A novel antimicrobial and remineralizing toothpaste containing CaCl₂/chitosan microspheres

LINFENG WU, PhD, FENG LI, PhD, BRIAN R. MORROW, MS, SI BO JIANG, MS, TIMOTHY L. HOTTEL, DDS, MS, MBA, FRANKLIN GARCIA-GODOY, DDS, MS, PhD & LIANG HONG, DDS, MS, PhD

ABSTRACT: Purpose: To investigate the feasibility of exploiting amorphous calcium phosphate (ACP) formed in situ from chitosan calcium microspheres and phosphate ions in water during brushing for caries control. Methods: A prototype toothpaste, namely Chi-ACP paste, was specially formulated containing CaCl₂/chitosan microspheres. The efficiency of Chi-ACP paste for remineralization on human tooth enamel was evaluated via an in vitro pH cycling approach. After 15 demineralization/remineralization cycles, the treated teeth were analyzed using scanning electron microscope (SEM)/energy dispersive X-ray spectroscopy (EDX), and polarized light microscope (PLM). Results: EDX analysis showed the treated enamel in the Chi-ACP paste group had statistically significantly higher calcium content and Ca/P weight ratios than those in the negative control group, while the MI plus group had a slightly higher Ca content and a slightly higher Ca/P weight ratio. PLM analysis revealed that the Chi-ACP paste group had a larger remineralization band in treated enamel than the negative control group, although there was no statistically significant difference on the demineralization depths in the enamel among the three groups. (Am J Dent 2018;31:149-154).

Clinical significance: Antibacterial chitosan could be used to encapsulate CaCl₂ and then formulated into toothpaste for caries control through in-situ formed amorphous calcium phosphate (ACP) during brushing.

Introduction

Recently, there has been a strong recommendation to manage dental caries using the medical model of disease prevention/control instead of the restorative/surgical model since the latter does not address the root causes of dental caries. Central to this medical model are caries risk assessment and cost-effective preventive strategies. With the advances in the knowledge of dental caries process/progression and preventive dentistry, different technologies have been investigated and developed for the medical model of caries prevention/management. Currently, fluoride-based technologies are still the cornerstone for dental caries control. However, despite the extensive use of fluoride, dental caries-free population reached a plateau in the 1990s, and at that time at least 60% of teenagers were still developing dental caries. Fluoride therapy alone cannot completely successfully deal with the high challenge of cariogenic bacteria, thus alternative/additional measures are needed to overcome such challenge. It is desirable to develop new effective agents for dental caries prevention in addition to fluoride.

Efforts have recently been made to develop calcium-based remineralizing agents as non-fluoride caries preventive agents, such as amorphous calcium phosphate (ACP), tricalcium phosphate, bioactive glass-calcium sodium phosphosilicate, and arginine bicarbonate-calcium carbonate complex. Although so far clinical evidence is still insufficient for a definitive recommendation for use for dental caries reduction, substantial laboratory and in situ evidence, and some clinical evidence have been reported for the enhanced remineralization on enamel by calcium-based remineralizing agents. The challenge facing these calcium-based anti-caries technologies is the low solubility of those calcium-based agents. This limits the bioavailability of calcium and/or phosphate ions, impairing their ability to diffuse/penetrate into enamel subsurface lesions for effective promotion of remineralization in vivo. There is an urgent need to develop better technologies to enhance the delivery of bioavailable calcium and phosphate ions.

ACP is a unique noncrystalline solid phase calcium phosphate and can be generated both in-vitro and in-vivo from a supersaturated solution containing calcium and phosphate ions. Chitosan is a deacetylated derivative of chitin which is a significant component in the shells/exoskeletons of crustaceans and is one of the most abundant natural polysaccharides. The deacetylation degree and molecular weight of chitosan are important to its physical and biological properties. Chitosan has a high number of hydroxyl and primary amino functional groups, which enable its various applications in drug delivery and pharmaceuticals, biotechnology, agriculture and environmental protection for its excellent adsorption, biosafety, carrier and antibacterial capabilities.

Novel toothpaste formulations based on chitosan, arginine, and in situ forming ACP might be a caries control choice by targeting multiple etiological factors contributing to dental caries. In this work, we investigated the feasibility of exploiting ACP in situ formed from chitosan calcium microspheres and phosphate ions in water during brushing for caries control. The calcium ions released from microspheres can freshly form ACP in situ during brushing with this toothpaste.

