Effectiveness of casein phosphopeptide–amorphous calcium phosphate and lysozyme, lactoferrin, and lactoperoxidase in reducing *Streptococcus mutans* counts in dentinal caries

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This study compared the capacity of casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) to that of a combination of lysozyme, lactoferrin, and lactoperoxidase (LLL) in root canal disinfectant for reducing the *Streptococcus mutans* counts from dentinal caries. Forty human permanent third molars were selected, and flat dentin surfaces were created. Carious lesions were induced using a microbiological model. The specimens were randomly divided into 2 groups (n = 20) according to the type of agent used: group 1, CPP-ACP; group 2, LLL. The *S mutans* counts were performed before application and after the first, second, and third applications of the agents. The duration of each application was 3 minutes. Carious dentin specimens were homogenized, diluted, and seeded onto mitis salivarius–bacitracin plates for viable counts of *S mutans*. Results showed that there was no significant reduction in the number of *S mutans* in group 1 after the applications of CPP-ACP ($P > 0.05$). In group 2, a significant reduction of *S mutans* was observed after the third application of LLL ($P < 0.01$). These results indicate that 3 applications of LLL enzymes can be used to reduce the number of *S mutans* in dentinal caries lesions.

Received: December 23, 2015
Accepted: February 24, 2016

GENERAL DENTISTRY
SELF-INSTRUCTION
CE CREDIT
Exercise 400, p. 51
Subject code: Cariology (011)

During the cariogenic process, toxins released by the bacteria diffuse into the enamel, initiating biochemical reactions in the dentin; as this infectious process progresses into the intact enamel, it causes the loss of minerals and changes the dentin matrix.[1] The reduction of pH through the acidic production of microorganisms actives metalloproteinases, which hydrolyze the extracellular matrix by degrading the organic phase of the dentin, thus contributing to the progression of caries.[2,3] Demineralization and remineralization are dynamic processes that are present at the onset, progression, and reversal of caries, and the balance between these processes is the key to preventing and treating the disease.[4,5]

Dentinal caries can be histologically divided into surface (infected dentin) and deep lesions (affected dentin). Infected dentin causes extensive decalcification and degeneration of the collagen fibers. Affected dentin causes intermediate decalcification, reversibly altered collagen fibers, and odontoblasts with an active process of recalcification.[6,7] There are 6 distinct layers of carious dentin: 1, external, irreversibly demineralized; 2, translucent; 3, subtransparent; 4, sclerotic; 5, healthy dentin; and 6, predentin. Layers 2, 3, and 4 correspond to areas that are reversibly changed by carious lesions.[8]

Various therapeutic possibilities that could assist with the arrest or reversal of caries have been presented in the literature, such as remineralization, the reorganization of affected dentin, and the reduction of cariogenic bacteria, particularly *Streptococcus mutans.* Casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) is a compound that locates and attracts calcium ions and the phosphate from the tooth surface, increasing the concentration gradient in the enamel subsurface by promoting remineralization in situ.[9-11] The anticariogenic potential of CPP-ACP has been attributed to its ability to attract the amorphous calcium phosphate on the surface of the tooth while maintaining a state of supersaturation of minerals on the surface.[9] It is a bioactive compound with a noncytotoxic effect, high cell adhesion, and kinetic stabilization.[11]

The use of the enzymes lysozyme, lactoferrin, and lactoperoxidase (known together as *LLL*) has also been studied as an alternative for reducing microorganisms in carious lesions. These enzymes are present in the saliva and exert antimicrobial activity against bacterial, viral, and fungal pathogens.[12,13] Lysozyme is an antimicrobial molecule capable of degrading peptidoglycan from

Table 1. Results of microbiological assessment: *Streptococcus mutans* counts (log_{10}) in group 1 (CPP-ACP).

Assessment (n = 5)	Mean	SD	Median	Interquartile deviation	*P*
Baseline (carious lesion)	4.68	0.72	4.98	0.91	0.1886
First application	3.93	0.17	4.00	0.07	
Second application	3.73	0.41	3.86	0.53	
Third application	3.96	0.34	4.05	0.36	

Abbreviation: CPP-ACP, casein phosphopeptide–amorphous calcium phosphate.

