterally given access to its most flattened portion of the root. So, the root was perforated using a trephine attached to a contra-angle positioned perpendicular to the teeth passing to mesial and distal dentin walls getting two blocks of dentin measuring 4 mm x 1.2 mm (diameter x thickness). These were adjusted using a polished with 500 and 800 grit SiC papers on the pulp surface to get it smooth. The smear layer formed, during preparation of dentine specimens, was removed by submerging them in 17% EDTA for 5 min and after this, they were sterilized by autoclaving at 121° for 20 minutes.

***Biofilm Growth***

The microbiological procedures and manipulation of the pastes were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). For *E. faecalis* biofilm, 15 µL standard strain (ATCC 4083) were put in 3 mL sterile BHI (Oxoid, Basingstoke, UK) for grown overnight at 37° in air. In dual species biofilm, the same procedure described was used with *E. faecalis* (ATCC 4083) and *P. aeruginosa* (ATCC 10145) each one apart and in different times for no contamination. After overnight, bacterial density was adjusted at $10^9$cells/mL for *E. faecalis* (ATCC 4083) and $10^7$cells/mL for *P. Aeruginosa* using a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density (OD) of 1 at 600 nm according to 0.5 MacFarland standard.

***Dentine Surface Infection***

After density adjustment, the dentine surface infection was performed. For *E. faecalis* biofilm a dentin block + 100µL of *E. faecalis* + 900 µL of BHI were put in each well of a 24-well multiwell plate. For dual species biofilms in each well were put a dentin block + 100µL of *E. faecalis* + 100µL of *P. aeruginosa* + 1500µL of BHI according to van der Waal et al, 2011. (11) For the grown of both biofilm all plates were incubated aerobically at 37°C for 21 days (mono specie biofilm) and 4 days (dual species biofilm). The BHI was refreshed every 2 days.

***Antimicrobial test for E. faecalis biofilm*** **and** ***E. faecalis + P. aeruginosa*** **biofilm**

The tests for biofilm were performed apart. After the incubation period, the infected samples were washed with 1 mL of distilled water to remove loosely adherent planktonic bacteria. So, they were randomly divided into six groups ($N$= 20) according to the experimental pastes + 1 control group that has no treatment. The contact test was performed by immersing dentin samples for 7 days in the experimental intracanal dressings and incubated at 37°C.

### *Microbiological Analysis*

Analysis of biofilm viability was performed by using the SYTO 9/propidium iodide technique (Live/Dead, Bacligth, Invitrogen, Eugene, OR). Acting like a dye, SYTO-9 is a green fluorescent nucleic acid stain, generally labeling both live and dead microorganisms and propidium iodide is a red fluorescent nucleic acid stain that only penetrates the cells with damaged membranes, signaling dead microorganisms After the contact time established with the pastes, the blocks were washed with phosphate-buffered saline (PBS) and stained with 15 µL of the dyes for 15 minutes in a dark environment. Then, they were washed again, and directly observed using an inverted confocal laser scanning microscopy. (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Four confocal "stacks" for random areas were obtained for each sample using 40x oil lens. In total we had 5 samples per group, so 20 stacks for each medication. For quantification purposes, bioImage_L software (www.bioImageL.com) was used to calculate the total biovolume and the percentage of red (dead cells) found after the antimicrobial treatment.

### *Statistical Analysis*

The data from all the analyses were subjected to the test of Shapiro-Wilks to verify the normality, where observed absent of normality of the data. However, for the n statistics comparisons between the groups, the Kruskal-Wallis and Dunn's tests were used. The significance level was established in 5%.

## Results

### *Experiment 1 and 2: pH and calcium ion release*

Table 1 presents the median, minimum and maximum values of the pH and calcium ion release proportioned by the experimental pastes in all analyzed periods. During all analyzed periods, the higher and lower values of pH and $Ca^{2+}$ ion release were observed in the CH/DAP and TAP ($P<0.05$) respectively, excepted for 30 days of CH/DAP pH. No statistical significant differences were observed between the groups CH/SS, CH/P, CH/CMCP and CH/CHX. ($P>0.05$).

***Experiment 3: micro-CT volumetric solubility***

Table 2 presents the median, minimum and maximum values of the percentage of solubility of the pastes in the analyzed periods. At 7, 15 and 30 day, CH/DAP and TAP presented the higher percentage of solubility values, with statistical significant differences in relation to the other groups ($P<0.05$). There was no statistical significant differences ($P>0.05$) between CH/SS, CH/P, CH/CMCP and CH/CHX.

