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Anti-cariogenic effect of a cetylpyridinium chloride-containing nanoemulsion

Valerie A. Lee, Ramalingam Karthikeyan, H. Ralph Rawls, and Bennett T. Amaechi

University of Texas Health Science Center at San Antonio, San Antonio, TX

Abstract

Objectives—The aim of this pilot study was to investigate the anticaries activity of a nanoemulsion composed of soybean oil, water, Triton X-100 and cetylpyridinium chloride.

Methods—Tooth blocks (3 mm length × 3 mm width × 2 mm thickness) were cut from smooth surfaces of selected molar teeth using a water-cooled diamond wire saw. The blocks were randomly assigned to three experimental groups, (A) nanoemulsion, (B) 0.12% chlorhexidine gluconate, and (C) no treatment. The formation of dental caries in human tooth enamel was tested using a continuous flow dual-organism (*Streptococcus mutans* and *Lactobacillus casei*), biofilm model, which acts as an artificial mouth and simulates the biological and physiological activities observed within the oral environment. Experimental groups A and B were treated with their respective solutions once daily for 30 seconds on each occasion, while group C received no treatment. 10% sucrose was supplied every 6 hours for 6 minutes to simulate meals and pH cycling. The experiment lasted for 5 days, and the tooth blocks were harvested and processed for demineralization assessment using transverse microradiography (TMR).

Results—For both lesion depth and mineral loss, statistical analysis indicated that Emulsion was significantly lower than Control and Chlorhexidine, and Chlorhexidine was significantly lower than Control.

Conclusions—We conclude that cetylpyridinium-containing nanoemulsions appear to present a feasible means of preventing the occurrence of early caries.

Keywords

artificial caries; nanoemulsion; artificial mouth; transverse microradiography (TMR)

INTRODUCTION

Biofilms are communities of microorganisms irreversibly attached to a surface and containing an exopolymeric matrix. Dental plaque, a well-recognized biofilm, is directly connected with the formation of dental caries. Despite the best efforts of dental health professionals, dental caries are still very prevalent in the general population. The average adult in the U.S. has from 10 to 17 decayed, missing or filled permanent teeth [1].

Corresponding author: Valerie A. Lee, 7703 Floyd Curl Dr., Comprehensive Dentistry, San Antonio, TX 78229-3900, Phone: 210-567-3676, Fax: 210-567-3669, leev@uthscsa.edu.

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Antimicrobial nanoemulsions are surfactant-containing oil and water emulsions (droplet size 100–300 nm) which have been shown to be non-toxic to animals, but very effective against many bacteria, viruses, fungi and spores in their free-floating or planktonic form [2]. Preliminary morphological studies suggest that nanoemulsions target bacterial cell membranes [3]. When growing as surface-adherent biofilms, microorganisms undergo phenotypic changes that make them very resistant to commonly used antimicrobial agents [4,5]. As many dental diseases, including dental caries, are associated with dental plaque, it is essential to test candidate antimicrobial agents against plaque biofilms, including cariogenic biofilm.

The nanoemulsion employed in this work contains cetylpyridinium chloride (CPC), a quaternary ammonium salt. CPC is an effective anti-plaque agent regulated by the Food and Drug Administration. It has been proposed for inclusion in a range of products for dental use, such as mouthrinses, toothpastes, varnishes, orthodontic adhesives and liners for glass ionomer cements[6–11]. The safety and efficacy of CPC have been evaluated extensively and proven based on cytotoxicity data collected from many animal studies[12–14].

The antimicrobial efficacy of CPC-containing nanoemulsions against planktonic and biofilm bacteria, coupled with low toxicity make this technology a candidate for control of dental plaque and caries. The objective of this pilot study was to determine if one nanoemulsion formulation effectively prevented the formation of early caries associated with oral biofilms. The null hypothesis was that CPC-containing nanoemulsion would not protect enamel better against demineralization compared to chlorhexidine gluconate, a potent antiplaque agent.

MATERIALS AND METHODS

Preparation of nanoemulsion

A nanoemulsion was prepared in our laboratory in the Division of Biomaterials at the University of Texas Health Science Center at San Antonio using 25 vol.% soybean oil, 65 vol.% deionized water, 10 vol.% Triton X-100, 1 wt.% cetylpyridinium chloride (SigmaAldrich, St.Louis, MO) and a 25G reciprocating syringe. The ratio of ingredients closely matches that described by Baker, inventor of the nanoemulsion, in his patent[2]. Droplet size measurement was carried out using a dynamic light-scattering method (Protein Solutions, DynaPro, St. Paul, MN). The preparation method resulted in a nanoemulsion with a narrow droplet size distribution with a mean diameter of 168nm, Fig. 1. The nanoemulsion had the consistency and appearance of whole milk.