There were no statistically significant differences between assessments (Kruskal-Wallis test).

the bacterial cell wall.[14,15] Lysozyme has non-muramidase activity due to the disruption of the bacterial membrane through amino acid cationic antimicrobial peptides.[16] Salivary lactoperoxidase, which plays a regulatory role in the incidence of caries, catalyzes the conversion of thiocyanate to hypothiocyanate.[17] Lactoferrin has an ability to reduce the availability of iron for bacteria, providing both bacteriostatic and bactericidal protection.[18]

The objective of the present study was to compare the effectiveness of CPP-ACP and LLL in reducing the numbers of *S mutans* in dentinal caries.

Materials and methods

The research was approved by the Research Ethics Committee of the State University of Campinas, Brazil (Protocol No. 088/2011).

Specimen selection

Forty healthy permanent third molars were selected from individuals at the Department of Oral and Maxillofacial Surgery, Hospital Celso Pierro, Campinas, Brazil, after they signed a tooth donation form. Inclusion criteria included healthy, permanent third molars; an absence of restorations; and an absence of cracks or fractures (verified by transillumination and low-power light microscopy).

Procedures

The extracted teeth were stored in 0.9% sodium chloride containing 0.02% sodium azide (Labcenter) at 4°C for a maximum of 1 month.[19] After this procedure, the teeth were washed with saline solution containing 10% sucrose (Labcenter). The occlusal third was removed using a double-sided diamond disc (KG Sorensen) at low speed with cooling. The dentin surfaces were polished with moistened 600-grit silicon carbide sandpaper (Norton Indústria Brasileira). The specimens were coated with epoxy resin (Araldite) and nail varnish (Colorama), except for a 4-mm-wide, 4-mm-long area of coronal dentin.

The teeth were sterilized by gamma rays and placed in sterile test tubes containing brain-heart infusion (BHI) supplemented with 0.5% yeast extract, 1% glucose, and 1% sucrose (Labcenter). The standard *S mutans* ATCC 25175 strain (Fundação André Tosello) was adjusted to 0.5 McFarland scale and introduced into the BHI. The specimens were incubated in a bacteriological incubator (Fanem) at 37°C for 14 days in jars containing anaerobic generator envelopes (Labcenter) in an atmosphere containing 85% nitrogen (N_2), 10% carbon dioxide (CO_2), and 5% hydrogen (H_2). During the 14 days, the BHI medium was renewed every 24 hours.[20,21] The pH level was measured at each exchange of the BHI medium.

The specimens were divided into 2 groups (n = 20) according to the type of agent used. Group 1 was treated with CPP-ACP. The *S mutans* counts were performed before application of CPP-ACP (baseline; n = 5) and after the first (n = 5), second (n = 5), and third applications (n = 5). The dentin surface was cleaned with 50 µL of saline solution and dried with sterile absorbent paper prior to the application of the product. A CPP-ACP nanocomplex was prepared using 1 g of toothpaste (Tooth Mousse, GC America) and 4 mL of distilled water. A sterile microbrush (KG Sorensen) was used for each application of CPP-ACP, and the duration of each application was 3 minutes. There was no interval between applications.

Group 2 was treated with LLL (Sigma-Aldrich). The LLL product contained 1% (0.018 mg) of the powder of each enzyme introduced in 1.8 mL of TergenForm root canal disinfectant (Fórmula & Ação). *Streptococcus mutans* counts and applications of the solution were performed in the same way as described for group 1.

The tissue from the dentinal caries was collected and immediately placed in the sterile BHI medium. The dentin material included in the BHI medium was homogenized for 3 minutes in shaker tubes (Vixar, VortexMixer) and diluted to 10^{-6}. Three aliquots of 25 µL were seeded on the surface of a mitis salivarius–bacitracin culture medium (Difco Mitis Salivarius Agar; Becton, Dickinson and Company). All plates were incubated in jars (Oxoid) at 37°C for 5 days in an atmosphere of 85% N_2, 10% CO_2, and 5% H_2, obtained by using anaerobic generator envelopes and anaerobic indicators (Anaerogen, Oxoid). After incubation, the number of viable bacteria was counted.