Materials and Methods

Materials - Chitosan (molecular weight 1k, 3k, 5k and 10k) was used. CaCl₂·2H₂O, hexane, light mineral oil (NF/FCC), arginine, XTT [2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolio-5-Carboxanilide], hydroxyethyl cellulose, KCl, glycerol, hydrochloric acid, KH₂PO₄, Na₂HPO₄, acetic acid, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
Span 80™ was also used. *S. mutans*, *Enterococcus faecalis* (*E. faecalis*), or *Streptococcus sanguis* (*S. sanguis*) were obtained from ATCC.³

**Bacteria culture and susceptibility** - Each bacteria species of *S. mutans* (ATCC 31377), *E. faecalis* (ATCC 29212), or *S. sanguis* (ATCC 31377) was grown in appropriate culture media and conditioned for 48 hours at 37°C according to the bacteria suppliers’ instructions. At the end of incubation, bacteria were centrifuged and re-suspended to a concentration of $3 \times 10^8$ cells/ml as the working bacteria suspension. The working bacteria suspension was added into the wells of 96-well plate at 100 µl per well. The 96-well plate was incubated at 37°C and 5% CO₂ for 24 hours. Chitosan solutions were added to the wells to treat the bacteria at a final chitosan concentration of 0 wt%, 0.625 wt%, 1.25 wt%, or 2.5 wt% chitosan. The 96-well plate was further incubated at 37°C and 5% CO₂ for 24 hours. TheXTT assay was performed according to the previous reports⁹,²⁰ to determine the bacteria viability by measuring their metabolic activity. The resulting colored solutions in the plate were measured at 492 nm using a microplate reader.

**Synthesis and characterization of chitosan microspheres** - CaCl₂/chitosan microspheres were synthesized from chitosan-10K, and CaCl₂ via water-in-oil (w/o) emulsion. Briefly, the emulsion system was prepared from 10 ml 80mg/ml chitosan solution, 2 ml 1M CaCl₂ solution, and 120 ml light mineral oil containing 1,200 mg surfactant Span 80 in a 250 ml beaker. Milky w/o emulsion was obtained by magnetically stirring the above mixture at 1,000 RPM for 50 minutes and then at 750 RPM for 3 hours. The resulting water-in-oil emulsion was then transferred into 1,000 ml beaker to evaporate water at 38°C overnight (~19 hours) followed by 45°C for 2-5 hours under magnetic stirring of 500 RPM. The resulting CaCl₂/chitosan microspheres were purified via precipitation into hexane and centrifuged, followed by washing two times using hexane. Finally, dry CaCl₂/chitosan microspheres were obtained after vacuum drying with the yield of 85%~95%. The loading content of CaCl₂ was about 16 wt% as determined using Ca²⁺ ion selective electrode after dissolving microspheres with water.

The synthesized CaCl₂/chitosan microspheres were characterized using scanning electron microscopy (SEM) and FT-IR (Fourier transform infrared spectroscopy). The SEM samples were prepared by mounting CaCl₂/chitosan microspheres on SEM sample holders using carbon double-adhesive tape, followed by gold sputtering. For FT-IR, CaCl₂/chitosan microspheres or chitosan polymer were tightly pressed on the crystal window of attenuated total reflection in FT-IR. FT-IR was recorded in the range of 650 to 4,000 cm⁻¹.

HEPES solution²¹,²² (pH 8.2) was used as the release medium to study the release of Ca²⁺ ions from the CaCl₂/chitosan microspheres. Briefly, CaCl₂/chitosan microspheres were suspended in the release medium at 2 mg/ml and kept in a 37°C shaker. At each pre-determined time point (5 minutes, 30 minutes, 55 minutes, 3 hours, or 6 hours), samples were collected and filtered through 0.22 µm filters. The Ca²⁺ concentrations in the release medium were determined using calcium ion selective electrode according to the manufacturer’s instructions.

The feasibility of using arginine to adjust the pH of CaCl₂-loaded chitosan microsphere suspension was studied. CaCl₂-loaded chitosan microspheres were weighed and suspended in DD water. Aliquots of arginine free base solution were added in sequences. After adding each aliquot, the pH of the CaCl₂-loaded chitosan microsphere suspension was measured using a pH meter.

The feasibility of forming ACP from water soluble calcium salt and phosphate salt in the presence of chitosan was also tested. The resulting precipitants were collected by filtration, washed with 30 mL of 2% ammonium followed by 40 mL 100% ethanol. The resulting filter cake was dried under vacuum. The dried powders were used as samples for X-ray diffraction experiments.

