Effect of the Association of Nonsteroidal Anti-inflammatory and Antibiotic Drugs on Antibiofilm Activity and pH of Calcium Hydroxide Pastes

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Abstract

Introduction: The objective of the present study was to evaluate the *in vitro* antibiofilm activity and pH of calcium hydroxide associated with different nonsteroidal anti-inflammatory drugs (NSAIDs). **Methods:** The groups analyzed were as follows: group 1, calcium hydroxide paste with propylene glycol; group 2, calcium hydroxide paste with propylene glycol + 5% diclofenac sodium; group 3, calcium hydroxide paste with propylene glycol + 5% ibuprofen; group 4, calcium hydroxide paste with propylene glycol + 5% ciprofloxacin; and group 6, positive control (without medication). For analysis of the pH, the pastes were inserted into tubes and immersed in flasks containing ultrapure water. At the time intervals of 3, 24, 72, and 168 hours, the pH was measured with a calibrated pH meter. For microbial analysis, biofilm was induced in 30 bovine dentin blocks for 21 days. Subsequently, the pastes were placed on the blocks with biofilm for 7 days. Afterward, the pastes were removed by irrigation with sterile water, and the specimens were analyzed with a laser scanning confocal microscope with the 50 μL Live/Dead BacLight Bacterial Viability solution L7012 Kit (Molecular Probes, Inc, Eugene, OR). Data were subjected to statistical analysis at a significance level of 5%. **Results:** The highest pH values were found for calcium hydroxide associated with ciprofloxacin in all periods analyzed. With the exception of pure calcium hydroxide paste, the other groups showed statistically significant differences ($P < .05$) in comparison with the positive control. **Conclusions:** The association of NSAIDs or antibiotic did not interfere with the pH of calcium hydroxide paste and increased the antimicrobial action of calcium hydroxide paste against *Enterococcus faecalis* biofilm formation. *(J Endod 2017;43:131–134)*

Key Words

Antibiofilm action, calcium hydroxide, diclofenac, ibuprofen, intracanal medication

Significance

The association of NSAIDs and ciprofloxacin increased the antimicrobial action of CH paste. The use of NSAIDS instead of antibiotics associated with CH paste would avoid the topical use of antibiotics, which could encourage the growth of resistant strains.

Calcium hydroxide is widely used in dentistry because of its antimicrobial and biologic effects (1). When used as an intracanal dressing, a vehicle is added to provide paste consistency. Different vehicles have been proposed as additives to calcium hydroxide (2). The dissociation of calcium hydroxide particles allows hydroxyl and calcium ion diffusion from the root canal walls through the dentinal tubules (3).

Calcium hydroxide acts by stimulating tissue mineralization (4), and its biologic and antiseptic effects rely on alkalinity and calcium ion release (3). The high pH of calcium hydroxide promotes microbial inhibition through an irreversible enzymatic reaction (5). Not only does calcium act by clearing the carbon dioxide used by bacteria for anaerobic respiration (6), but also calcium ions play an essential role in tissue mineralization, stimulating fibronectin gene expression (4). Another important property of calcium hydroxide is its ability to promote inactivation of bacterial lipopolysaccharides found in the outer membrane of gram-negative bacteria (7).

However, persistent infection might occur as a result of the presence of microorganisms that are refractory to endodontic procedures (8). Environmental alterations, such as increases in pH, might stimulate genetic cascades that modify the characteristics of the bacterial cell. Biofilm formation also represents a bacterial adaptation that increases the resistance of the microorganisms (8).

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Enterococcus faecalis, one of the microorganisms involved in persistent infections (predominantly in endodontic failures) (9), has shown resistance to calcium hydroxide therapy (10). For this reason, the association of calcium hydroxide with other antiseptic agents has been proposed to improve the action against this microorganism.

Several studies (11) have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) have proven antimicrobial activity. Diclofenac sodium has a highly bactericidal action against gram-positive and gram-negative bacteria by inhibiting the synthesis of bacterial DNA (11). Its antimicrobial effect is potentiated when associated with other drugs (12).

When antimicrobial activity was analyzed by means of radial diffusion assay, the effect of sodium diclofenac, ibuprofen, amoxicillin, and gentamicin, front-line drugs against *E. faecalis* ATCC 29212 (American Type Culture Collection, Rockville, MD), was verified and compared with the results provided by calcium hydroxide paste; a greater effectiveness of NSAIDS and antibiotics was observed in comparison with calcium hydroxide paste (13). In addition to *E. faecalis*, other microorganisms such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Mycobacterium tuberculosis* have shown sensitivity to NSAIDs (14).

