



Antimicrobial Activity and Physicochemical Properties of Antibiotic Pastes Used In Regenerative Endodontics

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The purpose of this study was to evaluate the pH, solubility and antimicrobial action of Calcium Hydroxide Paste (CH), Double Antibiotic Paste (metronidazole+ciprofloxacin-DAP), calcium hydroxide added to DAP (CH/DAP) and Triple Antibiotic Paste (metronidazole + ciprofloxacin+minocycline-TAP). pH (n=10) were measured by pHmeter. Root canals of acrylic teeth (n=10) were filled with the above-mentioned intracanal-dressings, immersed in ultrapure water, and solubility was measured by the difference between the initial and final volume (7,15 and 30 days) by using micro-computed tomography. *Enterococcus faecalis* biofilm was induced on bovine dentin disc surfaces (n=20), and treated with the pastes for 7 days. Percentage bacterial viability was verified by confocal microscope, with LIVE/DEAD dye. CH and CH/DAP presented the highest pH values. Regarding solubility, after 7 days, antibiotic groups presented significant volume loss. CH and CH/DAP showed no statistical difference compared with the Control in antimicrobial action against *E. faecalis* biofilm. However, TAP and DAP presented a significant percentage reduction in bacterial population. Due to high solubility of the pastes, renewing antibiotic dressings every 7 days, or using the medications for this period in regeneration protocols is recommended. DAP is indicated for killing *E. faecalis* in biofilm because it has antimicrobial action similar to TAP. Adding Calcium Hydroxide to DAP significantly decreased its antimicrobial action. In spite of its the low solubility and high pH values, the CH paste showed a low level of antimicrobial action against *E. faecalis* in biofilm.

Key Words: calcium hydroxide, antibiotic pastes, *Enterococcus faecalis*, solubility, hydroxyl ions

Introduction

Pulp necrosis of immature permanent teeth is a clinical situation in endodontics associated with traumatic injuries, anatomic anomalies and caries in young patients (1). Depending on the stage of tooth development, the dentin walls may be fragile, making the tooth more susceptible to fractures (2). The open-apex also increases the chances of extravasation of irrigant solutions and filling materials being over extended, causing chemical irritation, flare ups and an inappropriate obturation seal (3).

Clinically treatments have been proposed for artificial apical closure (apexification) performed in one visit, with mineral trioxide aggregate (MTA) that induced an artificial barrier, and treatment with calcium hydroxide (CH) in multiple-visits, which stimulated the formation of a hard tissue barrier (4). However, root development can be prevented or restricted by these techniques, leading to the risk of root canal fracture during masticatory efforts (5).

As an alternative technique, regenerative endodontic protocols (REPs) are intended to promote continued root maturation and apical closure. Local application of antibiotics as intracanal dressing has been used to eradicate endodontic pathogens during these procedures. Because of

its broad spectrum of action against oral microorganisms and its ability to disinfect dentin (6,7), Triple Antibiotic Paste (TAP), a combination of metronidazole, ciprofloxacin and minocycline has been widely used. Minocycline can cause tooth discoloration, but the use of only metronidazole and ciprofloxacin, called Double Antibiotic Paste (DAP), solved this problem (8). An alternative that has hardly been studied is the interaction of the antimicrobial action of DAP with the biological benefits of calcium hydroxide.

Based on the foregoing, the aim of the present study was to evaluate the pH, percentage of volumetric loss, the antimicrobial effect of different intracanal dressings used in regenerative procedures, on mono specie (*Enterococcus faecalis*) biofilms. The null hypothesis tested was that the pastes would have similar values of pH, volumetric loss and antimicrobial effect.

Material and Methods

Intracanal Dressings

Four intracanal dressings were evaluated:

CH - CH/polyethylene glycol (Calen; SS White Artigos Dentarios Ltd, Rio de Janeiro, RJ, Brazil); DAP-Double Antibiotic Paste (Metronidazole, Ciprofloxacin)/Saline (500

mg of each antibiotic/1 mL); CH/DAP-Double Antibiotic Paste (Metronidazole, Ciprofloxacin) /Ca(OH)₂ ((Merck & Co., NJ) /Saline (250 mg of each antibiotic/500 mg Ca(OH)₂ /1mL); TAP - Triple Antibiotic Paste (metronidazole, ciprofloxacin, minocycline)/Saline (500 mg of each antibiotic/1 mL).

