**In vitro study**

Capacity of a hydroxyapatite–lysozyme combination against *Streptococcus mutans* for the treatment of dentinal caries

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**Abstract**

**Background:** One current strategy for the treatment of carious lesions is the use of biomaterials with antimicrobial activity.

**Aims:** The aim of this study was to evaluate a combination of hydroxyapatite and lysozyme for the treatment of dentinal caries by measuring *Streptococcus mutans* counts before carious tissue sealing, and 24 h, 1 month, and 6 months after treatment.

**Materials and Methods:** Forty permanent third molars were selected, and flat dentin surfaces were prepared. The teeth were exposed to a cariogenic challenge with *S. mutans*. After challenge, the dentinal caries were collected from five specimens. The remaining specimens were treated with a mixture of hydroxyapatite and lysozyme in sodium laureth sulfate and sealed with composite resin. *S. mutans* counts were obtained 24 h, 1 month, and 6 months after sealing.

**Statistical Analysis:** The results were evaluated by descriptive statistics and Wilcoxon signed-rank test.

**Results:** A significant reduction in *S. mutans* (CFU/mL) was observed in dentinal lesions 1 month after treatment with hydroxyapatite/lysozyme in sodium laureth sulfate ($P = 0.0254$). Comparison of *S. mutans* counts obtained 24 h, 1 month, and 6 months after treatment revealed reductions only at the 1-month time point ($P = 0.0318$).

**Conclusions:** The combination of hydroxyapatite and lysozyme may be an alternative for reducing the *S. mutans* burden in dentinal caries.

**Keywords:** Dental caries; enzyme; *Streptococcus mutans*

**INTRODUCTION**

Dental caries are a very common condition caused by bacteria that form a biofilm on the surface of the teeth. In the presence of sugars or fermentable carbohydrates, the bacteria present in the biofilm produce acids, which demineralize the dental surface.[1] One current strategy for the treatment of carious lesions is the use of biomaterials with antimicrobial activity.[2,3]

From a histological standpoint, dentin caries can be classified as infected dentin or affected dentin.[4] The first layer exhibits extensive decalcification and degeneration of collagen fibers. The second layer is characterized by intermediate decalcification, reversible alterations in collagen fibers, and odontoblasts undergoing active re-calcification. Minimally invasive dentistry recommends the removal of infected dentin and preservation of the caries-affected dentin.[4,5]

Carious dentin can be regenerated by hydroxyapatite crystals, which penetrate and obliterate the dentinal tubules.[2-4] Carbonated hydroxyapatite nanocrystals are...
compatible in size, morphology, chemical composition, and crystal structure with native dentin, and can thus be used to re-mineralize enamel.[2]

In the same line, another agent that may help re-organize dentin affected by caries is lysozyme, which has antimicrobial properties. This agent has been reported to exert an inhibitory, bactericidal effect on oral pathogens, including Streptococcus mutans.[3]

Lysozyme induces bacterial lysis by breaking down linkages between N-acetylmuramic acid and N-acetylg glucosamine in the cell wall, thus neutralizing pathogenicity in Gram-positive and Gram-negative bacteria by degrading peptidoglycans in the bacterial cell wall.[7]

S. mutans, a Gram-positive oral pathogen, is considered the primary etiologic agent of dental caries. Some oral Gram-positive bacteria, including S. mutans, are resistant to direct lysis by lysozyme.[8] Instead, lysozyme may act on S. mutans by increasing cell permeability.[9]

The aim of this study was to evaluate the antimicrobial activity of a combination of hydroxyapatite and lysozyme on viable S. mutans counts before the sealing of caries-affected tissue, and after 24 h, 1 month, and 6 months after the sealing.

MATERIALS AND METHODS

This study was approved by the PUC-Campinas Research Ethics Committee (protocol #388.376).