***Experiment 4: Antimicrobial Test – Table 3***

Table 3 present the median, minimum and maximum values of the percentage of Live cells of different biofilms after contact with experimental pastes. In both biofilms, the weakest antimicrobial activity occurred in the CH/DAP, CH/CMCP and CH/P respectively, which did not present statistical significant differences ($P>0.05$) in relation to the control group. In the *E. faecalis* biofilm, TAP presented the greater antimicrobial activity followed by CH/SS and CH/CHX. In dual species biofilm CH/CHX and TAP presented the higher antimicrobial activity, in decreasing order.

**Table 1 -** shows the medians (Med), mininum and maximum (Min – Max) values for pH and calcium release (mg/dL) of the pastes in different studied periods.

| | **3 days** | | **7 days** | | **15 days** | | **30 days** | |
|---|---|---|---|---|---|---|---|---|
| | **Med (Min – Max)** | | **Med (Min – Max)** | | **Med (Min – Max)** | | **Med (Min – Max)** | |
| | pH | $Ca^{2+}$ release | pH | $Ca^{2+}$ release | pH | $Ca^{2+}$ release | pH | $Ca^{2+}$ release |
| G1 | 8,55 (8,03 - 9,6)$^{A}$ | 16,8 (11,4 – 31,9)$^{BC}$ | 7,5 (7,39 –7,61)$^{BC}$ | 7 (5,66 – 13,1)$^{BC}$ | 7,50 (7,29 –7,9)$^{AB}$ | 4,04 (2,08 -8,9)$^{BC}$ | 8,57 (8,24 -9,69)$^{A}$ | 5,02 (2,58 – 8,0)$^{BC}$ |
| G2 | 8,37 (7,75 -9,96)$^{A}$ | 22,6 (17,3 – 36,3)$^{AB}$ | 7,5 (7,27 –7,56)$^{BC}$ | 11,3 (9,2 –15,7)$^{AB}$ | 7,33 (7,08 –7,4)$^{AB}$ | 9,6 (7,01- 18,5)$^{AB}$ | 7,8 (7,68 - 8,12)$^{AB}$ | 9,94 (9,32 -13,1)$^{AB}$ |
| G3 | 8,26 (7,32 - 8,9)$^{AB}$ | 27,5 (20,2 – 34,7)$^{AB}$ | 7,62 (7,5 – 7,76)$^{AB}$ | 12,7 (8,4 – 17,2)$^{AB}$ | 7,06 (6,85 –7,5)$^{BC}$ | 13,5 (4,1 -35,5)$^{AB}$ | 8,4 (8,17 - 10,8)$^{A}$ | 10,8 (7,42 -17,7)$^{AB}$ |
| G4 | 8,3 (8,15 – 8,51)$^{A}$ | 21,3 (17,7 – 28,3)$^{AB}$ | 7,65 (7,44 7,74)$^{AB}$ | 6,93 (5,7 -16,6)$^{BC}$ | 7,09 (6,4 -7,32)$^{BC}$ | 3,63 (4,0 –8,9)$^{BC}$ | 7,64 (7,27 –7,8)$^{BC}$ | 3,79 (1,39 –8,7)$^{BC}$ |
| G5 | 9,26 (8,25 –10,3)$^{A}$ | 69,5 (58,5-84,8)$^{A}$ | 8,85 (7,87 –9,76)$^{A}$ | 46,8 (35,3 -55,2)$^{A}$ | 8,0 (7,31 – 8,64)$^{A}$ | 38,6 (25,4 –59)$^{A}$ | 7,11 (6,53 - 8,0)$^{BC}$ | 48,5 (30,4 – 140)$^{A}$ |
| G6 | 5,46 (4,84 –7,34)$^{B}$ | 0 (0 – 0)$^{C}$ | 6,17 (5,29 –6,54)$^{C}$ | 0 (0 – 0)$^{C}$ | 6,23 (5,78 –6,54)$^{C}$ | 0 (0 – 0)$^{C}$ | 5,39 (4,42 – 6,62)$^{C}$ | 0 (0 – 0)$^{C}$ |

*Kruskal-Wallis with Dunn's post-hoc p-value < 0.05; Different capital letters in columns indicate statistically significant intergroup differences in the same time period.
G1: $Ca(OH)^2$ + Saline; G2: $Ca(OH)^2$ + polyethilene glycol; G3: $Ca(OH)^2$ + polyethilene glycol + PMCC; G4: $Ca(OH)^2$ + CLX 2%/ + propylene glycol; G5: DAP + $Ca(OH)^2$; G6: TAP

**Table 2 -** Median (Med), minimum and maximum (Min - Max) values of the percentage of lost (solubility) comparing 7, 15 and 30 days volume with the initial.

