Antibacterial Properties of Chitosan Nanoparticles and Propolis Associated with Calcium Hydroxide against Single- and Multispecies Biofilms: An *In Vitro* and *In Situ* Study

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Abstract

Introduction: The aim of this study was to evaluate the efficacy of chitosan nanoparticles (CNPs) and ethanolic propolis extract (EPE) incorporated into a calcium hydroxide paste ($Ca[OH]_2$) to kill bacterial biofilms. **Methods:** Human root canal dentin was infected with *Enterococcus faecalis* for 21 days and also intraorally for 48 hours followed by incubation in brain-heart infusion for 48 hours to standardize biofilm growth. $Ca(OH)_2$ pastes associated or not with CNPs or EPE were tested on biofilms for 7 and 14 days. Distilled water was used for control purposes. After the treatment procedures, microbiological analysis was performed to determine the reduction in *E. faecalis* colonies. Confocal microscopy was used to determine the percentage of cell viability in polymicrobial biofilms before and after the exposure to the experimental intracanal medications. **Results:** All experimental pastes were able to significantly reduce the *E. faecalis* colony-forming units (CFU) after 7 or 14 days ($P < .05$). However, the CFU reduction was significantly improved when CNPs were incorporated into the $Ca(OH)_2$ paste ($P < .05$). The multispecies biofilms treated with $Ca(OH)_2$ showed similar percentages of bacterial viability to the control regardless of the exposure time ($P > .05$). The viable cell count significantly dropped in the $Ca(OH)_2$/CNPs groups for both 7 and 14 days ($P < .05$), whereas the $Ca(OH)_2$/EPE groups were only effective in eliminating bacteria during the first 7 days of treatment ($P < .05$). **Conclusions:** Incorporating CNPs into pastes of $Ca(OH)_2$ could potentially be beneficial when using interappointment intracanal medications because of their ability to kill bacteria in short- and long-term exposure. *(J Endod 2017;■:1–5)*

Key Words

Biofilm, calcium hydroxide, chitosan, dentin, propolis, root canal dressings

Significance

Incorporating chitosan nanoparticles into a $Ca(OH)_2$-based paste has the potential of increasing its antibacterial ability on multispecies biofilms, even after prolonged times of interappointment intracanal medications.

Bacterial biofilms and their products in avascular and necrotic root canal systems are the main etiologic factor of apical periodontitis (1). Chemomechanical preparation is used to eliminate as much as possible bacteria lodged inside the root canal system (2). Nonetheless, it has been shown that it is impossible to achieve complete disinfection through cleaning and shaping (3). Therefore, the remaining bacteria could multiply between the appointments and reach similar levels to the initial treatment (3, 4). The difficulty in eliminating microorganisms will determine the use of appropriate interappointment intracanal medication after biomechanical preparation.

Calcium hydroxide ($Ca[OH]_2$) is commonly used as an intracanal medication because of its alkaline pH and its ability to damage the bacterial DNA (5). Nevertheless, the buffering action of dentin neutralizes the action of $Ca(OH)_2$ (6). Consequently, many bacterial species, which mainly belong to the genus *Enterococcus*, can remain alive because they are alkali resistant at pH = 9 and/or pH = 10 in infected root canals (7).

The limitations of $Ca(OH)_2$ have prompted researchers to look for new alternatives. Propolis is a flavonoid-rich resinous product of honeybees, which is known for its antibacterial, antifungal, and healing properties (8, 9). In addition, it has been shown that it is 10 times less cytotoxic than $Ca(OH)_2$ (10). Similar properties are shown by chitosan, which is a nontoxic cationic natural biopolymer that is usually obtained by the alkaline deacetylation of chitin. This biopolymer also possesses activity against a wide variety of fungi and bacteria (11, 12). Antibacterial nanoparticles, like chitosan nanoparticles (CNPs), have a significant antibacterial activity when compared with other antibacterial powders. This is because of a higher surface area and charge density that enables CNPs to react with the negative charge surface of bacterial cells, resulting in bacterial cell death (13).

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Currently, there is limited information in the literature regarding the use of propolis and CNPs as intracanal dressings, and no studies were conducted under *in situ* conditions, which is a more realistic scenario because it simulates *in vivo* conditions. The goal of the present study was to evaluate the antibacterial properties of $Ca(OH)_2$ alone or in combination with CNPs or ethanolic propolis extract (EPE) powder as an intracanal medication against uni- and multispecies bacterial biofilms using an *ex vivo* and *in situ* experimental model.

Material and Methods

All of the chemicals used in this study were of analytic grade and were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Chitosan was obtained from CarboMer Inc (San Diego, CA), and propolis was obtained from Apiter Laboratories (Montevideo, Uruguay). CNPs and EPE powder were synthesized according to the protocols published by Kishen et al (14) and Franchi et al (15), respectively. The institutional ethical committee of the university approved this study.

First Stage: Antibacterial Effect of the Experimental Intracanal Pastes—An *In Vitro* Study

Sectioning of the Samples. Human mandibular premolars with a single canal were instrumented using the ProTaper Gold system (Dentsply Tulsa Dental, Tulsa, OK) up to F3 file and irrigated with 5% sodium hypochlorite, 17% EDTA, and distilled water. Subsequently, the roots were sectioned horizontally to obtain 40 discs of 4 mm. The 3 mm below the cervical third and the 4 mm above of the apical third were discarded. The specimens were autoclaved, and the external surfaces were sealed with nail varnish.

