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Analysis of the reaction of subcutaneous tissues in rats and the antimicrobial activity of calcium hydroxide paste used in association with different substances

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ABSTRACT

The aim of this study was to evaluate the subcutaneous tissue response in rats and the antimicrobial activity of intracanal calcium hydroxide dressings mixed with different substances against *E. faecalis*. Fifty four rats were divided into three experimental groups according to the vehicle in the calcium hydroxide treatment: 0.4% chlorohexidine in propylene glycol (PG), *Casearia sylvestris* Sw in PG and calcium hydroxide+PG (control group). The pastes were placed into polyethylene tubes and implanted into the subcutaneous tissue. After 7, 14 and 30 days, the samples were processed and histologically evaluated (hematoxylin and eosin). The tissue surface in contact with the material was analyzed, and the quantitative analysis determined the volume density occupied by the inflammatory infiltrate (giant cells, polymorphonuclear cells and mononuclear cells), fibroblasts, collagen fibers and blood vessels. For the antimicrobial analysis, 20 dentin blocks infected with *E. faecalis* were treated with calcium hydroxide pastes in different vehicles; 0.4% chlorhexidine in PG, PG, extract from *Casearia sylvestris* Sw in PG and a positive control (infection and without medication) for 7 days. The efficiency of the pastes was evaluated by the live/dead technique and confocal microscopy. The results showed that 0.4% chlorhexidine induced a higher inflammatory response than the other groups. The *Casearia sylvestris* Sw extract showed satisfactory results in relation to the intensity of the inflammatory response. In the microbiological test, there were no statistical differences between the evaluated intracanal dressings and the percentage of bacterial viability was between 33 and 42%. The control group showed an 86% viability. Antimicrobial components such as chlorhexidine or *Casearia sylvestris* Sw did not improve the antimicrobial activity against *E. faecalis* in comparison to the calcium hydroxide+PG treatment. In addition, the incorporation of chlorhexidine in the calcium hydroxide paste promoted the highest inflammatory response.

Key words: Calcium hydroxide. Chlorhexidine. Endodontics. Materials testing.

INTRODUCTION

In the presence of pulp necrosis, the whole root canal system presents contamination by microorganisms and their products[16]. The removal of these irritants is usually performed with a biomechanical preparation and antimicrobial solutions[4,28]. Due to the limitations of this technique in consistently eliminating the root canal infection, the use of an intracanal medication is necessary to continue the antisepsis process[4]. The use of calcium hydroxide (CH) is advantageous and accepted because of its antiseptic and biological actions. The antimicrobial and biological properties

of CH occurred due to its high pH level, which provides microbial inhibition[8]. Another important factor is related to lipopolysaccharide inhibition[24]. In addition, the calcium release plays an important role in the formation of mineralized tissue[20].

Several substances have been mixed with calcium hydroxide to improve some of its properties such as viscosity, radiopacity, antimicrobial spectrum action and ionic dissociation rate[3,9,15]. The alkaline pH level is approximately 12.6, however, some types of bacteria such as *Enterococcus faecalis* is considered resistant to the antimicrobial action of calcium hydroxide inside the root canals[18].

Chlorhexidine digluconate is a bisguanide which is known for its antimicrobial action against bacteria[26]. The association of chlorhexidine to calcium hydroxide seems to increase the efficiency of calcium hydroxide paste against *Enterococcus faecalis*[11]. However, chlorhexidine used alone has caused damage to the DNA in oral mucosa cells and leukocytes[14], and the association of calcium hydroxide with chlorhexidine increases the production of reactive oxygen[2], which can result in increased tissue aggression[28].

The search for herbal medicines for use in medicine and dentistry is intense, including its uses as an intracanal dressing[13]. One of the herbs currently being studied is *Casearia sylvestris* Swart (Sw). It is a plant that originates from Latin America and can be found from Mexico to Argentina. In Brazil, it is found in abundance, it is a species very common in the State of São Paulo[25]. This plant extract has shown anti-inflammatory[7] and antimicrobial actions[22]. In relation to its chemical composition, essential oils (terpenes and triterpenes), saponins, fatty acids, tannins, resins, anthocyanosides and flavonoids were found[25]. Some chemical components in particular can take direct action against *E. faecalis*, such as tannins, flavonoids and essential oils.