**Toothpaste formulation** - In order to simplify this proof of concept study by formulating toothpaste containing CaCl₂/chitosan microspheres, while adding phosphate in the brushing water instead of using phosphate/chitosan microspheres in the toothpaste formulation in order to test the feasibility. A homogeneous viscous thickening mixture was prepared by mixing non-ionic thickener hydroxyethyl cellulose with glycerol/water co-solvents. The resulting aqueous mixture was lyophilized to remove the water, leaving a water-free viscous thickening mixture behind. Experimental toothpaste was formulated right before use by mixing the chitosan microspheres with the above viscous thickening mixture at a weight ratio of 15:85.

**Demineralization of human teeth** - Thirty non-carious human third molars were selected from de-identified teeth extracted in the College of Dentistry clinic at the University of Tennessee Health Science Center, in Memphis UTHSC IRB 12-01866 JM. They were painted with two layers of acid-resistant nail polish on all surfaces except for a window (2×7 mm) for each tooth after cleaning. The painted teeth were randomly divided into three groups (10 teeth per group): negative control (labeled as Contr), experimental group (labeled as Chi-ACP), and MI Plus Paste® group (positive control group, labeled as MI+). Featherstone’s pH cycling model²³,²⁴ was used to study the remineralization potential of the experimental toothpaste. The whole process consisted of 15 demineralization steps with 6 hours per stage and 15 remineralization stages with 16.5 hours per stage (one demineralization followed by one remineralization per day). The teeth were treated twice a day. During the remaining 1.5 hours each day, the teeth were soaked in double distilled (DD) water if not actively receiving treatment. The tooth windows were treated according to their group assignments during the transition between the two stages. The Negative Control group received no treatment except for washing with DD water for 60 seconds; Chi-ACP group (i.e. experimental group) was treated by manually brushing the exposed tooth surface window in the presence of a slurry of the selected toothpaste (~40 mg toothpaste and 150 µl Na₂HPO₄/arginine solution per tooth) for 1 minute; and MI+ group was treated with MI Plus Paste according to the manufacturer’s instructions. Each tooth specimen was washed with DD water for 60 seconds both before and after the treatments. After the treatments, the teeth were suspended in the corresponding demineralization solutions (i.e. 2.2 mM CaCl₂, 2.2 mM KH₂PO₄ and 50 mM acetic acid at pH 4.3) or re-mineralization solutions (1.5 mM CaCl₂, 0.9 mM KH₂PO₄, and 150 mM KCl at pH 7.0) at room temperature.
Fig. 1. Antimicrobial activity of chitosan: A. Inhibition/killing of *S. mutans* after incubation with different molecular weight chitosans at a concentration of 1.25 mg/ml; B. Chitosan 10K significantly killed *S. mutans*, *E. faecalis*, and *S. sanguis*.

**Characterization of treated enamel** - After the 15-day de/remineralization, the teeth were first analyzed for the elemental compositions on the treated enamel surface using EDX coupled to SEM. After the EDX analysis, the teeth were then sectioned bucco-lingually to 120-150 µm thick per section using hard tissue microtome to expose the cross sections beneath the treated enamel surfaces. These thin sections were examined using polarized light microscopy (PLM).25,26

**Statistical analysis** - All experiments were conducted in triplicate unless otherwise noted. Numerical data were expressed as mean±SD in the figures. One-way ANOVA and post hoc Dunnett t-test were used to evaluate the difference among/ between data groups with P< 0.05 indicating significant difference among/between the groups.

**Results**

**Antibacterial chitosan** - Figure 1A shows the antimicrobial capability of chitosan with different molecular weights (MW) against *S. mutans*. At a concentration of 1.25 wt%, chitosan with MW of 10kDa (chitosan 10K) showed the greatest capability to inhibit/kill *S. mutans* among the chitosans (deacetylation degree 90%) investigated. The bacterial viability for *S. mutans* treated with chitosan 10K was about 10% of the control (i.e. 0 wt% chitosan). Chitosan 5K had a moderate inhibition/killing capability with a bacterial viability of 55% as compared to the control. No significant antimicrobial effect was observed for chitosan with a molecular weight of 1kDa or 3kDa. Figure 1B shows the antimicrobial capability of chitosan 10K against three common oral bacteria, *S. mutans*, *S. sanguis*, and *E. faecalis*. As shown in Fig. 1B, the antimicrobial effects are concentration dependent, increasing as the chitosan concentration increases. Chitosan 10K showed the greatest capability in the inhibition/killing of *S. mutans* (IC50 = 0.655 mg/mL).

**Chitosan microspheres** - Figure 2 shows the typical SEM images for the synthesized CaCl2/chitosan microspheres scanned at magnifications of ×5000 and ×100. The microspheres were less definitive (Fig. 2A) with some nanostructures on the surface of micron-sized particles (less than 10 µm). EDX analysis showed that both those nanostructures and the micron-sized particles had calcium and nitrogen elements, indicating the existence of CaCl2 and chitosan (Data not shown). These microspheres were prone to form aggregates less than 100 µm (Fig. 2B).