Sample selection

Forty permanent third molars were selected at the PUC-Campinas dental clinic. All donor patients signed an informed consent form. The criteria for inclusion were permanent third molars with no cracks or fractures. After sample selection, the occlusal third was removed from each specimen using a double-sided diamond disc (KG Sorensen Indústria e Comercio Ltda, São Paulo, Brazil) using a low-speed handpiece (KaVo Dental Excellence Ind. Com. Ltda, Joinville, Santa Catarina, Brazil) under water cooling for dentin exposure, and the dentin surfaces were polished with wet silicon carbide sandpaper sheets, P600 grit (Água T223 advance, Norton, São Paulo, Brazil). A 4 mm × 4 mm paper label (Kalunga, São Paulo, Brazil) was placed onto each specimen to standardize the location of the carious lesion.

Under a laminar flow hood (Veco, Campinas, São Paulo, Brazil), the specimens were sealed using epoxy resin (Araldite, São Paulo, Brazil) and nail polish (Colorama, São Paulo, Brazil), except on the coronal dentin. After sealing, the label was removed from the occlusal third of each specimen to enable generation of the carious lesion.

Teeth were then exposed to a cariogenic challenge in brain–heart infusion (BHI) broth (LabCenter, São Paulo, Brazil), supplemented with 0.5% yeast extract (LabCenter, São Paulo, Brazil), 1% glucose (LabCenter, São Paulo, Brazil), 1% sucrose (LabCenter, São Paulo, Brazil), and S. mutans type strain ATCC 25175 (Fundação André Tosello, Campinas, São Paulo, Brazil), standardized to 0.5 McFarland turbidity (Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil). Samples were incubated in anaerobic jars (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C and subsequently stored in a bacteriological incubator (Fanem Ltda, São Paulo, Brazil) for 14 days. During this period, BHI broth (LabCenter, São Paulo, Brazil) was replaced for every 48 h.

After the cariogenic challenge, dentinal carious lesions were collected from ten specimens, placed in BHI broth (LabCenter, São Paulo, Brazil) and homogenized for 3 min in a vortex mixer (Prolab, São Paulo, Brazil). Five decimal dilutions were performed, and three 25 µL aliquots from each dilution were seeded onto the surface of mitis-salivarius-bacitracin medium (LabCenter, São Paulo, Brazil). All plates were incubated in the anaerobic jars (Oxoid Ltd., Basingstoke, Hampshire, England) with gas-generating envelopes (Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil) for 5 days at 37°C. After incubation, the viable bacterial count was determined.

The remaining samples were treated with hydroxyapatite and lysozyme in sodium laureth sulfate, applied with a microbrush (KG Sorensen Indústria e Comercio Ltda, São Paulo, Brazil) for 1 min onto the carious lesion. Specimens were then sealed with a composite resin (Dentsply, São Paulo, Brazil). The mixture of lysozyme and hydroxyapatite in sodium laureth sulfate was compounded as follows: 0.018 mg of 1% lysozyme (Sigma-Aldrich, São Paulo, Brazil) and 0.045 mg of 3% hydroxyapatite (Sigma-Aldrich, São Paulo, Brazil), both in powder form, were mixed in 1.8 mL sodium laureth sulfate (Tergentol®, Fórmula and Ação, São Paulo, Brazil) as vehicle. S. mutans counts were obtained 24 h, 1 month, and 6 months after sealing (n = 10 specimens per time point). For this procedure, the composite resin seal was removed with a round diamond bur (KG Sorensen Indústria e Comercio Ltda, São Paulo, Brazil) in a high-speed handpiece (Kavo Dental Excellence Ind. Com. Ltda, Joinville, Santa Catarina, Brazil), under saline cooling (Dauf, Fortaleza, Ceará, Brazil), to enable removal and collection of the carious lesions. All microbiological procedures (homogenization, dilution, and seeding) were described for counts obtained before sealing.

The results were evaluated by descriptive statistics and the Wilcoxon signed-rank test.