To obtain the antibiotic powders (ciprofloxacin, metronidazole and minocycline), their tablets were ground by using a pestle and mortar, and then weighed on a high precision balance.

Experiment 1 and 2: pH of the Pastes

The pH measurement test was done in two ways. In Test 1 the pH of the 4 pastes was measured (with a pH meter). In Test 2 we tried to simulate the pH of the solubilized root canal pastes in the periapical tissues. Test 1 : 1 mL of the pastes were manipulated as described below and immersed in 10 mL distilled water. After being agitated for 5 min the solutions were measured with a pH meter (model 371; Micronal, São Paulo, SP, Brazil). Test 2 and 3: Based on the Duarte et al. (9) methodology, 40 acrylic teeth (n=10), with an artificial foramen standardized to a diameter of 800 µm were filled with the antibiotic pastes, and the crown access was sealed. Afterwards, the teeth were individually immersed in a plastic flask containing 10 mL of ultrapure water. After time intervals of 7, 15 and 30 days, the teeth were moved to a new plastic flask with an equal volume of new ultrapure water. The pH in the solutions was analyzed with a pH meter (Model 371; Micronal, São Paulo, SP, Brazil).

Experiment 3: Micro-CT Volumetric Solubility

The canals of 40 artificial maxillary central incisors fabricated of resin acrylic, with a foramen standardized to a diameter of 800 µm, were filled immediately after mixing the experimental pastes (n=10) Subsequently, the samples were scanned using an X-ray desktop microfocus CT scanner (SkyScan 1174v2; SkyScan, Kontich, Belgium). The acrylic teeth were stored, individually immersed in plastic flasks containing 10 mL of deionized water in a warm room at 37° C. After time intervals of 7, 15 and 30 days, the acrylic teeth were removed from the flasks and scanned again. The image capture parameters used were the same for all stages: voxel size 19.70 µm; 0.5° rotation step and 360° rotation. Each scan consisted of 373.tif images with 1024 x 1304 pixels. Digital data were further elaborated by reconstruction software (NRecon v1.6.4.8, SkyScan) and the volume (mm³) of the pastes was obtained by means of CTan software (CTan v1.11.10.0, SkyScan). The solubility of each specimen was considered the difference between the initial and final volume scanning, showing the proportion of the dissolved material.

Experiment 4: Antimicrobial Test and Microscopic Analysis

Sample Preparation

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

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 *Enterococcus faecalis* (*E. faecalis*) biofilm, 15 µL standard strain (ATCC 4083) was put into 3 mL sterile BHI (Oxoid, Basingstoke, UK) for growth overnight at 37° in air. After this, bacterial density was adjusted at 10⁹ cells/mL for *E. faecalis* (ATCC 4083) in a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density (OD) of 1 at 600 nm according to the 0.5 MacFarland standard.

Dentin Surface Infection

After density adjustment, the dentin surfaces were infected. For *E. faecalis* biofilm a dentin block + 100 µL of *E. faecalis* + 900 µL of BHI was put into each well of a 24-well multiwell plate. For biofilm growth, all plates were incubated aerobically at 37 °C for 21 days. The BHI was refreshed every 2 days.

Antimicrobial Test for *E. faecalis* Biofilm

After the incubation period, the infected samples were washed with 1 mL of distilled water to remove loosely adherent planktonic bacteria. Then they were randomly divided into four groups (n=20) according to the experimental pastes + 1 control group that received no treatment. The contact test was performed by immersing dentin samples for 7 days in the experimental intracanal dressings and incubating them at 37°C.

Microbiological Analysis

Biofilm viability was analyzed by using the SYTO 9/propidium iodide technique (Live/Dead, BacLight, Invitrogen, Eugene, OR). SYTO-9 is a green fluorescent nucleic acid

stain that acts as a dye, 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 established time of contact with the pastes, the blocks were washed with phosphate-buffered saline (PBS) and stained with 15 µL of the dyes for 15 min in a dark environment. Then they were washed again, and directly observed using an inverted confocal laser scanning microscope (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Four confocal "stacks" of random areas were obtained for each sample using a 40x oil lens. In total, there were 5 samples per group, therefore, 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 Shapiro-Wilks test to verify normality, which was shown to be absent. Then, for the global and individual comparisons, the Kruskal-Wallis and Dunn tests, respectively, were used. The significance level was established at 5%.