Dentin Infection. *Enterococcus faecalis* (American Type Culture Collection 29212) was suspended in brain-heart infusion (BHI) to obtain 1×10^7 colony-forming units (CFUs). The bacterial suspension was adjusted to an optical density of 0.5/mL. Each sample was transferred into a 1.5-mL Eppendorf tube and inoculated with 1 mL *E. faecalis* suspension. The tubes were centrifuged 4 times for 5 minutes at 5000*g* to facilitate the bacterial penetration. A fresh cell suspension was added between centrifugations. Subsequently, each sample was incubated under continuous agitation at 37°C in 1 mL BHI for 21 days. The broth was refreshed every 48 hours. After the infection period, the samples were washed with distilled water to remove nonadherent bacteria.

Paste Preparation and Root Canal Treatments. A 30%–35% $Ca(OH)_2$ paste (Ultracal XS; Ultradent Products, Salt Lake City, UT) was used as a single vehicle through the experiment. The addition of 5 mg CNPs to $Ca(OH)_2$ was based on an antibacterial nanoparticle-based study (16); the $Ca(OH)_2$ + EPE paste was prepared with the same proportions (5 mg) as the $Ca(OH)_2$ + CNPs for standardization purposes because no information is available regarding the association of $Ca(OH)_2$ and EPE in powder. The infected specimens were divided into 4 groups (n = 10/group) and treated as follows: group 1, the samples were immersed in distilled water for 30 minutes (n = 10); group 2, the root canals were filled with $Ca(OH)_2$ (n = 10); group 3, the root canals were filled with $Ca(OH)_2$ + CNPs (n = 10); and group 4, the root canals were filled with $Ca(OH)_2$ + EPE (n = 10). Immediately after the treatment procedures, the samples were incubated at 37°C and 100% humidity for 7 (n = 5/subgroup) and 14 (n = 5/subgroup) days.

The pH of the experimental pastes was also determined. The pH was measured immediately after their preparation (0 days) and after 1, 2, 7, and 14 days in order to evaluate if the CNPs or EPE were able to vary the pH of the $Ca(OH)_2$.

Microbiological Analysis. After the incubation periods, the root canals were irrigated with distilled water and sonic agitation (tip 25/.04, medium) to remove the intracanal medications. The canals were dried with sterile paper points, and then Largo Peeso Reamers size 5 (ISO size 150) (Dentsply Maillefer, Ballaigues, Switzerland) were used to remove dentinal shavings (approximately 300 μm into the dentin) (17), which were immediately transferred into a microcentrifuge tube containing 1 mL BHI and vortexed for 10 seconds. After serial dilutions, 100 μL of the solution was inoculated onto BHI agar and incubated for 48 hours at 37°C. CFUs were calculated (log CFU/mL). The experiment was performed in triplicate.

Second Stage: Microscopic Analysis of the Bacterial Viability after the Experimental Treatments—An *In Situ* Study

***In Situ* Dentinal Infection.** The sectioning and intraoral infection of the samples were performed according to the protocol described by del Carpio-Perochena et al (17). Briefly, 32 sterile human dentin blocks (approximately 5 × 5 × 3 mm) were fixed in cavities of a palatal orthodontic device to allow intraoral dentin infection for 48 hours. After that, the samples were incubated in BHI broth for 48 hours at 37°C to standardize the bacterial colonization. A single volunteer used the orthodontic device throughout the experiment. The infected specimens were irrigated with distilled water to remove nonadherent bacteria.

Treatment of the Infected Dentin. The experimental medications were prepared in the same way as described in the first stage of the study. The infected dentin blocks were divided into 4 groups (n = 8/group) and individually immersed into the experimental intracanal pastes using 96-well tissue culture plates. Then, the plates were incubated for 7 (n = 4/subgroup) and 14 (n = 4/subgroup) days at 37°C and 100% humidity.

Post-treatment Bacterial Viability. After the experimental periods, the pastes were washed with distilled water for 5 minutes. Later, the remaining biofilm was dyed with the Live/Dead *Bac*Light Bacterial Viability Kit (Invitrogen, Eugene, OR) (excitation/emission ~495/515 nm) and analyzed using a multichannel confocal microscope (Nikon Ti Eclipse; Nikon, Melville, NY). Four samples per period of time (7 and 14 days) were stained and analyzed immediately after infection to determine the percentage of live cells before any treatment. These data were combined to obtain a single control group. Five stacks per sample were scanned at ×40 and 1-μm step size. The area of each image represented 275 × 275 μm. The level of laser penetration into the biofilm was 30–80 μm from the top of the biofilm to the entrance of the dentinal tubules. The percentage of viable bacterial cells pre- and post-treatment was determined using the Bioimage_L program (The MathWorks, Natick, MA) (18).

Statistical Analysis. One-way analysis of variance followed by the Tukey test was used for multiple comparisons in the first stage of the study. The nonparametric Kruskal-Wallis and Dunn tests were used in the second stage because the data did not show a normal distribution. Significance levels were fixed at $P < .05$. Prisma 5.0 (GraphPad Software Inc, La Jolla, CA) was used as the analytical software.

Results

First Stage: *In Vitro*

There was not a significant pH variation of the pastes through time (0, 1, 2, 7, and 14 days), which suggests that the addition of CNPs or EPE into $Ca(OH)_2$ did not interfere with the pH of $Ca(OH)_2$. Consequently, the data were combined to provide a single mean per group. The means ± standard deviations (95% confidence interval [CI]) of the pH variation of the $Ca(OH)_2$, $Ca(OH)_2$/CNPs, and $Ca(OH)_2$/EPE groups