The addition of *Casearia sylvestris* extract and chlorhexidine to calcium hydroxide does not interfere with its physico-chemical properties[6]. The aim of this study was to analyze the biocompatibility and the antimicrobial action of *Casearia sylvestris* Sw (Guaçatonga) and chlorhexidine mixed with calcium hydroxide paste.

MATERIAL AND METHODS

This study evaluated three calcium hydroxide pastes: Group 1 – calcium hydroxide (Merck KGaA, Darmstadt, Germany) mixed with 0.4% chlorhexidine in propylene glycol (Specific Pharmacy, Bauru, SP, Brazil); Group 2 – calcium hydroxide (Merck KGaA, Darmstadt, Germany) mixed with the extract from *Casearia sylvestris* Sw in propylene glycol (Max Pharma Trade and Import of Pharmaceutical Ingredients/Inputs, São Paulo, SP, Brazil); Group 3 (control) – calcium hydroxide (Merck KGaA, Darmstadt, Germany) mixed with propylene glycol (Specific Pharmacy, Bauru, SP, Brazil).

The *Casearia sylvestris* Sw extract utilized in this study was obtained from the Max Pharma pharmaceutical laboratory (Max Pharma Trade and Import of Pharmaceutical Ingredients/Inputs, São Paulo, SP, Brazil) and was produced in accordance with the Brazilian pharmacopeia. The *Casearia sylvestris* Sw leaves were subjected to a drying process in a circulating air, anatomical oven under a controlled temperature until a constant weight was achieved. The leaves were ground with grinder knives before being used in the preparation of the extract. The crushed product was subjected to maceration for propylene glycol extraction applications, where the powder's proportion was 25 grams for each 200 mL of propylene glycol. This extract solution remained in contact with the powder for 8 days with sporadic agitation in an amber glass bottle, to avoid possible interference from light, at a room temperature of approximately 25°C.

The pastes were prepared on a sterile glass plate using 3.0 g of calcium hydroxide powder (Merck KGaA, Darmstadt, Germany) for each 1.75 mL of the substances tested in each group.

Tissue analysis

This study was approved by the Ethical Committee for Teaching and Research on Animals. A total of 54 Wistar rats (*Rattus norvegicus albinus*) were divided into three experimental groups each of 18 animals, according to the division of the groups mentioned above.

The animals were anesthetized with a combination of Ketamine hydrochloride (Dopalen® - Vetbrands, CEVA, Paulínia, SP, Brazil) and Xylazine hydrochloride (Anasedan® - Vetbrands, CEVA, Paulínia, SP, Brazil). These substances were used in a concentration of 0.1 mL of solution for every 100 grams of animal weight. A manual trichotomy was performed on the dorsal region and was disinfected with an iodized alcohol solution. A longitudinal incision was made in the middle of the back of each region, with a no. 15 scalpel blade (Embramac, - Empresa Brasileira de Materiais Cirúrgicos, Campinas, SP, Brazil).

After manipulation, the pastes were placed in polyethylene tubes measuring 1 mm in internal diameter, 2.0 mm in external diameter and 10 mm in length (Embramed Ind. Com. Ltda., São Paulo, SP, Brazil) which were sterilized with ethylene oxide. Then each polyethylene tube, filled with one of the pastes, was placed in an exchange cannula, which was inducted into the open space until it reached a

depth of 18 mm in the subcutaneous tissue, causing tissue dilatation. Two tubes form the same group, one for the left side and one for the right side, were deployed into the back of each animal.

The implants were carefully deposited perpendicular to the line of incision, to avoid deportation and to decrease mobility. The incisions were sutured with 4.0 silk thread (Ethicon - Professional Products, Johnson & Johnson Ltda., São Paulo, SP, Brazil).

Six animals were used for each experimental period (7, 14 and 30 days). After the experimental time, the animals were killed with an excessive dose of Ketamine hydrochloride (Dopalen® - Vetbrands, CEVA, Paulínia, SP, Brazil). A new trichotomy was made on the dorsal region and a dissection of the implant area with a normal safety margin of the surrounding tissue. The tissue containing the implanted tubes was placed on paper and maintained fixed in a 10% formalin buffer (Merck KGaA, Darmstadt, Germany) for 7 days.

Next, the tissue samples were subjected to conventional histotechnical processing. Five micrometer thicknesses of serial sections were obtained from the capsule area.