Calcium hydroxide paste showed low antibiofilm action (15), and the association of NSAIDs with calcium hydroxide paste has not yet been evaluated. Therefore, the objective of the present study was to analyze the effect of the association of diclofenac sodium, ibuprofen, or ciprofloxacin with calcium hydroxide paste on pH and antimicrobial action against the biofilm of *E. faecalis*. The null hypotheses tested were that NSAIDS and ciprofloxacin would not interfere with antibiofilm action of calcium hydroxide paste and would not interfere with the pH of calcium hydroxide paste.

Material and Methods

Preparation of Bovine Dentin Specimens

Thirty sterilized bovine dentin blocks were used. The blocks of bovine incisor teeth were obtained by using an IsoMet metallographic cutting machine (Buehler Ltd, Lake Bluff, IL). Cuts were made until specimens measuring 4 mm in diameter and 0.8 thickness were obtained.

Afterward, the blocks were fixed in a polishing machine and polished. The dentin segments were treated with 1% sodium hypochlorite for 30 minutes and 17% EDTA for 5 minutes to remove organic waste and possible presence of the smear layer. To verify the cleaning method, 3 blocks were observed by scanning electron microscopy. The blocks were sterilized in autoclave at 121°C.

Contamination of the Dentin

For inducing the biofilm on the dentin blocks, *E. faecalis* ATCC 29212 was used according to the methodology recommended by Guerreiro-Tanomaru et al (16). After confirming the purity of the microorganism by Gram stain, morphology of the colony, and biochemical identification, the microorganism was reactivated in 4 mL sterile brain-heart infusion (BHI) broth and kept in an oven at 37°C for 12 hours.

After this, the optical density of the medium was measured and adjusted in a spectrophotometer (Model 600 Plus; Femto, São Paulo, SP, Brazil) with a wavelength of 600 nm. The cell density of 1.5×10^8 colony-forming units per milliliter (cfu/mL) was adjusted. The dentin blocks were placed into two 24-well cell culture plates within an area marked with a pencil, facing down. Then, the blocks were submerged in 3.6 mL sterile BHI broth with the addition of 0.4 mL standardized bacterial inoculum. The plates were placed in a bacteriological oven at 37°C for 21 days. To avoid nutrient deficiency in the bacterial cells, the BHI culture medium of each specimen was completely replaced every 48 hours, without the addition of new microorganisms.

Antibiofilm Activity Test

After the biofilm induction period, all blocks were removed from culture broths, rinsed 3 times with sterile buffered saline solution to remove planktonic cells, dried with a sterile needle, and randomly distributed on the surface of sterile Petri dishes according to each tested antibiotic as follows: group 1, calcium hydroxide paste with propylene glycol; group 2, calcium hydroxide paste with propylene glycol + 5% diclofenac sodium; group 3, calcium hydroxide paste with propylene glycol + 5% ibuprofen; group 4, calcium hydroxide paste with propylene glycol + 5% ciprofloxacin; and group 5, positive control (without medication). The powders of substances (NSAIDs and antibiotics) were added to the calcium hydroxide powder at the rate of 5% by weight. To manipulate the pastes, 1 g powder was mixed with 1 mL propylene glycol.

In groups 1 to 4, the block surfaces were covered with the calcium hydroxide pastes tested (0.1 mL). In the control group, the block containing biofilm was placed in contact with saline solution. The blocks were incubated again at 37°C for 7 days at 100% humidity produced by sterile cotton soaked with sterile ultrapure water. After this period, the blocks were removed from the incubation chamber; the pastes were removed by irrigation with 2 mL sterile water, and then the blocks were dried with a sterile needle.

Confocal Microscopic Analysis

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

All specimens were examined under confocal laser scanning microscopy (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany) at 40× magnification. Six specimens were photographed in each

TABLE 1. The Mean and Standard Deviation pH Values of the Calcium Hydroxide (CH) Pastes Tested in the Different Time Intervals

Pastes	3 h	24 h	72 h	168 h
CH + diclofenac	10.62 ± 0.14^{aA}	11.87 ± 0.08^{aB}	11.58 ± 0.21^{aC}	12.18 ± 0.14^{aD}
CH + ibuprofen	11.36 ± 0.43^{bA}	11.96 ± 0.18^{aB}	12.20 ± 0.19^{bC}	12.32 ± 0.10^{acC}
CH + ciprofloxacin	11.99 ± 0.49^{cA}	12.75 ± 0.13^{bB}	12.40 ± 0.15^{bC}	12.84 ± 0.15^{bB}
CH	11.33 ± 0.27^{bA}	11.85 ± 0.18^{aB}	12.23 ± 0.11^{bC}	12.45 ± 0.07^{cD}

Different lowercase letters show statistically significant differences between CH pastes in the same period, and different capital letters represent statistically significant differences between periods in a same CH paste ($P < .05$).