Figure 3A shows the FT-IR spectra of the raw chitosan 10K, and CaCl2/chitosan microspheres. Chitosan 10K had the characteristic peaks of polysaccharides at 1,153 cm⁻¹ (vC–O-C in the glycosidic bridges),28 1,061 cm⁻¹ (vC–O near the primary hydroxyl groups) and 1,017 cm⁻¹ (coupled vC–C and vC–O),28 and amide band I at 1,633 cm⁻¹ and amide band II at 1,538 cm⁻¹ due to N-acetyl groups. After the incorporation of CaCl2 into
chitosan to form microspheres, several new peaks at 1,527 cm\(^{-1}\), 1,450 cm\(^{-1}\), and 1,378 cm\(^{-1}\) were observed in addition to the characteristic peaks observed for chitosan. Figure 3B shows the release profiles of Ca\(^{2+}\) ions from CaCl\(_2\)/chitosan microspheres in HEPES buffer solutions (pH 8.2) at 37°C. Almost 100% of Ca\(^{2+}\) ions were released out of the microspheres within the first 5 minutes. Figure 3C shows the pH change of CaCl\(_2\)/chitosan suspension with the addition of arginine solution (pH 9.6). With the addition of arginine, the pH was rapidly increased to around 8.3, and then gradually approached around 9 below the pH 9.6 of arginine solution used. Figure 3D shows the X-ray Diffraction (XRD) spectrum of the synthesized CS-ACP powder, which clearly confirmed the successful formation of ACP.

**Elemental analysis of enamel surface by EDX** - Figure 4 shows the results of elemental analysis and the typical SEM images for the treated enamel surfaces in all three groups. Compared with the negative control group (Contr group, treated with DD water), the Chi-ACP group had a statistically significantly higher Ca content (37.23±4.25 wt% vs. 33.89±3.28 wt%, P<0.05) and a statistically significantly higher Ca/P weight ratios (2.02±0.19 vs. 1.88±0.11, P<0.05) on the treated enamel surfaces (Figs. 4A, B). Compared with the negative control group (Contr group, treated with DD water), the positive control group (i.e. MI+ group) had a slightly higher Ca content (36.51±4.14 wt% vs. 33.89±3.28 wt%, P>0.05) and a slightly higher Ca/P weight ratio (1.99±0.14 vs. 1.88±0.11, P>0.05) (Figs. 4A, B). There was no statistically significant difference on the phosphate contents (P>0.05) of the three groups of teeth: 17.97±0.82 wt% for the negative control group, 18.41±0.59 wt% for the experimental group Chi-ACP, and 18.30±0.80 wt% for the positive control MI+ group (Fig. 4C).

Figure 5 shows the typical SEM images for the treated enamel surfaces in all three groups. For all three groups, no apparent deposits were observed (Figs. 5A, B, C).

**De/Remineralization depths from PLM** - There was no significant difference on the demineralization depth among all three groups and between any two groups (Fig. 6A). The de-mineralization fronts\(^{25}\) were penetrating about 650 μm beneath the enamel surface. The negative control group (Contr groups) had the lowest remineralization band (73.8±24.9 μm) (Fig. 6B). The experimental group (Chi-ACP group) had the highest remineralization band (131.4±63.6 μm), followed by positive control group (MI+ group, 115.2±26.8 μm) (Fig. 6B). The difference among the three groups was statistically significant at the level of P<0.05. Post Hoc test (Dunnett t) showed that there were statistically significant differences on remineralization depth between Contr and Chi-ACP groups or between Contr and MI+ groups at the level of P=0.05. Further analysis showed that no statistical difference existed between the Chi-ACP group and MI+ group on the remineralization band, although the Chi-ACP group had a slightly larger remineralization band than MI+ group.