Table 1. Medians (Med), minimum and maximum (Min-Max) values for pH of the pastes in different studied periods

	3 days	7 days	15 days	30 days
CH	9.52 (8.6-12.3)Aa	8.1 (7.6 - 9.07)Aab	7.9 (7.4-8.15)ABb	8.9 (7.9-11.4)Aa
DAP	5.3 (4.8-7.2)BCa	6.1 (5.2-6.6)Bab	6.2 (5.9 -6.6)BCab	5.5 (4.3-6.7)BCb
DAP/CH	9.2 (8.2-10.3)ABa	8.85 (7.8-9.76)Aa	8.0 (7.3-8.64)Aab	7.1 (6.5 - 8.0)ABb
TAP	5.46 (4.8-7.34)Ca	6.17 (5.29-6.54)Ba	6.23 (5.78-6.54)Ca	5.39 (4.4-6.6)Ca

*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.

Table 2. Median (Med), minimum and maximum (Min - Max) values of intracanal dressing volume, initial and after 7, 15 and 30 days of immersion in 10 mL of deionized water and the percentage of lost volume (solubility) comparing 7, 15 and 30 days volume with the initial

Groups	Initial	7 days		15 days		30 days	
	Med (Min-Max)	Med (Min-Max)		Med (Min-Max)		Med (Min-Max)	
	Volume	Volume	% of lost V (7d-I)	Volume	% of lost V (15d - I)	Volume	% of lost V (30d - I)
CH	9.7 (8.34-11.96) ^A	8.3 (5.5-11.6) ^{AB}	2.35 (0-44.95) ^b	8.05 (5.2-11.6) ^B	5.23 (1.25-47.72) ^b	8.05 (5.2 - 11.3) ^B	16.13 (3.41-47.4) ^b
DAP	9.8 (8.21- 11.3) ^A	6.17 (3.9 - 8.0) ^{AB}	41.0 (22.35-58.0) ^a	5.2 (2.2-7.12) ^{BC}	52.3 (32.25-75.84) ^a	3.3 (2.2-5.1) ^C	65.8 (51.8-75.8) ^a
DAP/CH	9.7 (7.4 - 11.5) ^A	5.6 (0.05 - 8.9) ^{AB}	41.71 (17.11 -99.41) ^a	4.1 (0.05-7.71) ^{BC}	57.9 (31.13-99.41) ^a	3.4 (0.04-6.16) ^C	63.5 (44.99-99.4) ^a
TAP	10.5 (8.5-12.12) ^A	6.3 (4.5-9.3) ^{AB}	41.70 (20.5-57.09) ^a	6.0 (0.5-8.4) ^B	42.31 (24.3-93.38) ^a	3.5 (0.5-6.9) ^B	63.89 (39.8- 93.3) ^a

*Friedman p-value <0.05; Different capital letters in rows indicate statistically significant intragroup differences; *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.

Results

Table 1 presents the OH⁻ and Ca²⁺ ion release results provided by the experimental pastes in all time intervals analyzed. The CH and DAP/CH presented the highest values, with no statistically significant differences (p<0.05) between the two. DAP and TAP showed acid pH values. pH measured in direct contact with the pastes showed higher pH values for CH- 12.51, DAP/CH-12.36, and lower for DAP-5.49 and TAP-4.32.

Table 2 presents the percentage of solubility of the pastes in 7, 15 and 30 days. At all periods there was high solubility with no statistically significant differences (p>0.05) between DAP/CH, DAP and TAP. For CH, lower values were shown (p<0.05). For all groups in 30 days the volume of solubilized pastes was higher than 7, with no statistical difference intragroup for CH (p>0.05).

Table 3 presents the percentages of live cells in the biofilms after contact with experimental pastes. The weakest antimicrobial activity occurred in Group CH followed by DAP/CH, showing no statistical differences compared with the control group (p>0.05). Groups DAP and TAP showed the best antimicrobial activity (p<0.05).

Figure 1 presents micro-CT 3D reconstructions of the superimposed intracanal dressings at the root canal thirds and cross-sections in all groups.

Figure 2 presents confocal laser scanning microscopy images of biofilms treated with the four intracanal dressing materials and control.

