IN VITRO STUDY

Antibiofilm Activity of Five Different Endodontic Filling Materials Used in Primary Teeth Using Confocal Laser Scanning Microscopy

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Abstract: ***Purpose:*** *The purpose of this study was to evaluate the effect of five endodontic materials for primary teeth on in vitro mixed-species biofilms.* ***Methods:*** *Zinc oxide eugenol (ZOE) cement, Vitapex, Calen paste thickened with zinc oxide (ZO), pure calcium hydroxide ($Ca(OH)_2$) paste, and iodoform were evaluated. Sterile water was used as a control. Mixed-species biofilm was incubated in anaerobic conditions for 21 days. The biofilm specimens were placed in contact with the endodontic materials for periods of seven and 30 days. The biofilm was studied by using confocal laser scanning microscopy. The cell viability ratio was calculated. The results were analyzed using analysis of variance and Tukey tests.* ***Results:*** *There was a statistically significant difference between groups at both seven and 30 days (F equals 73,073, P=0.00). After 30 days, 69 percent, 51 percent, and 35 percent of the biofilm volume fluoresced red, indicating the proportion of bacteria killed by iodoform, Vitapex, and ZOE cement, respectively. Calen plus ZO and pure $Ca(OH)_2$ paste were the least effective materials against the biofilms.* ***Conclusions:*** *Pure iodoform paste and iodoform containing Vitapex were the most effective materials against the biofilms. Vitapex appears to be a suitable endodontic material for primary teeth.* *(Pediatr Dent 2017;39(2):145-9) Received February 19, 2016 | Last Revision November 23, 2016 | Accepted November 25, 2016*

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The tortuous and bizarre morphology of root canal systems of primary teeth, as well as the difficulty with patient management and isolation, continue to present challenges for dental practitioners in performing root canal therapy. The apical foramen, large accessory canals (lateral and furcation canals), and dentinal tubule exposure due to physiological root resorption may cause structural alteration and increase the permeability of the root surface to microbial toxins.[1] Consequently, eliminating biofilms from an infected primary tooth root canal system by instrumentation and irrigation alone is a challenge. Endodontic filling materials with antimicrobial activity could play an important role in further facilitating the elimination of the microflora.

Several materials have been used as endodontic fillings in primary teeth. In the primary dentition, zinc oxide eugenol cement (**ZOE**) in particular has been widely used.[2,3,4] Due to ZOE's irritating potential[4] and low resorption capacity,[5] materials containing iodoform and/or calcium hydroxide (**$Ca(OH)_2$**) have been used because of their antibacterial activity, biocompatibility, and easy resorption.[2,4,5] Other studies have also suggested using combinations of zinc oxide and $Ca(OH)_2$[6,7] or $Ca(OH)_2$,[6] and iodoform[5] to improve the chances of success of root canal therapy in primary teeth.

Agar diffusion test (**ADT**) has been extensively used in the past to evaluate the antimicrobial efficacy of endodontic materials,[2,3] despite its well-known limitations. The inhibition zone is directly influenced by two factors: (1) the solubility and diffusibility of the material in the agar medium; and (2) the chemical interactions between the medium and the disinfecting agents. The buffering capacity of the agar plate can also influence the size of the inhibition zone, since high pH, for example, can affect the antimicrobial effect of a material.[8]

While ADT is a standardized method for susceptibility testing of systemic antibiotics, corresponding standardization regarding reliability and interpretation of ADT results with endodontic materials does not exist. Thus, it is important to evaluate the antimicrobial activity of the root filling materials for primary teeth using appropriate methods. In recent years, a technique involving viability staining and confocal laser scanning microscopy (**CLSM**) has become a widely used method for the measurement of the antimicrobial effect of different materials against single species and polymicrobial biofilms.[9-11]

Bacteria that grow in biofilms are responsible for diverse persistent infections.[10,12,13] A biofilm is a community of microorganisms embedded in a matrix of extracellular polymeric substance and attached to a solid surface. Within this community, the biofilm bacteria express a wider variety of different phenotypes than the same bacteria in a planktonic state.[14] Microbial invasion of the root canal system eventually leads to pulpal necrosis and apical periodontitis. As the bacteria in the necrotic root canal grow mostly in sessile forms, the success of endodontic treatment will depend on the effective elimination of such biofilms.[12]