The histological sections were stained with the hematoxylin and eosin technique and evaluated using a light microscope (Aristoplan, Leitz Wetzlar, Germany). A calibrated examiner analyzed the sections in a quantitative manner.

To evaluate the inflammatory response, the tissue surfaces in contact with the material were analyzed, and those areas were analyzed in four microscopical fields of two sections.

Quantitative analysis using an optical microscope (Aristoplan, Leitz Wetzlar, Germany) with a 100× objective and 8× Zeiss Kpl eyepiece containing a Zeiss II integration grid with 100 points symmetrically distributed over a quadrangular area, determined the volume density occupied by the inflammatory infiltrate (giant cells, polymorphonuclear cells and mononuclear cells), fibroblasts, collagen fibers and blood vessels[19,21].

The Kruskal–Wallis and Dunn tests were utilized for the analysis of the quantitative analysis. The level of significance was set at $P<0.05$ and the Prisma 5.0 software (GraphPad Software Inc, La Jolla, CA, USA) was utilized as the analytical tool.

Microbiological analysis

Twenty blocks of sterile bovine dentin were used in the experimental procedures. The measurements of the blocks were approximately 4x4x2 mm. The dentin blocks were made from bovine incisors, sterilized by autoclaving. The dentin segments were treated with 1% sodium hypochlorite for 30 minutes and 17% EDTA for 5 minutes. After that the blocks were immersed in BHI broth (Brain Hearth Infusion – BD – Becton, Dickinson and Company, Sparks MD, USA) culture containing *Enterococcus faecalis* ATCC 25912 (American Type Culture Collection, Manassas, VA) standardized by the 0.5 McFarland scale ($1.5x10^8$ bacteria *per* mL) and incubated for 21 days. The new culture broth of *E. faecalis*, obtained by overnight incubation and standardized with the 0.5 McFarland scale was refreshed every 3 days.

At the end of the experimental period, the infected blocks were removed from the culture broths, rinsed 3 times with a sterile, buffered saline solution then dried with absorbent paper and randomly distributed over the surfaces of sterile Petri dishes in accordance with each test group: Group 1 - Calcium hydroxide (Merck KGaA, Darmstadt, Germany) mixed with 0.4% chlorhexidine in propylene glycol (Specific Pharmacy, Bauru, SP, Brazil); Group 2 - calcium hydroxide mixed with the extract from *Casearia sylvestris* Sw in propylene glycol (Max Pharma Trade and Importer of Pharmaceutical Ingredients/Inputs. Ltda., São Paulo, SP, Brazil); Group 3 - calcium hydroxide (Merck KGaA, Darmstadt, Germany) mixed with propylene glycol (Specific Pharmacy, Bauru, SP, Brazil); Group 4 - positive control (with infection and without medication).

The blocks from groups 1-3 had their surfaces filled with their respective calcium hydroxide paste using the different vehicles. The blocks were again maintained at 37°C for one week at 100% humidity. After this period, the pastes were removed by irrigation with 2 mL of sterile water and then dried with sterile paper point cones.

For the bacterial viability analysis a confocal laser scanning microscope was used. The samples were stained using the Syto-9/Propidium iodide (PI) technique (Live/Dead, Baclight; Invitrogen, Carlsbad, CA, USA). SYTO-9 is a green, fluorescent nucleic acid stain which generally labels all live microrganisms. PI is a red, fluorescent nucleic acid stain that penetrates only cells with damaged membranes, highlighting the dead micro-organisms. The treated dentine samples were stained with 50 µL of the dyes for 10 min at room temperature in a dark environment. Four 40X confocal pictures were obtained from each sample using a 40X oil lens (Leica TCS-SPE; Microsystems GmbH, Mannheim, Germany). The scanning was performed by using a 1 µm step-size in a 512x512 pixel format, for a total of 20 images analyzed for each medication. The obtained biofilm images represented an area of 275x275 μm^2. For quantification purposes, the Bioimage-L software (http://www.bioimagel.com) was used to determine the percentage of live cells[5]. The Kruskal–Wallis and Dunn tests were utilized for the analysis of the antimicrobial activity. The significance level was set at $P<0.05$ and the Prisma

5.0 software (GraphPad Software Inc, La Jolla, CA, USA) was used as the analytical tool.