Discussion

The null hypothesis tested was rejected because the pastes presented differences in pH,

solubility and antimicrobial action.

As the dentin walls of infected immature teeth are fragile, the revitalization approach is the treatment that allows root development to continue, in terms of both length and thickness (5). In this method, hemorrhage is induced in the cleaned and disinfected canal space and the blood clot formed serves as a fibrin scaffold, allowing the formation of new living tissue. For effective antiseptics in root canals, care must be taken to perform the copious irrigation without extruding the irrigant; and also when performing instrumentation, because this is capable of weakening the dentin walls. For microbial control, it is necessary to place an intracanal medication with crown restoration to prevent recontamination (10).

Intracanal applications of Triple Antibiotic Paste (TAP) have been used as intracanal medication endodontics in regeneration procedures due to the broad spectrum antimicrobial activity by the combination of metronidazole, ciprofloxacin and minocycline. This has been beneficial because of the polymicrobial nature of root canal infections, (8) since TAP has been shown to be capable of eradicating anaerobic, gram-positive and gram-negative microorganisms (6). The Triple Antibiotic Paste has caused color change in teeth due to the minocycline in its formulation, which is incorporated into the tooth matrix and binds to the calcium ions via chelation, causing the discoloration (11). Thus, the use of metronidazole and ciprofloxacin only - called a Double Antibiotic Paste (DAP) was tested. Hoshino clarified the antibacterial effect of a mixture of antibiotics (TAP) in 1996 (6) but the efficacy of each agent alone needed to be studied, to investigate the real necessity of the mixture. Some studies have shown no difference between antimicrobial activity of DAP and TAP, which significantly reduced the viability of cells in a biofilm (12,13) according to the results of these studies.

CH is known to amplify release of the growth factor (TGF- β 1), which modulates pulpal stem cell migration,

proliferation, and differentiation, and has been shown to be beneficial for regenerative endodontic therapies.(14). It is also preferred for the survival of stem cells from apical papilla when compared with antibiotic pastes used in high concentrations (15,16). However, it may be ineffective for killing *E. faecalis* in biofilm, a species commonly used for microbiological tests in endodontics (Table 3). The presence of microorganisms and their antigens has a profound effect on the final phenotype of stem cells transferred to root canals during REPs (17). Thus, the presence of residual bacteria that lead to periapical and intracanal inflammation has been a major risk to the success of this therapy (18). Further antimicrobial tests are necessary with other bacterial species found in immature teeth to evaluate the antimicrobial action of CH in REPs.

Therefore, the purpose of using CH associated with DAP was to associate their biologic and antimicrobial action, respectively, as suggested by Maniglia-Ferreira (19). The pH measurements in direct contact with these pastes showed an acid pH, which may have had a demineralizing effect on dentin (2). DAP/CH, showed the highest OH⁻ ion release favoring an alternative biological approach, with no statistical difference when compared with the alkaline CH paste. However, instead of any increase in antimicrobial activity, the association of CH to the DAP had the same behavior as that of CH alone in the antibacterial action against *E. faecalis*, without differing from the Control (Fig. 2). The alkaline environment provided by CH could probably influence the permeability of the antibiotic into the bacterial cells. Thus, this combination is unnecessary and should be avoided. Maniglia-Ferreira (19) found that CH did not alter the antimicrobial effects of DAP, but these conflicting results may be due to the different methodologies; in the present study the antibacterial action was tested in a biofilm model, and the above-mentioned study used the radial diffusion method in agar that involved planktonic cells.

The solubility of pastes was measured based on a novel methodology using microcomputed tomography to produce their volumetric losses in the different time intervals (20). Acrylic teeth with standardized root canals and foraminal diameter of 600 μ m were used to simulate immature teeth. CH paste had the lowest solubility values. However, approximately 40% of the antibiotic pastes were solubilized in 7 days and 65% in 30 days (Fig. 1). The favorable outcomes of the treatment depend on effective antiseptics of the root canal. In the antimicrobial test, the DAP and TAP in the period of contact analyzed (7 days) showed the highest level of antimicrobial activity, demonstrating that 1 week was sufficient to promote effective antibacterial action. High solubility leads to a higher ionic dissociation of the molecules of intracanal pastes, which can favor

Table 3. Median and minimum and maximum values of the percentage of live cells of *E. faecalis* biofilm after contact with the experimental medicaments for a week