TABLE 2. The Median, Minimum, and Maximum Values of the Biovolume (mm^3) and Percentage of Live Bacteria after Being in Contact with the Calcium Hydroxide (CH) Pastes Tested

	CH + diclofenac	CH + ibuprofen	CH + ciprofloxacin	CH	Control
Biovolume	33.03[a] (1.88–102.9)	20.12[a] (1.26–193.56)	23.38[a] (0.83–156.37)	20.02[a] (1.94–277)	90.29[a] (10.65–422.36)
Percentage of live bacteria	19.46[a] (0.02–71.72)	52.33[a] (1.87–99.5)	27.02[a] (0.5–73.83)	55.88[ab] (13.46–84.61)	83.37[b] (63.54–95.14)

Different lowercase letters show statistically significant differences between CH pastes and the control in comparison with biovolume and the percentage of live bacteria ($P < .05$).

group, and 4 images were taken per specimen, totaling 24 pictures per group.

The images were analyzed by bioImage_*L* (Chávez de Paz, Farmington, CT) software for structural analysis of biofilm formed on the dentin blocks and the percentage of live cells according to Chávez de Paz (17).

pH Test

For pH analysis, the manipulated pastes were handled in the same proportion as that used for the antibiofilm test and inserted into polyethylene tubes with the aid of a syringe and needle. Immediately afterward, the tubes were placed in flasks containing 10 mL ultrapure water. After the time intervals of 3, 24, 72, and 168 hours, the pH of the water was measured with a pH meter that had previously been calibrated in pH solutions 4, 7, and 14. Flasks containing only water without material were also measured. Ten specimens were analyzed per group.

Statistical Analysis

Data obtained on antibiofilm analysis and pH tests were submitted to normality analysis using the Shapiro-Wilk test. Because of the absence of normality of the data on antibiofilm action, the Kruskal-Wallis and Dunn tests were used for global and individual comparisons. The pH data showed normal distribution, and the analysis of variance and Tukey tests were used for global and individual comparisons. The significance level was 5%.

Results

Table 1 presents the values of the mean and standard deviation of pH of the tested calcium hydroxide pastes in the different time intervals and also shows the differences between pastes in the same period (lowercase) and between periods in the same paste (capital letters). All the tested pastes provided pH values above 10.0 in all periods analyzed. The highest pH values were found for the calcium hydroxide paste containing ciprofloxacin, and with the exception of the 24-hour time interval, the lowest values were found for the calcium hydroxide paste containing sodium diclofenac.

Table 2 presents the median, minimum, and maximum values of the biovolume and the percentage of live bacteria in the biofilm after contact with the CH pastes tested. There were no statistically significant differences between the CH pastes with respect to the biovolume. Regarding the percentage of live bacteria, there were statistically significant differences between the calcium hydroxide pastes containing the NSAIDs and antibiotic and the control ($P < .05$). In the comparisons between the calcium hydroxide paste and the control and in the comparisons of the calcium hydroxide paste with NSAIDs and calcium hydroxide paste with antibiotic, there were no statistically significant differences ($P > .05$). Figure 1 shows representative images of the biofilm of each calcium hydroxide paste tested and the control group.

Discussion

The null hypotheses tested in the present study were rejected because the groups that presented the addition of NSAIDs and ciprofloxacin increased the effectiveness of the antimicrobial action in biofilm and interfered in the pH of the calcium hydroxide paste.

The methodology used for testing the antimicrobial action of the calcium hydroxide pastes and substances using confocal microscopy with live/dead dye was similar to that used previously (18).

Dissolution of biofilms by dressings is crucial because a significant area of the root canal system is inaccessible to endodontic instruments. The effectiveness of calcium hydroxide against strains of *E. faecalis* and other microorganisms has been widely discussed in the literature (19–23).

Frequent recovery of *E. faecalis* in root canals with endodontic treatment failure has been widely reported (24, 25). *E. faecalis* has shown high resistance to medications used during treatment and is 1 of the few microorganisms that has been shown to resist the *in vitro* antibacterial effect of calcium hydroxide (10, 26). The resistance of this microorganism to calcium hydroxide is related to the presence of a proton pump (10).