RESULTS

Inflammatory reaction

The values and statistical comparisons concerning the intensity of the inflammatory cells, collagen fibers, fibroblasts and blood vessels between the groups in each experimental period and between periods for each group are presented in Table 1 and Table 2. At the initial periods, it was possible to observe amorphous tissue in the contact region with the material, suggestive of coagulation necrosis,

Table 1- Median, maximum and minimum values obtained from the inflammatory reaction analysis in the three experimental periods

	Inflammatory cells								
	Giant cells			Mononuclear cells			Polymorphonuclear cells		
	7 days	14 days	30 days	7 days	14 days	30 days	7 days	14 days	30 days
CH+CLX 0.4 %	1.125$^{A;1,2}$ (0.0000-3.500)	1.563$^{A;1}$ (0.0000-8.250)	0.0000$^{A;2}$ (0.0000-1.250)	14.44$^{A;1}$ (6.250-19.50)	11.25$^{AB;1}$ (7.500-24.75)	5.000$^{A;2}$ (4.250-7.750)	0.6250$^{A;1}$ (0.0000-1.750)	0.2500$^{AB;1,2}$ (0.0000-1.000)	0.0000$^{A;2}$ (0.0000-0.2500)
CH+CS	0.1250$^{A;1}$ (0.0000-3.250)	0.0000$^{B;1,2}$ (0.0000-0.5000)	0.0000$^{A;2}$ (0.0000-0.0000)	14.32$^{A;1}$ (9.250-42.50)	9.875$^{A;1}$ (3.250-15.50)	1.563$^{B;2}$ (0.5000-4.750)	0.0000$^{AB;1}$ (0.0000-0.5000)	0.0000$^{A;1}$ (0.0000-0.2500)	0.0000$^{A;1}$ (0.0000-0.2500)
CH+PG	2.000$^{A;1}$ (0.0000-5.500)	0.6250$^{AB;1,2}$ (0.0000-2.000)	0.0000$^{A;2}$ (0.0000-0.2500)	17.25$^{A;1}$ (7.750-28.00)	13.75$^{B;1}$ (5.500-16.75)	1.188$^{B;2}$ (0.2500-4.000)	0.0000$^{B;1}$ (0.0000-1.500)	0.6875$^{B;1}$ (0.0000-1.750)	0.0000$^{A;1}$ (0.0000-0.5000)

Different uppercase letters in columns represent statistically significant differences between the materials from each period. Different numbers represent statistically significant differences between periods for each material

CH: Calcium hydroxide; CLX: Chlorhexidine; CS: *Casearia sylvestris*; PG: Propylene glycol

Table 2- Median, maximum and minimum values obtained from the histological events shown in connective tissue analysis in the three experimental periods

	Histological events								
	Collagen fibers			Fibroblasts			Blood vessels		
	7 days	14 days	30 days	7 days	14 days	30 days	7 days	14 days	30 days
CH + CLX 0,4 %	52.07$^{A;1}$ (43.50-58.00)	42.38$^{A;1}$ (17.50-54.75)	63.75$^{A;2}$ (57.75-71.75)	5.255$^{A;1}$ (3.500-6.750)	3.625$^{A;1}$ (1.000-7.500)	7.938$^{A;2}$ (4.500-11.00)	0.8750$^{A;1}$ (0.0000-5.130)	0.5000$^{A;1}$ (0.0000-3.500)	1.125$^{A;1}$ (0.5000-1.750)
CH+CS	46.50$^{A;1}$ (20.25-61.25)	56.63$^{B;1}$ (42.25-70.00)	53.06$^{B;1}$ (39.25-69.50)	7.875$^{B;1}$ (2.500-10.00)	7.500$^{B;1}$ (4.250-10.75)	7.563$^{A;1}$ (6.250-8.750)	1.250$^{A;1}$ (0.0000-2.750)	0.7500$^{A;2}$ (0.0000-1.250)	0.8125$^{A;1,2}$ (0.5000-1.500)
CH+PG	48.50$^{A;1}$ (36.25-52.25)	51.75$^{B;1}$ (45.50-67.50)	68.00$^{A;2}$ (53.50-79.00)	8.565$^{B;1}$ (4.000-10.75)	5.063$^{A;2}$ (2.000-8.750)	8.125$^{A;1}$ (4.500-10.50)	0.2500$^{B;1}$ (0.0000-1.500)	0.0625$^{A;1}$ (0.0000-1.250)	0.3750$^{B;1}$ (0.0000-0.5000)