Group	% live cells
Control	75.59 (42.9-96.54)A
CH	44.64 (4.08-92.03)A
DAP	0.05 (0.0-31.56)BC
DAP/CH	59.98 (11.46-80.33)AB
TAP	0.06 (0.001- 1.23)C

Kruskal-Wallis with Dunn's post-hoc p-value < 0.05; On % of live cells, different capital letters in columns indicate statistically significant intergroup differences in the same biofilm.

antimicrobial properties such as OH⁻ ion release from the Calcium Hydroxide paste (20). Antibiotics induce bacterial cell death by physical or chemical interaction between a drug molecule and its bacterial-specific target. Nevertheless, the high percentage of volume loss found in

solubility test of antibiotic pastes may lead to an empty root canal, blocking their direct contact with bacteria and consequently their antimicrobial action.

Furthermore, when the volume of water in the mixture of pastes was increased, higher solubility was observed (21). At present, there are no defined protocols for endodontic regeneration. Recommendations of the American Association Endodontics (AAE), namely "Considerations for regeneration procedures" based on clinical evidences and recent articles, have indicated that the placement of an intracanal dressing with CH or TAP in the patient first appointment, with the treatment time required varying between 1-4 weeks, depended on patient's signs and symptoms (22). The proportion of powder/liquid in regenerative procedures varies in concentration of up to a million-fold (0.001-1000 mg/mL) (23). Due to the high solubility, in cases of low medication concentration, the root canal is probably empty for the greater part of time, in periods longer than 7 days. Thus, in regeneration protocols it is important to consider renewing antibiotic pastes every 7 days, or using the medication for this period, and thereby induce clot formation. The DAP is indicated because it has a percentage of bacterial reduction similar to that of TAP at 7 days.

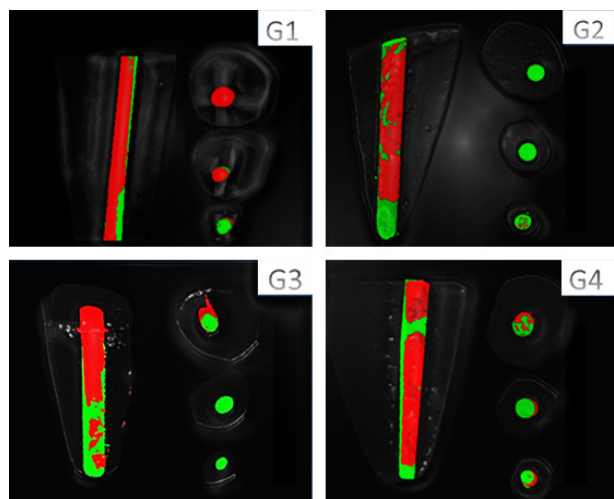


Figure 1. Representative micro-CT 3D reconstructions of the initial (green) and final (red) images of the superimposed intracanal dressings in root canals and cross-sections at the coronal (c), middle (m), and apical (a) thirds. G1: CH, G2: DAP, G3: DAP/CH, G4: TAP.