| ***Groups*** | ***% of lost V (7d-I)*** ***Med (Min – Max)*** | ***% of lost V (15d - I)*** ***Med (Min – Max)*** | ***% of lost V (30d - I)*** ***Med (Min – Max)*** |
|---|---|---|---|
| *G1* | 2,35 (0,0 – 17,17)[b] | 2,51 (0,0 – 17,17)[b] | 5,7 (0,0 – 17,17)[b] |
| *G2* | 5,86 (0,0 – 19,48)[ab] | 12,62 (2,06 – 24,75)[ab] | 21,30 (13,2-35,2)[ab] |
| *G3* | 7,01 (0,29 – 12,75)[b] | 9,81 (6,0 – 18,60)[ab] | 13,42 (8,40 – 19,90)[ab] |
| *G4* | 1,54 (0,0 – 7,98)[b] | 4,90 (0,0 – 7,98)[b] | 4,9 (0,50 – 9,02)[b] |
| *G5* | 41,71 (17,11 -99,41)[a] | 57,99 (31,13 – 99,41)[a] | 63,5 (44,99 – 99,43)[a] |
| *G6* | 41,70 (20,51 – 57,09)[a] | 42,31 (24,38 – 93,38)[a] | 63,89 (39,89– 93,3)[a] |

*Kruskal-Wallis with Dunn's post-hoc p-value < 0.05; Different lowercase letters in columns indicate statistically significant intergroup differences in the same time period.

**Table 3-** Median (Med), minimum and maximum (Min - Max) values of the Percentage of Live Cells of different biofilms after Contact with the Experimental Medicaments for a week . B1 represents *E. Faecalis* biofilm and B2 a dual-species biofilm. (*E. faecalis + P. aeruginosa*)

*Green (% )*

| | HC + SS | Calen | Calen PMCC | HC + CHX 2% | DAP | TAP | Control |
|---|---|---|---|---|---|---|---|
| B1 | 13,15 (0,21 – 53,10) $^{CD}$ | 44,64 (4,08 – 92,0)$^{ABC}$ | 52,94 (8,14 – 82,45)$^{AB}$ | 35 (8,44 - 77,65)$^{BC}$ | 59,98 (11,46 – 80,33)$^{AB}$ | 0,06 (0,001- 1,23)$^{D}$ | 75,59 (42,9 – 96,54)$^{A}$ |
| B2 | 52,91 (5,63 – 51,24)$^{BC}$ | 46,26 (6,85 – 96,87)$^{AB}$ | 50,28 (4,75 – 95,29)$^{AB}$ | 9,67 (0 – 92,1)$^{CD}$ | 67,76 (17,99 – 96,81)$^{AB}$ | 11,92 (0,12 - 51,24)$^{D}$ | 85,14 (29,76 - 99,3)$^{A}$ |

*Kruskal-Wallis with Dunn's post-hoc p-value < 0.05; Different capital letters in rows indicate statistically significant intergroup differences in the same biofilm.