Statistical analysis

The data were analyzed with BioEstat 4.0 software and submitted to descriptive statistical analysis and the Kruskal-Wallis test complemented with Student-Newman-Keuls.

Results

In group 1, there was no significant reduction of *S mutans* ($P > 0.05$) after the applications of CPP-ACP (Table 1). A statistically significant reduction of *S mutans* ($P < 0.01$) was observed in group 2 after the third application of the solution containing LLL and TergenForm (Table 2).

Table 2. Results of microbiological assessment: *Streptococcus mutans* counts (log_{10}) in group 2 (LLL).

Assessment (n = 5)	Mean	SD	Median	Interquartile deviation	*P*
Baseline (carious lesion)	5.14[a]	0.43	5.12[a]	0.18	0.0011
First application	4.64[a]	0.32	4.60[a]	0.15	
Second application	5.72[a]	0.16	5.79[a]	0.04	
Third application	4.18[b]	0.37	4.12[b]	0.61	

Abbreviation: LLL, lysozyme, lactoferrin, and lactoperoxidase.

Values with a different lowercase superscript letter are significantly different (Kruskal-Wallis test complemented with Student-Newman-Keuls).

Discussion

Antimicrobial compounds can control the growth and development of bacteria remaining in the affected dentin, thus favoring dental tissue repair, particularly in cases of deep dentin caries that may present low pulp response due to advanced inflammation. The most common antimicrobials described in the literature are antibiotics, propolis, chlorhexidine, triclosan, cetrimide, cetylpyridinium chloride, CPP-ACP, and enzymes.[22-30]

The results of the present study have shown that topical applications of CPP-ACP do not reduce the number of *S mutans* from dentinal caries, which is in agreement with the results reported by Erdem et al, who pointed out that the action of CPP-ACP may be related to its high ability to help remineralization rather than a bactericidal/bacteriostatic action against *S mutans*.[31] However, Pukallus et al emphasized that CPP-ACP may interfere with the growth and adhesion of the *Streptococcus* species.[32] Mehta et al observed that CPP-ACP produced a significant amount of remineralization in artificial white-spot enamel lesions.[29]

The LLL solution significantly reduced *S mutans* after the third application. The results of the present study are in agreement with those reported by Oliveira et al, who noted that the salivary antimicrobial enzymes lactoferrin and lysozyme are effective in inhibiting the main cariogenic microorganisms (*S mutans* and *Lactobacillus casei*), thus playing an important role in regulating oral microbiota.[33]

The enzymes that were used in this study are present in saliva, and they exert a bacteriostatic and bactericidal effect against the pathogens in the oral cavity. The presence of enzymes such as lysozyme may be related to the etiology and development of caries as well as its prevention.[30] The association of lysozyme and lactoferrin has synergistic antimicrobial effects.[34] The LLL enzymes, due to their antimicrobial properties, assist in the reorganization of the affected dentin.[14,17,30] Lactoferrin and lysozyme inhibit the adhesion of *S mutans* to hydroxyapatite, the consumption of glucose, and lactic acid synthesis.[35] Salivary peroxidase also prevents the ingestion of glucose by the bacteria.[35] Jyoti et al observed an increase of thiocyanate ions in saliva after use of a toothpaste containing lactoperoxidase and a consequent reduction of cariogenic microbiota in children with early onset of caries.[36]

In vitro lactoferrin has anti-inflammatory and antimicrobial activity. The cationic peptide residue is released from lactoferrin after a combined cleavage of pepsin and trypsin, and it has broad-spectrum bactericidal activity.[37,38] Lactoferrin also binds to salivary agglutinin, and both proteins may remove microorganisms.[38] Salivary peroxidase also contributes to decreased bacterial growth as it prevents the accumulation of lysine and glutamic acid, the transport of bacterial amino acids, and the lysis of the cell wall.[35]

Conclusion

It may be concluded that 3 applications of the solution containing LLL and TergenForm can be used to reduce the number of *S mutans* in dentinal caries, suggesting clinical applications for the control of dental caries.

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