The presence of bacteria organized in biofilms in endodontic infections of primary teeth was demonstrated by Rocha et al.[15] The known difficulty to eradicate biofilms could lead to the persistence of the inflammatory process, delaying periapical healing. Bacteria identified in necrotic primary teeth are similar to those found in permanent teeth, consisting predominantly of cocci and bacilli.[16] Filaments and spirochetes have also been detected. While planktonic microorganisms can

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be readily eliminated by several disinfecting solutions and medicaments used in endodontics, the killing and removal of biofilm bacteria from the root canal system remain a major challenge.[12] The resistance of microbes in biofilms to antimicrobial agents can be 100 to 1,000 times greater than in planktonic culture.[10,14] To obtain clinically relevant results, it is, therefore, important to evaluate the ability of endodontic materials to kill biofilm bacteria in direct contact using multispecies biofilm models.

The purpose of the present study was to measure the antibiofilm effect of commonly used endodontic materials for primary teeth using viability staining of the exposed biofilms and confocal laser scanning microscopy.

Methods

Five different endodontic materials were tested: (1) group one—zinc oxide eugenol cement (**ZOE**; S.S. White Artigos Dentários Ltda., Rio de Janeiro, Rio de Janeiro, Brazil); (2) group two—a premixed $Ca(OH)_2$ and iodoform paste (Vitapex; Neodental International Inc., Federal Way, Wash., USA); (3) group three—1.0 g of a commercial $Ca(OH)_2$ and polyethylene glycol-based paste (Calen; S.S. White Artigos Dentários Ltda.) thickened with 1.0 g zinc oxide (**ZO**; Biodinâmica Química e Farmacêutica Ltda., Ibiporã, Paraná, Brazil); (4) group four—1.0 g $Ca(OH)_2$ mixed with one mL sterile water (pure $Ca(OH)_2$ paste); and (5) group five—1.0 g iodoform mixed with one mL sterile water (pure iodoform paste). Sterile water was used as a control.

Biofilm growth. Thirty-six sterile hydroxyapatite (**HA**) discs (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, South Williamsport, Pa., USA) were used as substrate to grow biofilms, as previously described.[10,11,13] The HA discs were coated overnight at four degrees Celsius with bovine dermal collagen type one (10 mg/mL collagen in 0.012-M HCl in water; Cohesion, Palo Alto, Calif., USA).[10] Subgingival plaque was collected from a healthy adult volunteer and suspended in brain-heart infusion broth (**BHI**; Becton Dickinson, Sparks, Okla., USA). The cell density was adjusted in a spectrophotometer at 405 nm (model no. 3350; Bio-Rad Laboratories, Richmond, Va., USA) to a density of approximately 7.5 by 10^7 colony forming units per milliliter in BHI broth. The discs were incubated in BHI in 24-well cell culture plates, with the plaque suspensions under anaerobic conditions using an anaerobic bag and anaerobic indicator (AnaeroGen, OXOID, Hampshire, Winchester, UK) at 37 degrees Celsius. Each well contained 1.8 mL sterile BHI broth and 0.2 mL inoculum, in which the samples were kept submerged. The BHI medium was replaced with a fresh BHI medium once a week without addition of new microorganisms.

Bacterial viability assay on biofilm. A thin layer of each material (0.1 g) was carefully placed on the three-week-old biofilms. The specimens were then placed in the incubator for experimental periods of seven and 30 days (n equals three for each).

The material on each of the discs was gently washed three times with a phosphate-buffered saline (**PBS**) solution. Live/Dead BacLight Bacterial Viability kit L-7012 for microscopy and quantitative assays (Molecular Probes, Eugene, Ore., USA) containing separate vials of the two component dyes (SYTO 9 and propidium iodide in a one-to-one mixture) in solution was used for staining the biofilm, following the manufacturer's instructions. The excitation/emission maxima for these dyes are approximately 480 out of 500 nm for the SYTO 9 stain and 490 out of 635 nm for propidium iodide. Fluorescence from the stained cell was viewed using a CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, Ontario, Canada). Simultaneous dual-channel imaging was used to display green and red fluorescence. SYTO 9 (green fluorescence) stains the viable bacteria, while bacteria with impaired membranes are stained with propidium iodide to produce red fluorescence.