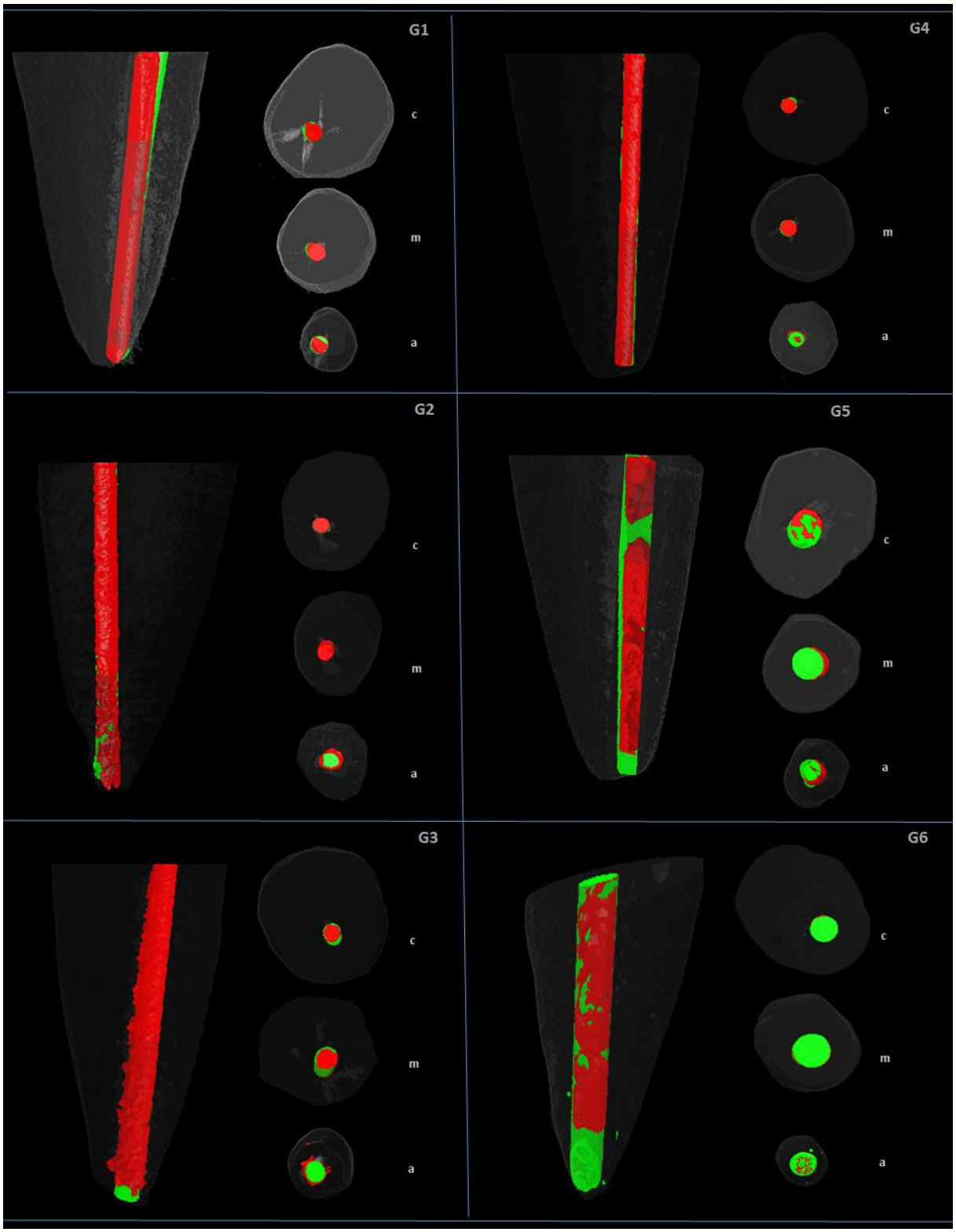


Figure 1. Representative micro-CT 3D reconstructions of the inicial (green) and final volume (red) of the superimposed intracanal dressings in root canals and it cross-sections at the coronal (c), middle (m), and apical (a) thirds.