**Discussion**

Dental caries is a multi-factorial disease due to imbalanced demineralization/remineralization processes which favor net mineral loss in teeth.\(^{30}\) Dentifrices which simultaneously target multiple etiological factors contributing to dental caries might be able to offer synergistic anti-caries benefits to their users.\(^{4}\) In this study, chitosan was chosen as an anti-bacterial ingredient and delivery system for calcium in the experimental toothpaste. The antimicrobial capability of chitosan against *S. mutans* was significantly influenced by its molecular weight. The chitosan with molecular weight of 10k (chitosan 10K) showed the great-
est capability to inhibit/kill S. mutans. Its antimicrobial activity was also concentration-dependent. Therefore, chitosan 10K was used to encapsulate CaCl₂ salt. The release of Ca²⁺ ions from the CaCl₂-loaded chitosan microspheres was very fast, almost completed within the first 5 minutes. This fast release of Ca²⁺ ions has been reported for CaCl₂-loaded phosphorylcholine group bearing hydrogels.³¹ The fast release should be attributed to the high solubility of CaCl₂ in water and the fast swelling of the microspheres in water. If slower release of Ca²⁺ is desired or needed, low soluble calcium salts should be loaded into the chitosan microspheres. ACP is generally synthesized from water soluble calcium salts and phosphate salts at pH higher than 7.²³ In order to achieve the desired pH suitable for in-situ formation of ACP, we used arginine to buffer the chitosan microspheres. In addition to its pH buffering capability, arginine as a natural amino acid could be catabolized to ammonia via arginine deiminase system in arginolytic bacteria associated with dental health.²⁴ This alkali generation from arginine has been explored for dental caries prevention in combination with 1,450 ppm fluoride.³³,³⁴ The feasibility of forming ACP from water soluble calcium salt and phosphate salt in the presence of chitosan was further confirmed.

The ultimate goal is to develop a novel antibacterial/ remineralizing toothpaste for anti-caries via in-situ formation of ACP from Ca²⁺ and phosphate ions released from chitosan microspheres. In this proof of concept study, we simplified the process by using CaCl₂/chitosan microspheres, and phosphate solution that was used to replace the phosphate/chitosan microspheres in order to test the feasibility of this concept. The experimental non-aqueous toothpaste group apparently had the highest Ca content and Ca/P weight ratio among all the three groups. However, no apparent deposits were observed on the treated enamel surface for all three groups. This observation seems to be different from the observations in many anti-hypersensitivity studies which usually reported that the dentin tubule was occluded by deposits from toothpaste.³⁵ This difference might be attributed to the fact that most of those studies on dentin did not expose the treated dentin to pH cycling, and instead the treated dentin was evaluated almost immediately after the treatments. The tooth specimens in the present study were soaked in de/remineralization solutions for 22.5 hours per pH cycle for 15 days before SEM examination.

The demineralization of enamel is a complicated process induced by the acid generated by the cariogenic bacteria such as S. mutans. It is generally believed that demineralization is a dissolution process consisting of several sequential reactions producing solid intermediate calcium phosphate compounds such as Ca₅(PO₄)₂ and CaHPO₄, instead of spontaneous dissolution of HA to Ca²⁺ ions, phosphate ions and water. Therefore, the continuous attack from acid reduces the mineral density, Ca and P contents, and the ratio of Ca/P in the enamel. This mechanism well explains the observations in low Ca and P contents, and low ratio of Ca/P for demineralization of enamel reported in the literature.³⁶,³⁷ In this study, EDX analysis showed that the Contr group in this study had the lowest Ca and P contents among all three groups, and also a lower ratio of Ca/P in the enamel compared with sound enamel reported.³⁸,³⁹

The ideal remineralization should be viewed as the reincorporation of Ca and phosphate back to the demineralized enamel to maintain the contents of hydroxyapatite (HA) or fluoridated hydroxyapatite (FHA) closer to those for sound teeth, instead of simple precipitation of insoluble calcium phosphate compounds. From this point of view, successful treatment for remineralization should reduce the loss of Ca and P contents, and reduce the decrease of the Ca/P ratios in the enamel. In this work, significantly higher Ca content and weight ratio of Ca/P were indeed observed for the experimental group than the Contr group. There was no statistically significant difference on the P contents for all the three groups. This might be attributed to the fact that during remineralization, Ca²⁺ ions start to be released out from enamel at the very beginning before phosphate ion could be released under acid attack. Thus, less phosphorus was actually needed for repairing the demineralized enamels.

In the present study, the remineralization fronts penetrated about 650 μm beneath the enamel surface. This was higher than the previous report of 400 μm.²⁵,²⁶ The difference may be related to the duration of pH cycling. Although there is no significant difference on the demineralization depth among three groups, the remineralization band widths for all three groups were statistically different. The negative control group (Contr groups) had the lowest remineralization band width, which was consistent with a previous report.²⁵ The experimental group (Chi-ACP group) had the highest remineralization band width, followed by positive group (MI+ group). Given the fact that MI+ contains 900 ppm fluoride, the newly-formulated toothpaste had apparent advantages over MI paste plus for remineralizing demineralized enamel.

In conclusion, this study demonstrated the feasibility of enhancing remineralization of demineralized tooth enamel through in-situ freshly formed amorphous calcium phosphate from the prototype toothpaste with antimicrobial properties.

References