The confocal laser scanning microscopic images of 512 by 512 pixels were captured using a EZ-C1 3.40 camera and constructed using 691 software (Nikon Corp., Tokyo, Japan). Five randomly selected areas on the disc surface of each specimen were scanned (1.2 mm by 1.2 mm for each area) by CLSM and three-dimensional reconstruction analysis. For each group and period, three specimens were assigned, totaling 15 measurements per group and time period. Five separate scans, 20-μm deep (0.5 μm step size, 40 slices/stack), of each sample were performed to standardize the area and volume of the biofilm scanned.

Live/dead ratios of HA disks were analyzed using Imaris 7.2 software (Bitplane Inc., St. Paul, Minn., USA). The volume ratio of red fluorescence to green and red fluorescence indicated the proportion of killed cells. The proportions of dead cell volume after exposure to different root dental material were subjected to analysis of variance and Tukey tests using SPSS 17.0 software (SPSS Inc., Chicago, Ill., USA).

Results

A total of 180 scanned biofilm areas were analyzed for the proportions of red and green fluorescence (Figure). There were significant differences in the microbial killing efficacy between different endodontic materials (F equals 73.073, P=0.00). The proportion of killed microbes was also dependent on exposure time. In the negative control group, 93 percent of bacterial cells were viable after seven days, and 94 percent after 30 days. Iodoform and Vitapex were the most effective materials against the biofilm (Figure).

After seven days, Vitapex and pure iodoform paste had killed 46 percent and 41 percent of the biofilm bacteria, respectively, followed by ZOE, which killed 35 percent of bacteria. While pure $Ca(OH)_2$ paste killed 16 percent of biofilm, in the Calen plus ZO group, only nine percent of biofilm bacteria was killed. After 30 days of incubation with pure iodoform, the volume of killed biofilm was 69 percent, while 51 percent of biofilm bacteria were killed after the use of Vitapex. ZOE was weaker than the iodoform-containing materials, killing 34 percent of the biofilm microbes. Calen plus ZO and pure $Ca(OH)_2$ paste were the least effective against biofilm microbes. After 30 days, only 27 percent and 19 percent of the biofilm bacteria were killed by Calen plus ZO and pure $Ca(OH)_2$ paste, respectively (Table).

Discussion

The antimicrobial effect of endodontic filling materials used in primary teeth has been much studied using planktonic bacteria in agar diffusion or direct contact tests, which both have great limitations and are not suited to study killing of biofilm microbes.[2,3] Consequently, little is known about the effect of these materials on endodontic biofilms. To date, only a few studies have been performed using CLSM and the two-component BacLight staining to evaluate the antimicrobial effect of root canal filling materials for primary teeth.[18] This method is frequently used currently to evaluate the antimicrobial effect of different materials against microbes found in endodontic infections[9-11] and presents several advantages as a rapid and relatively

easy-to-use method that combines both viable and total counts in one step. The two stains in the kit differ in their ability to penetrate normal and damaged bacterial cells; live bacteria with intact membranes fluoresce green (SYTO9), whereas bacteria with damaged cell membranes fluoresce red (propidium iodine).[13] A variety of different bacteria organized as biofilms are known to be present in inaccessible areas of the root canal system,[15] and the microbes organized in communities generally have a low metabolic rate and tend to be resistant to antimicrobial agents.[10,13] Consequently, endodontic filling materials should always be tested for their antimicrobial capacity using multispecies biofilm models.