Because of the resistance of *E. faecalis* to calcium hydroxide, substances have been associated with calcium hydroxide paste, such as chlorhexidine (19, 20, 27–29), iodine (27), camphorated

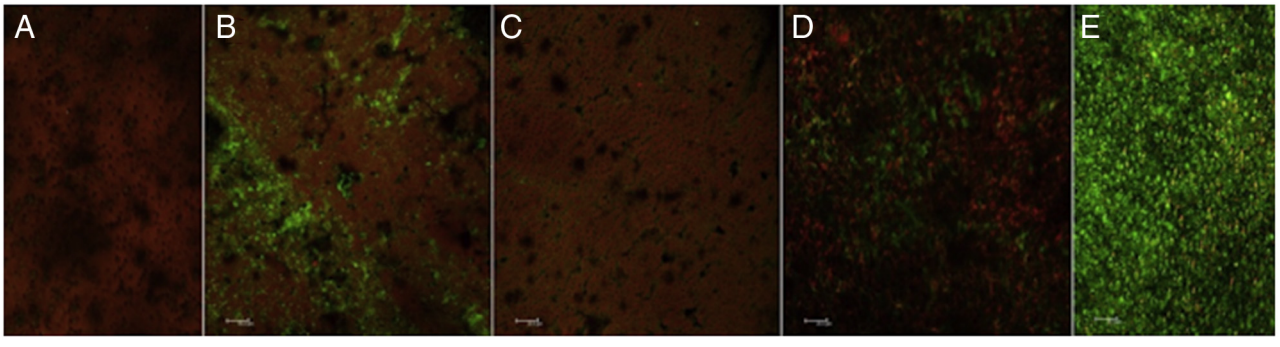

Figure 1. Confocal laser scanning microscopy of biofilms treated with (*A*) sodium diclofenac, (*B*) ibuprofen, (*C*) ciprofloxacin, (*D*) calcium hydroxide, and (*E*) control. Live cells are seen in *green*, and dead cells are seen in *red*. Each picture represents an area of 275 × 275 μm. Bars represent 20 μm.

paramonochlorophenol (29, 30), iodoform (28), propolis (31), and linezolid (32), in order to enhance the antimicrobial action. Several recent studies have revealed that NSAIDs have proven antimicrobial activity (11, 13, 14). However, the association of NSAIDs with calcium hydroxide paste and the effect of this association on biofilm had not been tested.

The results showed that calcium hydroxide pastes containing the drugs significantly reduced the percentage of live bacteria in the biofilm, a fact not noted with pure calcium hydroxide pastes; indeed, the results of pure calcium hydroxide paste corroborate the findings of another study (18). In relation to pastes containing drugs, diclofenac reduced the pH of the paste at the end of 7 days, maintained a more effective antimicrobial action, and showed that the antimicrobial action is not related to the alkalinity of the paste only. Diclofenac sodium has a highly bactericidal action against gram-positive and gram-negative bacteria by inhibiting the synthesis of bacterial DNA (11). The antimicrobial action of sodium diclofenac, ibuprofen, and ciprofloxacin has been shown by the radial diffusion assay (13). Another study using *in vivo* and *in vitro* tests proved that the antimicrobial action of diclofenac was increased when associated with another drug (12).

With respect to pH, the association of diclofenac reduced the pH when compared with the pure paste at the time intervals of 3, 72, and 168 hours; however, in all periods, the pH remained above 10. Alkalinity is important to antimicrobial and biological action. The alkaline pH favors the activation of alkaline phosphatase, an enzyme that allows the reaction of phosphate with calcium ions from the bloodstream, forming calcium phosphate, which corroborates the biomineralization process (33).

Another important clinical factor in the association of anti-inflammatory drugs with calcium hydroxide paste would be a topical action in the control of inflammation, which could reduce postoperative pain in patients after endodontic intervention. However, further studies are necessary to confirm this efficiency. Noting the effectiveness of pure calcium hydroxide paste revealed that its associations with diclofenac sodium, ibuprofen, and ciprofloxacin strengthened the antimicrobial action, with the best performance being observed in association with diclofenac sodium.

Conclusion

The authors concluded that the association of NSAIDs or antibiotic did not interfere with the pH of calcium hydroxide paste and increased the antimicrobial action of calcium hydroxide paste against the biofilm of *E. faecalis*.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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