RESULTS

A significant reduction in S. mutans counts (CFU/mL) was observed in dentinal lesions 1 month after the
treatment with hydroxyapatite/lysozyme in sodium laureth sulfate (P = 0.0254). S. mutans counts obtained 24 h after treatment were not significantly reduced as compared with baseline counts or those obtained at 6 months (P > 0.05). Comparison of S. mutans counts obtained 24 h, 1 month, and 6 months after the treatment revealed reductions at the 1-month time point (P < 0.05) [Table 1].

DISCUSSION

Several substances with antimicrobial activity that may be used as adjuncts in the re-mineralization process of caries-affected teeth have been described in literature, including lysozyme and hydroxyapatite, as used in the present study. Combining antimicrobial agents broadens their spectrum of activity to inactivate many of the bacterial species present in the carious lesion, thus halting disease progression. [10]

A significant reduction in S. mutans counts was observed 1 month after treatment. This may be explained by the antimicrobial activity of lysozyme and by the effect of cavity sealing. This significant reduction in S. mutans 1 month after sealing is consistent with previous reports in the literature, [7-11] which state that lysozyme has a beneficial effect on the re-organization of caries-affected dentin by exerting antimicrobial activity.

In this study, the bactericidal effect of lysozyme was observed only 1 month after treatment. There are three possible explanations for this finding. The first possible explanation for this late effect of lysozyme is that S. mutans can synthesize extracellular polysaccharides such as glucan, which may reduce the permeability to lysozyme of the biofilm present in the carious lesion. Several active enzymes have been identified among the components of the dental pellicle, including lysozyme itself, [12] bacterial glycosyltransferase, and fructosyltransferase. [13] The lysozyme component of the pellicle has bactericidal activity and reduces the adhesion of S. mutans to hydroxyapatite. [14] The presence of lysozyme affects the amount of glucan produced by glycosyltransferase B, but it has no effect on the glucan structure. [15] Another possible explanation for an effect of lysozyme being observed only at the 1-month time point could be the acidic pH of the caries environment; this hinders enzyme activity, which is immediate at neutral pH. [16] The last hypothesis is that lysozyme acts as a cationic, low-molecular weight protein that is capable of inducing bacterial lysis by catalyzing the breakage of glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine in the polysaccharide backbone of the bacterial cell wall. [12] Curiously, many Gram-positive bacteria in the oral flora, including S. mutans, are resistant to direct lysis by lysozyme. [8] Therefore, lysis does not necessarily have to be the primary cause of cell death induced by lysozyme. Lysozyme may have a bactericidal mechanism of action similar to that of the other cationic peptides, which act on the cell membrane to produce a loss of selective permeability and increasing membrane permeability to electrolytes, which is followed by osmotic changes within the cell. [9] Bacterial membranes work as proton barriers and are capable of maintaining a relatively basic cytoplasm even in an acidic environment by proton extrusion, generally through proton translocation driven by membrane-bound ATPase. [17] In S. mutans, lysozyme causes massive potassium loss, which may lead to a marked decline in membrane potential, as K+ is the major monovalent cation in bacterial cells; loss of the energy-dependent membrane transport function; inactivation of potassium-dependent cellular enzymes; loss of turgor pressure; cessation of growth; and cell death. [17] This is consistent with the findings of this study at the 1-month time point.

After 6 months, S. mutans counts had increased, possibly due to degradation of the tooth restoration interface and bacterial infiltration and growth, as the samples were submerged in BHI, a nutrient-rich medium.

CONCLUSION

The results of this study show that incorporation of lysozyme and hydroxyapatite into composite resin reduced S. mutans counts 1 month after sealing, which is justified by the antimicrobial and re-mineralizing properties of these compounds. This combination may be a feasible alternative for the reduction of S. mutans burden in dentinal caries.

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Conflicts of interest
There are no conflicts of interest.

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Pinheiro, et al.: Antimicrobial activity of lysozyme against S. mutans

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