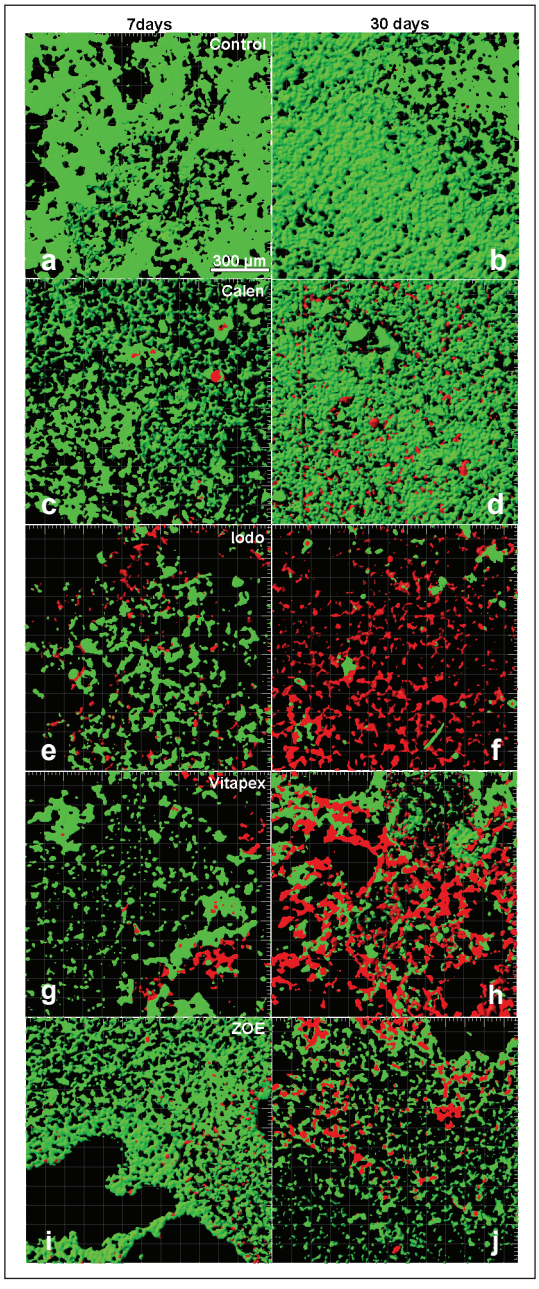

Figure. Three-dimensional constructions of confocal laser scanning microscopy scans of multispecies biofilms after incubation with different root canal filling materials for seven and 30 days: (a) Control group, seven days; (b) Control group 30 days; (c) Calen+ZO, seven days; (d) Calen+ZO, 30 days; (e) Iodoform, seven days; (f) Iodoform, 30 days; (g) Vitapex, seven days; (h) Vitapex, 30 days; (i) ZOE, 7 days; (j) ZOE, 30 days. Green indicates viable cells; red indicates dead cells.

Previous in vitro studies with the same multispecies biofilm model used in the present study have shown that the biofilm microbes are relatively sensitive to common endodontic disinfecting agents during the first two weeks of biofilm growth but become much more resistant after two weeks.[10,13] After three weeks of growth and maturation, the biofilms remained resistant to the disinfecting agents for the whole follow-up period of several months.[13] This, together with the fact that most endodontic biofilms in the root canal system are likely to be older than two weeks, was the basis of using three-week-old biofilms in the present study.

The results of the present study showed that there were significant differences between different endodontic materials in terms of their effectiveness in killing biofilm microbes. Pure $Ca(OH)_2$ paste as well as Calen thickened with ZO demonstrated only weak effects against the biofilm. $Ca(OH)_2$ is well-known for its antimicrobial potential on endodontic bacteria grown in planktonic culture. However, its effect against biofilms is inconclusive.[19,20] In addition, meta-analysis on clinical trials with $Ca(OH)_2$ root canal dressing has suggested its limited efficacy in the eradication of microorganisms from infected root canals, even after instrumentation and irrigation with NaOCl solution.[21] Since the antimicrobial action of $Ca(OH)_2$ is related to its high pH, which depends on the release and diffusion of hydroxyl ions,[22] such results may be explained by the difficulty of maintaining the high concentration of hydroxyl ions. Some bacteria can increase their tolerance to high pH by the activation of specific proton pumps, specific enzymatic systems, and/or buffering mechanisms, which help to keep the internal pH practically constant.[23] In addition, metabolic end products such as organic acids generated during microbial growth may help them to neutralize high environmental pH.[23]

Pure $Ca(OH)_2$ products are strongest in maintaining high pH in the presence of neutralizing factors.[22] Also, viscous vehicles can interfere with the ability of $Ca(OH)_2$ to release hydroxide ions in comparison to $Ca(OH)_2$ in a water vehicle.[24]