Different uppercase letters in columns represent statistically significant differences between the materials in each period. Different numbers represent statistically significant differences between periods for each material

CH: Calcium hydroxide; CLX: Chlorhexidine; CS: *Casearia sylvestris*; PG: Propylene glycol

Table 3- Minimum, median, maximum and mean of the percentage of living cells after contact with the experimental substances

Live cells (%)	CH + CLX 0,4 %	CH + CS	CH + PG	Positive control
Minimum	3.149	4.968	1.953	53.58
Median	42.45	34.58	33.59	86.62
Maximum	77.84	80.96	81.22	93.18
Mean	43.08	40.55	36.89	83.65

CH: Calcium hydroxide; CLX: Chlorhexidine; CS: *Casearia sylvestris*; PG: Propylene glycol

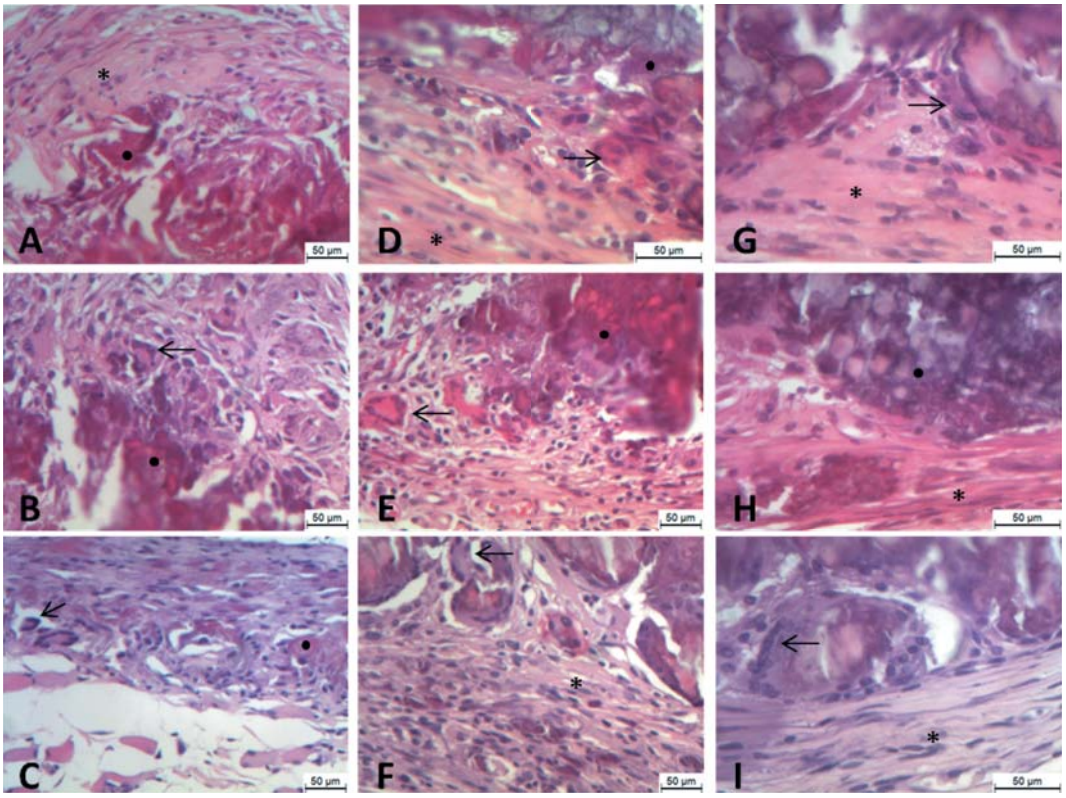

Figure 1- Week one subcutaneous tissue response of the evaluated intracanal dressings (7 days). Chlorhexidine 0.4%+calcium hydroxide (A); *Casearia sylvestris* Sw+calcium hydroxide (B); Propylene glycol+calcium hydroxide (C). Fibrocellular tissue (*), macrophage infiltration (arrows) and material remnants (●) can be observed. At 14 days, a decrease in the inflammatory infiltrate (arrows) and a discrete increase in fibrocellular tissue (*) in comparison to the 7 day period were observed. Chlorhexidine 0.4%+calcium hydroxide (D); *Casearia sylvestris* Sw+calcium hydroxide (E); Propylene glycol+calcium hydroxide (F). After 30 days, organized subcutaneous tissue is evident. Chlorhexidine 0.4%+calcium hydroxide (G) presenting giant cells (arrow). Fibrocellular tissue (*) is present in *Casearia sylvestris* Sw+calcium hydroxide (H) and propylene glycol+calcium hydroxide (I)