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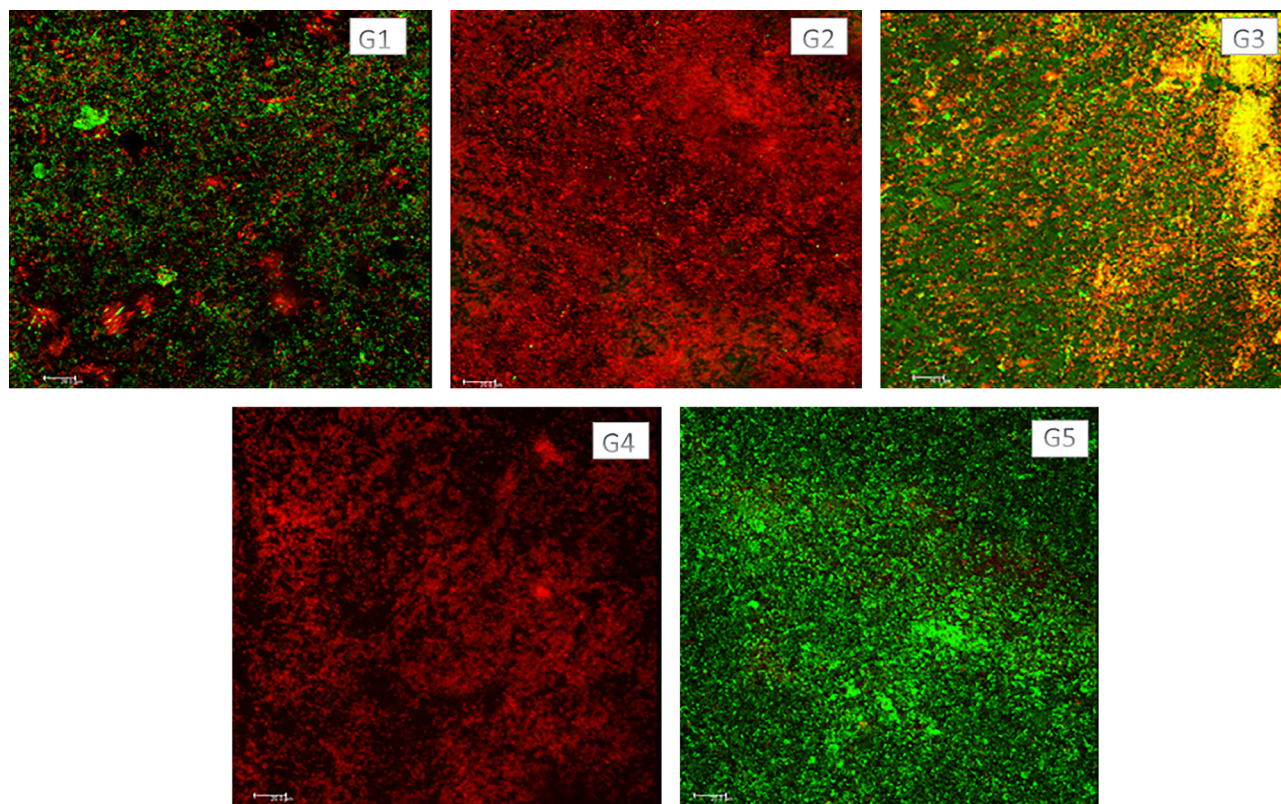


Figure 2. Confocal laser scanning microscopy of biofilms treated with CH (G1), DAP (G2), DAP/CH (G3), TAP (G4) and control (G5). Live cells are seen in green, and dead cells are seen in red. Each picture represents an area of 275 x 275 μm.

Resumo

A proposição deste estudo foi avaliar o pH, solubilidade e ação antimicrobiana da pasta de Hidróxido de Cálcio (HC), Pasta Diantibiótica (metronidazol+ciprofloxacina-DAP), Hidróxido de Cálcio adicionado a DAP (HC/DAP) e pasta Triantibiótica (metronidazol + ciprofloxacina + miociclina - TAP). a medição do pH e liberação de íons cálcio (n=10) foram avaliadas através de peagômetro e espectrofotômetro de absorção atômica. Canais de dentes de acrílico (n=10) foram preenchidos com as medicações de canal mencionadas, imersos em água ultrapura e a solubilidade foi aferida pela diferença entre o volume inicial e final (7,15 e 30 dias) usando micro tomografia computadorizada. O biofilme de *Enterococcus faecalis* foi induzido em blocos de dentina bovina (n=20) e tratados com as pastas em questão por 7 dias. A porcentagem de bactérias vivas foi verificada usando o microscópio confocal com corante LIVE/DEAD. HC e HC/DAP apresentaram os maiores valores de pH e liberação de íons cálcio. A respeito da solubilidade, depois de 7 dias, os grupos com pastas antibióticas apresentaram perda de volume significativa. HC e HC/DAP não tiveram diferenças estatísticas com o grupo controle na ação antimicrobiana contra biofilme. No entanto, a TAP e a DAP mostraram porcentagem significativa de redução bacteriana. Conclusões: Devido à alta solubilidade das pastas, renovar as medicações antibióticas a cada 7 dias ou usa-las por esse período em protocolos regenerativos é recomendado. DAP é indicada para matar *E. faecalis* na forma de biofilme porque tem ação antimicrobiana similar a TAP. A adição do CH à DAP reduz significativamente sua ação antimicrobiana. Apesar da baixa solubilidade e altos valores de pH, a pasta de CH apresenta baixa ação antimicrobiana contra *E. faecalis* em biofilme.

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