Table. PROPORTION OF DEAD BACTERIAL VOLUME (MEAN±SD) IN THE BIOFILMS, CALCULATED AS THE PROPORTION OF THE DEAD CELL VOLUME (RED COLOR) TO THE ENTIRE BACTERIA VOLUME (RED + GREEN) AFTER EXPOSURE TO THE DIFFERENT ROOT CANAL FILLING MATERIALS*

Group	Material	7 days	30 days
I	Zinc oxide eugenol	0.35±0.64[a]	0.34±0.08[c]
II	Vitapex	0.47±0.17[b]	0.51±0.14[a]
III	Calen + ZO	0.09±0.53[c]	0.27±0.67[c,d]
IV	Calcium hydroxide	0.16±0.83[c]	0.19±0.10[d]
V	Iodoform	0.41±0.10[a,b]	0.69±0.12[b]
Control	Saline	0.08±0.32[c]	0.06±0.04[c]

* Different small letters indicate statistically significant difference ($P<0.05$) between materials within the two experimental times (in vertical columns).

However, in the present study, there was no significant difference in the results obtained with Calen paste thickened with ZO and pure $Ca(OH)_2$.

The superior effectiveness of ZOE in killing biofilm, in comparison to Calen plus ZO, may be due to the absence of eugenol release in this association, which is mainly responsible for the ZOE antimicrobial effect over a short period of time.[2,25] Although several studies have shown a strong antimicrobial effect by ZOE-containing materials,[3,25] other studies have shown only limited antimicrobial activity.[2,26] Such differences could be explained by the confounding factors involved in the agar diffusion methodology, different species of bacteria tested, and the concentration of eugenol.[25] The results of the present study show that ZOE exhibited moderate activity against biofilm microbes. ZOE is not particularly antibacterial once it has set,[25] which may explain why ZOE did not kill additional biofilm bacteria after seven days of exposure.

Our results show that iodoform paste, followed by Vitapex, another iodoform-containing material, were the most effective material against the biofilms. Vitapex presents in its composition 40.4 percent iodoform and 30 percent $Ca(OH)_2$, and it seems that $Ca(OH)_2$ reduced the antibacterial effectiveness of iodoform, the main agent responsible for the antibiofilm effect of Vitapex. This finding agrees with Ordinola-Zapata et al.[27] and is likely to be attributed to the release of iodine ion.[28] Iodine has high reactivity in precipitating proteins and oxidizing essential enzymes and has, therefore, been used as the antimicrobial agent in primary teeth with necrotic pulps.[28,29] Although some studies demonstrate that $Ca(OH)_2$ together with iodoform present weak antibacterial activity,[2,3] high clinical and radiographic success rates with Vitapex in root canal therapy for primary teeth have been reported.[30,31]

Pure iodoform and pure $Ca(OH)_2$, both mixed into pastes with sterile water, were included in the present study in order to better understand the relative role of these compounds in the antimicrobial properties of the combination products Vitapex and Calen plus ZO. No previous studies have reported the use of pure iodoform as an endodontic filling material for primary teeth. Walkhoff originally described the formula for iodoform paste in 1928 and used it as a root canal dressing in permanent teeth.[25,28] In primary teeth; however, the iodoform had always been studied in combination with other materials.[2,5,25,28]

The high level of clinical and radiographic success of pulpectomy with Vitapex in primary teeth may be related to its antibacterial properties and the material's distinctive property of rapid resorption when in contact with living tissue.[30,31] Our results suggest, in accordance with other studies, that the good performance of Vitapex was due to iodoform, since iodoform alone also resulted in the killing of nearly 70 percent of the biofilm bacteria.

While the antimicrobial efficacy of an endodontic filling material may be vital to achieving success in endodontic therapy, it is not the only property required of an ideal material. Additional studies should be performed to evaluate other biological properties of materials used in endodontic treatment of primary teeth.

Conclusions

Based on this study's results, the following conclusions can be made:

1. Pure iodoform paste and iodoform containing Vitapex were the most effective materials against the biofilms.
2. Vitapex appears to be a suitable root canal filling material for primary teeth.

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