probably due to the alkaline action of the calcium hydroxide. Overall, the lowest values were found at the 30 day period for all pastes tested. In the 7 day period there were no statistically significant differences ($P>0.05$) among the tested pastes ($P>0.05$), however, during the periods of 14 to 30 days, statistically significant differences were found ($P<0.05$). A representative picture of the inflammatory reaction is shown in Figure 1.

Microbiological analysis

Table 3 shows the minimum, median, maximum and mean of the percentage of living cells after contact with the different calcium hydroxide pastes. All the pastes presented a significantly ($P<0.05$) lower percentage of live cells than the control. There were no statistically significant differences ($P>0.05$) between the medications. Figure 2 represents images obtained from the biofilms after treatment with the different pastes.

DISCUSSION

The objective of this study was to compare the effects of the addition of chlorhexidine or the extract from *Casearia sylvestris* Sw in propylene glycol to calcium hydroxide on the biocompatibility and antimicrobial activity of the pastes. In endodontic therapy, chlorhexidine is used in concentrations of 0.2% to 2.0%[29]. Chlorhexidine has an antimicrobial action in low concentrations (0.006%), and its effectiveness decreases in line with the reduction in concentration. This shows that there is a consensus among the choice of chlorhexidine concentrations used both as an irrigant solution or intracanal medication[1]. The findings for the inflammatory reaction of all groups tested, including the control group at 7 days, was more intense than at 14 and 30 days, as reported by a previous study[30].

For the period of 14 days, the inflammatory reaction intensity values for all groups showed significant differences. For the tested materials, the group of *Casearia sylvestris* Sw extract in propylene glycol proved to be less irritating than