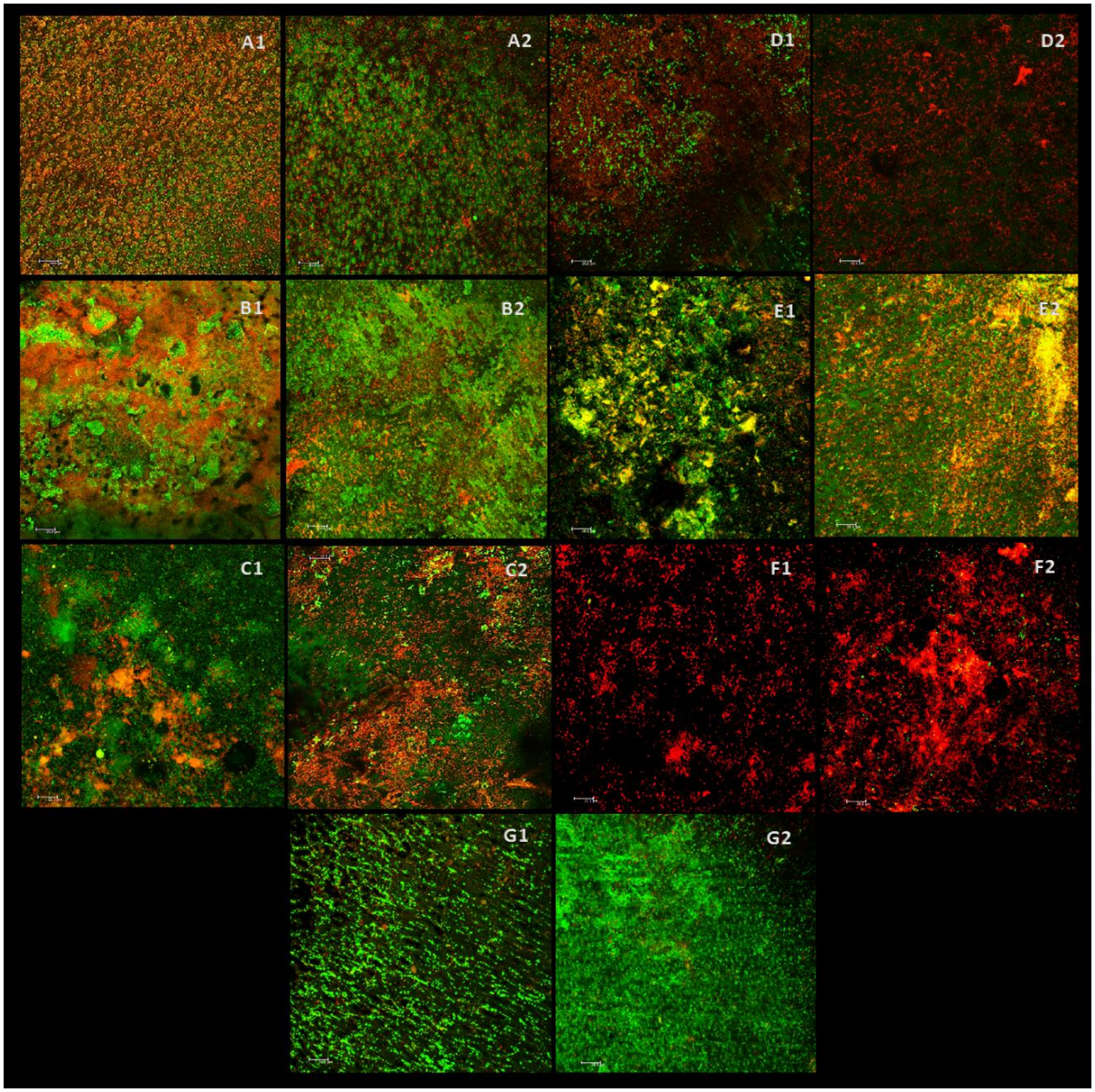


Figure 2. Confocal laser scanning microscopy of biofilms treated with (A) calcium hydroxide + saline solution, (B) calcium hydroxide + polyethilene glycol (C) calcium hydroxide + polyethylene glycol + CMCP (D) calcium hydroxide + saline solution + chlorhexidine (E) calcium hydroxide + saline solution + double antibiotic paste, (F) calcium hydroxide + saline solution + triple antibiotic paste, and (G) control group. Number 1 corresponds to mono species biofilm and 2 to dual species. Live cells are seen in green, and dead cells are seen in red. Each picture represents an area of 275 x 275 µm.

**Discussion**

The null hypothesis tested was rejected once the pastes presented differences in the pH and calcium release, solubility and antimicrobial action.

$Ca(OH)_2$ paste remains the medication of choice between appointments to reduce bacteria in root canals system. (12, 13) It is a strong base, with a high pH (12.5), that releases $Ca^{2+}$ and OH- ions when mixed with a vehicle. (2, 14) Elevated pH values are directly proportional to the maintenance of a high concentration of $OH^-$ ions which participate in the biological and antimicrobial actions. $Ca^{2+}$ ion release plays an essential role for anaerobic microbial inhibition clearing it breathing gas ($CO_2$) and on tissue mineralization, encouraging the fibronectin gene expression. (10, 15) The faster diffusion of this ions into dentine, cement barriers and tissue remnants, favor a better antimicrobial action of the $Ca(OH)_2$ pastes. (16)

The literature has demonstrated that aqueous vehicles have a high solubility and rapidly ion release, while the viscous presents an intermediate action, with the lowest values of pH and ion release. (2, 3) These finds are not in agreement with the present study. The results of pH, $Ca^{2+}$ release and solubility (percentage of lost volume) shown no difference between CH/SS (aqueous vehicle - saline), CH/P (viscous vehicle - polyethilene glycol), CH/CMCP (viscous vehicle -polyethilene glycol/oil - CMCP) and CH/CHX (viscous vehicle - propylene glycol/ aqueous - CHX) in 3,7 and 15 days periods. In the period of 30 days the CH/CHX presented the lowest values. A dryness of the pastes manipulated with aqueous vehicles was verified in a short time after manipulation, which probably caused the slowed ionic release compared with other studies. The pastes had a good consistence and the increase of vehicle would make it fluid. CH/DAP that is a mixture of antibiotics, $Ca(OH)_2$ and an aqueous vehicle (saline) has the higher values of pH, $Ca^{2+}$ release and solubility. This fact show a correlation between solubility and ionic dissociation. Although the G6 presented the higher solubility, the pH was lower and no calcium was evidenced due the absence of $Ca(OH)_2$ in the composition.

The methods of solubility applied in most studies, ANSI/ADA no.57 or ISO 6876, are based on the difference between weights before and after placing substances into ultrapure water. (17, 18) Particles of the pastes might detach from the specimen during storage having an influence on results, besides that $Ca(OH)_2$ may have an hygroscopic action increasing weight values. In relation to solubility of calcium hydroxide pastes, several studies claim that aqueous, viscous and oily vehicles, present different solubility, with the higher values to the aqueous pastes and the lowest values to the pastes which contains oily vehicles, based on

chemical analyzes of the liberation of $Ca^{2+}$ and $OH^-$ ions from calcium hydroxide paste. (2, 3) Based on a novel methodology proposed for MTA, the solubility of pastes is unprecedentedly measured using a microcomputed tomography to produce the volumetric lost of the pastes in different periods. (19) It is notice in this study an influence of powder-to-liquid ratio in the physical and chemical properties of the pastes. For an adequate consistence of aqueous pastes, vehicle/powder proportion of CH/SS and CH/CHX is 0,5mL/1g, and for CH/DAP and TAP is 1mL/1,5g, having an increase in the amount of water. Comparing all periods of test, the CH/SS and CH/CHX presented the lowest values of solubility, and the CH/DAP and TAP presented the higher values. The viscous paste have an intermediate value, without statistical differences with the other groups. So it's notice that hydration of the pastes may influence on solubility even more than the type of vehicle. Further studies using the $Ca(OH)_2$ manipulated with different proportions of the same and other vehicles are necessary to prove this hypothesis.

Development of mono species (*E. faecalis*) and dual species (*E. faecalis* + *P. aeruginosa*) biofilm allows evaluation of the antimicrobial action of intracanal dressings. *E. faecalis* is a gram-positive anaerobic facultative cocci, commonly isolated in persistent root canal infections to its ability to resist high pH stress (11,5) and form biofilm. (20) *P. aeruginosa* is a gram-negative aerobic motile road which can survive on anaerobic conditions, (21) related to failure in endodontics treatment. (22) Comparing both biofilms, resistance of intracanal dressing was higher in dual species for the majority of the analyzed paste. This occurrence can be related to the capacity of these bacteria to exchange of genetic material, which can lead to a spread of antibiotic resistance genes between inter species, favored by the biofilm organization. (20)

Time needed for $Ca(OH)_2$ pastes optimally disinfect the root canal system reaching a rapid and significant increase in the pH is still unknown. When measurements of ionic release are made directly in pastes or in distilled water with immersed pastes, high values of pH (11-12) are shown (23) simulating pastes action on direct contact with biofilm. Extremely alkaline environment unfeasible the survival of most of bacteria, but when in form of biofilm $Ca(OH)_2$ pastes shown a limited antimicrobial ability. (20) In our study, the direct contact of CH/P, even when associated to CMPC, on both biofilms, shown no statistic difference with control group (no treatment). So, even with a high pH, 7 days may be an insufficient time for these pastes to kill bacterial cells into the biofilm.

The standardization of root canals apical foramens with diameters that mimic clinic situation in tests of pH and $Ca^{2+}$ release, evidence the antimicrobial and biological action of

$Ca(OH)_2$ pastes in regions with indirect contact with the pastes, as apical and periapical regions. In concordance with others studies (10, 14), the results shown lower pH values of pastes (7-9). This reduction of antimicrobial effectiveness of the paste in critical areas can be related to endodontic failures.

In the other hand, association of HC and CHX have demonstrated effectiveness against biofilms in concordance with the results of the present study. (6) CHX presents a broad antimicrobial spectrum having excellent antimicrobial activity between pH 5,5 and 7. (24) Although pH of CH/CHX in 7 days was 8.3, in alkaline environment, the CHX may decompose into reactive by-products such as reactive oxygen compose, (22) which may kill *E. faecalis* and *P. aeruginosa*, explaining the great results.

The large spectrum of TAP activity by the combination of metronidazole, ciprofloxacin and minocycline is benefic because of the polymicrobial nature of root canal infections. Minocycline can cause discoloration of teeth, but the use of only metronidazole and ciprofloxacin called double antibiotic paste (DAP) solve this problem. Researches found no difference between antimicrobial activity of these antibiotic pastes. (25, 26) In the present study, TAP reduced significantly the viability of cells in a biofilm, different of CH/DAP that resembles to control. Probably, the alkaline environment proportionated to addition of $Ca(OH)_2$ in DAP can influence on the permeability of antibiotic into the bacterial cells. Antibiotics induce bacterial cell death by physical interaction between a drug molecule and its bacterial-specific target. The high percentage of lost volume in TAP may block the direct contact of pastes with bacteria and consequently the antimicrobial action of pastes.

This study found that the vehicle did not interfere in the pH, calcium release and solubility of calcium hydroxide pastes. None of the pastes killed 100% of the bacteria into the biofilm. Even with a high pH, 7 days may be an insufficient time for CH/P and CH/CMCP pastes to kill bacterial cells into the biofilm. Calcium hydroxide in addition to the Double Antibiotic paste did not favor it antimicrobial action. The Trianbiotic paste presented the bigger solubility and antimicrobial action.