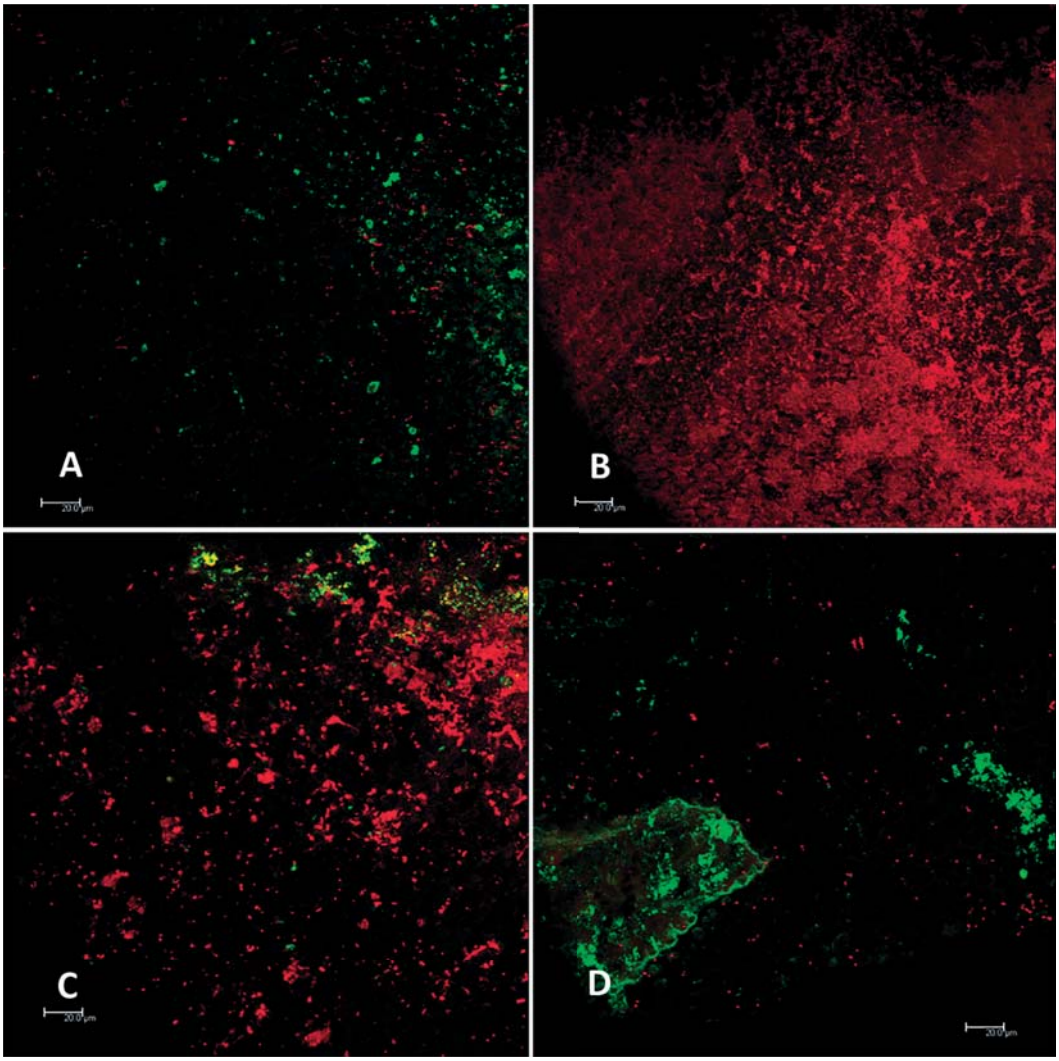

Figure 2- Representative confocal pictures after treatment with chlorhexidine 0.4%+calcium hydroxide (A); *Casearia sylvestris* Sw+calcium hydroxide (B); Propylene glycol+calcium hydroxide (C); Positive control (D). Dead cells are observed as red and live cells are observed as green

the others, which is contrary to another study[26]. The differences in the results can be attributed to the fact that the previous study[26] used a pure extract, and in our study the association with the calcium hydroxide paste could have reduced the cytotoxic effect. The characteristics of the inflammatory infiltrate during this period were also defined by the presence of giant cells and macrophages, however, the beginning of some tissue organization was detected in the three groups.

At the 30 day period, there was a decrease in the intensity of the inflammatory response in all the groups. The 0.4% chlorhexidine group reaction remained high compared to the other groups, establishing a statistically significant difference between groups 1 and 3. Such differentiation can be linked to the severity of the tissue injury by the chlorhexidine substance[12]. The increase in the percentage of chlorhexidine mixed with calcium hydroxide was noted; proportionally increasing the intensity caused an inflammatory reaction. Concentrations greater than 0.5%, chlorhexidine can produce tissue necrosis and the inflammatory process is more intense in slowing the healing process[12,23,27]. For the *Casearia sylvestris* extract group, a reduction of the inflammatory infiltrate occurred, probably due to its anti-inflammatory potential originating from its essential oils[22] and, in addition, the extract from this plant does not have a genotoxic effect[17].

With regards to the antimicrobial analysis of the paste, the *Casearia sylvestris* Sw extract group showed the lowest performance against *E. faecalis* compared to the other pastes used in the experiment, suggesting that this vehicle would not be adequate for the preparation of calcium hydroxide, especially to provoke an antimicrobial action, although it had a satisfactory result in the inflammatory response.

The 0.4% chlorhexidine in the propylene glycol group had a poor performance with the paste, suggesting that the use of propylene glycol as a

vehicle reduces its antimicrobial efficiency, probably due to a reduction of the ability of the hydroxyl ion's diffusion of calcium hydroxide in this vehicle. A possible reason is that pastes treated with propylene glycol promote a slower release of hydroxyl ions[30]. It should be stressed that *Enterococcus faecalis* is a microorganism resistant to pHs up to the magnitude of 11.0[10,18], which would explain the smaller antimicrobial action of the pastes with propylene glycol that favor a lower pH level[6].

CONCLUSION

Antimicrobial components such as chlorhexdine and *Casearia sylvestris* did not improve antimicrobial activity against *E. faecalis* in comparison to the calcium hydroxide + propylene glycol medication. In addition to this, the incorporation of chlorhexidine in the calcium hydroxide paste gave the highest inflammatory response.

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