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# 3- Discussion

# 3 DISCUSSION

$Ca(OH)_2$ paste remains the medication of choice between appointments to reduce bacteria in root canals system. (BYSTROM; CLAESSON; SUNDQVIST, 1985; PETERS et al., 2005) It is a strong base, with a high pH (12.5), that releases $Ca^{2+}$ and OH- ions when mixed with a vehicle. (ESTRELA; PECORA; et al., 1999; SIMON; BHAT; FRANCIS, 1995) Elevated pH values are directly proportional to the maintenance of a high concentration of $OH^-$ ions which participate in the biological and antimicrobial actions. $Ca^{2+}$ ion release plays an essential role for anaerobic microbial inhibition clearing it breathing gas ($CO_2$) and on tissue mineralization, encouraging the fibronectin gene expression. (DUARTE et al., 2009; MIZUNO; BANZAI, 2008) The faster diffusion of this ions into dentine, cement barriers and tissue remnants, the better antimicrobial action of the $Ca(OH)_2$ pastes. (ESTRELA; PIMENTA; et al., 1999)

The literature has demonstrated that aqueous vehicles have a high solubility and rapidly ion release, while the viscous presents an intermediate action, with the lowest values of both. (ESTRELA; PECORA; et al., 1999; FAVA; SAUNDERS, 1999) These finds are not in agreement with the present study. The results of pH, $Ca^{2+}$ release and solubility (percentage of lost volume) shown no difference between CH/SS (aqueous vehicle - saline), CH/P (viscous vehicle - polyethilene glycol), CH/CMCP (viscous vehicle -polyethilene glycol/oil - CMCP) and CH/CHX (viscous vehicle - propylene glycol/ aqueous - CHX) in 3,7 and 15 days periods. In the period of 30 days the CH/CHX presented the lowest values. A dryness of the pastes manipulated with aqueous vehicles was verified in a short time after manipulation, which probably caused the slowed ionic release compared with other studies. The pastes had a good consistence and the increase of vehicle would make it fluid. CH/DAP that is a mixture of antibiotics, $Ca(OH)_2$ and an aqueous vehicle (saline) has the higher values of pH, $Ca^{2+}$ release and solubility. This fact shows a correlation between solubility and ionic dissociation. Although G6 presented the higher solubility, the pH was lower and no calcium was evidenced due the absence of $Ca(OH)_2$ in the composition.

When measurements of ionic release are made directly in pastes or in distilled water with immersed pastes, high values of pH (11-12) are shown (PACIOS et al., 2004) simulating pastes action in direct contact with biofilm. In this study, the standardization of root canals apical foramens with diameters that mimic clinic situation, simulates the ions release to the

apical and periapical regions demonstrated the antimicrobial and biological actions in these areas. In concordance with others studies (DUARTE et al., 2009; SIMON et al., 1995) , the results shown lower pH values around 7-9, which may reduces the antimicrobial effectiveness of the paste.

Under this conditions, the solubility of pastes plays an important role. A new design of root canal with smaller apical diameters and great tapers it's becoming common with mechanized instrumentation. When dense pastes were used, apically regions may be difficult to achieve and antimicrobial action can be committed. (PETERS et al., 2005) On the other hand, tissue fluids can access root canal from foramen and dissolve fluid pastes which may have a great cytotoxic potential on tissue area in direct contact with the paste, what can permit the bacterial leakage. (REHMAN et al., 1996)

The methods of solubility applied in most studies, ANSI/ADA no.57 or ISO 6876, are based on the difference between weights before and after placing substances into ultrapure water. (HUNGARO DUARTE et al., 2012; VIVAN et al., 2010) Particles of the pastes might detach from the specimen during storage having an influence on results, besides that $Ca(OH)_2$ may have an hygroscopic action increasing weight values. In relation to solubility of calcium hydroxide pastes, several studies claim that aqueous, viscous and oily vehicles, present different solubility, with the higher values to the aqueous pastes and the lowest values to the pastes which contains oily vehicles, based on chemical analyzes of the liberation of $Ca^{2+}$ and $OH^-$ ions from calcium hydroxide paste. (ESTRELA; PECORA; et al., 1999; FAVA; SAUNDERS, 1999) Based on a novel methodology proposed for MTA, the solubility of pastes is unprecedentedly measured using a microcomputed tomography to produce the volumetric lost of the pastes in different periods. (CAVENAGO et al., 2014) It is notice in this study an influence of powder-to-liquid ratio in the physical and chemical properties of the pastes. For an adequate consistence of aqueous pastes, vehicle/powder proportion of CH/SS and CH/CHX is 0,5mL/1g, and for CH/DAP and TAP is 1mL/1,5g, having an increase in the amount of water. Comparing all periods of test, the CH/SS and CH/CHX presented the lowest values of solubility, and the CH/DAP and TAP presented the higher values. The viscous paste have an intermediate value, without statistical differences with the other groups. So it's notice that hydration of the pastes may influence on solubility even more than the type of vehicle. Further studies using the $Ca(OH)_2$ manipulated with different proportions of the same and other vehicles are necessary to prove this hypothesis.

Development of mono specie (*E. faecalis*) and dual species (*E. faecalis* + *P. aeruginosa*) biofilm allows evaluation of the antimicrobial action of intracanal dressings. *E.*

*faecalis* is a gram-positive anaerobic facultative cocci, commonly isolated in persistent root canal infections to its ability to resist high pH stress (11,5) and form biofilm. (JHAJHARIA et al., 2015) *P. aeruginosa* is a gram-negative aerobic motile road which can survive on anaerobic conditions, (YOON et al., 2002) related to failure in endodontics treatment. (SIREN et al., 1997) Comparing both biofilms, resistance of intracanal dressing was higher in dual species for the majority of the analyzed paste. This occurrence can be related to the capacity of these bacteria to exchange of genetic material, which can lead to a spread of antibiotic resistance genes between inter species, favored by the biofilm organization. (JHAJHARIA et al., 2015)

Time needed for $Ca(OH)_2$ pastes optimally disinfect the root canal system reaching a rapid and significant increase in the pH is still unknown. When measurements of ionic release are made directly in pastes or in distilled water with immersed pastes, high values of pH (11-12) are shown (PACIOS et al., 2004) simulating pastes action on direct contact with biofilm. Extremely alkaline environment unfeasible the survival of most of bacteria, but when in form of biofilm $Ca(OH)_2$ pastes shown a limited antimicrobial ability. (JHAJHARIA et al., 2015) In our study, the direct contact of CH/P, even when associated to CMPC, on both biofilms, shown no statistic difference with control group (no treatment). So, even with a high pH, 7 days may be an insufficient time for these pastes to kill bacterial cells into the biofilm.

In the other hand, association of HC and CHX have demonstrated effectiveness against biofilms in concordance with the results of the present study. (DELGADO et al., 2010) CHX penetrates into bacteria and exerts toxic effects, presenting a broad antimicrobial spectrum. It had an excellent antimicrobial activity between pH 5,5 and 7. (GOMES et al., 2003) Although pH of CH/CHX in 7 days was 8.3, in alkaline environment, the CHX may decompose into reactive by-products such as reactive oxygen compose, (SIREN et al., 1997) which may kill *E. faecalis* and *P. aeruginosa*, explaining the great results.

The large spectrum of TAP activity by the combination of metronidazole, ciprofloxacin and minocycline is benefic because of the polymicrobial nature of root canal infections. Minocycline (derivative from tetracycline) is associated with difficulties on bacteria repopulation on biofilms treaties with TAP for it capacity of substantiality. An inconvenient of this antibiotic is teeth discoloration, been harmful to esthetic.(KIM et al., 2010), but the use of only metronidazole and ciprofloxacin called Double Antibiotic Paste (DAP) solve this problem.

Metronidazole has a large spectrum of antimicrobial action against restrict anaerobic gram-positive, gram-negative and protozoa, however is not effective against facultative

bacteria. (TANEJA; KUMARI, 2012) Ciprofloxacin has antimicrobial activity against aerobic gram-positive (*Staphylococcus aureus, S epidermidis, Streptococcus spp*) and gram-negative ((*Escherichie coli, Enterobacter soo e Pseudomonas*) bacteria (FERREIRA, 2008), complementing metronidazole action.

Researches found no difference between antimicrobial activity of these antibiotic pastes. (ALGAMY et al., 2015; SABRAH et al., 2013) In the present study, TAP reduced significantly the viability of cells in a biofilm, different of CH/DAP that resembles to control. Probably, the alkaline environment proportionated to addition of $Ca(OH)_2$ in DAP can influence on the permeability of antibiotic into the bacterial cells. Antibiotics induce bacterial cell death by physical interaction between a drug molecule and its bacterial-specific target. The high percentage of lost volume in TAP may block the direct contact of pastes with bacteria and consequently the antimicrobial action of pastes.

# 4-Conclusions

# 4 CONCLUSIONS

This study found that the vehicle did not interfere in the pH, calcium release and solubility of calcium hydroxide pastes. None of the pastes killed 100% of the bacteria into the biofilm. Even with a high pH, 7 days may be an insufficient time for CH/P and CH/CMCP pastes to kill bacterial cells into the biofilm. Calcium hydroxide in addition to the Double Antibiotic paste did not favor it antimicrobial action. TAP presented the bigger solubility and antimicrobial action.

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