Preparation of teeth and experimental grouping

Human third molar teeth extracted due to impaction were autoclaved (120°C for 15 minutes), cleansed of soft tissue debris and pumiced with non-fluoridated toothpaste slurry using a Braun Oral-B Plaque Remover 3D electric toothbrush. The teeth were examined by transillumination, and thirteen teeth without cracks, hypoplasia, white spot lesions and other malformations were selected. Three tooth blocks (each 3 mm length × 3 mm width × 2 mm thickness) were cut from the smooth surfaces (buccal, lingual (palatal), mesial or distal) of each tooth using a water-cooled diamond wire saw (Walter Ebner, Switzerland). The blocks were randomly assigned to three experimental groups, 13 blocks/group: (A) nanoemulsion, (B) 0.12% chlorhexidine gluconate (Peridex, 3M ESPE Dental Products, St. Paul, MN), and (C) no treatment (Control).. The three groups were subjected to demineralization by plaque growth in an Artificial Mouth described below, to test the ability of a CPC-containing nanoemulsion to inhibit the formation of biofilms responsible for dental caries in human tooth enamel.

The “artificial mouth” system

This system is composed of multiple-station continuous flow culture chambers, Fig. 2, which acts as an artificial mouth and simulates the biological and physiological activities observed within the oral environment. Each station consists of a chamber bearing (i) a cylindrical clear-acrylic rod with vertical grooves (**C, D, E**) for mounting either whole tooth or tooth blocks (**I**), (ii) a head assembly with two lines for supply of simulated oral fluid (SOF), nutrients, experimental reagents, and inoculation of the chamber with either single- or mixed-organism bacterial consortium (**J, K**), and (iii) access for plaque sampling and electrode insertion for pH monitoring. The SOF used in this system is Bacto™ Todd Hewitt Broth (Fisher Scientific, Pittsburgh, PA) and was adjusted to pH 7.0 (**F**). This was continuously circulated to simulate saliva. Continuous circulation through the chambers at individually controlled flow rates (2 ml/min) via a digital programmable pump (**A**) was maintained from a reservoir (**F**). A complete circulatory system was established by a return-flow line (**H**) from the chamber back into the reservoir. The reservoir content was changed daily. The flow rate of the SOF was varied in accordance with the oral condition being simulated (e.g. stimulated or unstimulated salivary flow). 10% sucrose was supplied every 6 hours for 6 minutes to simulate meals and pH cycling (**G**) using pump (**B**). All fluids flowed uniformly as a thin film over the surface of the rod. The entire assembly was housed inside a reach-in CO₂ incubator maintained at 5% CO₂ and at a constant physiological temperature of 37°C. A micro-esophageal glass pH electrode and micro-reference electrode connected through a pH meter were installed in each chamber at the plaque growth surface to monitor the intra-plaque pH. The completely assembled system with reservoirs and contents were sterilized using ethylene oxide gas prior to each experiment.

Experimental procedure

The three experimental groups were randomly assigned to three culture chambers in the artificial mouth system (13 blocks/chamber). Using heavy duty putty, these blocks were embedded in the vertical grooves on the surface of the cylindrical rod in the culture chamber. The blocks were embedded such that their surfaces were flush with the surface of the cylinder to permit streamlined flow of fluids, and the exposed enamel was available for plaque growth and subsequent demineralization. Caries development on the experimental blocks was initiated by inoculation of the chambers by 1-hour circulation of mixed *Streptococcus mutans* (NCTC 10449, ATCC, Manassas, VA) and *Lactobacilli casei* (NCIB 8820, ATCC, Manassas, VA) culture in Todd Hewitt broth (broth to inoculum ratio 10:1) through the chambers. Inoculation was repeated once daily for two consecutive days. Plaque biofilm formation was confirmed by scanning the surface of two blocks from each group using confocal microscopy on Day 5. The system was then operated as described above by continuous circulation of Todd Hewitt Broth separately through the three chambers to simulate saliva, and 10% sucrose was supplied every 6 hours for 6 minutes to simulate meals and pH cycling. The pH of plaque in each chamber was monitored at non-feeding time to check maintenance of neutrality by CO₂. All fluids, including inoculation, were delivered at a flow rate of 2ml/min. Change in plaque pH following sucrose supply was monitored on two occasions on the third day to confirm exhibition of Stephan-like curve of pH fall under sucrose challenge. The chambers were accessed individually from the tops. The pH at non-feeding time remained at approximately 6.8; following the supply of sucrose it decreased gradually to as low as 5.2, remained at this level for the remaining part of the 6 minutes. Upon withdrawal of sucrose and re-circulation of broth after 6 minutes, the pH rose gradually to neutrality (6.8) after 45 minutes, but was below 5.5 for 20 minutes. Experimental groups A and B were treated with their respective solutions once daily for 30 seconds on each occasion, while group C received no treatment. The experiment lasted for 5 days, and the tooth blocks were harvested and processed for demineralization assessment using transverse microradiography (TMR).

Effect of nanoemulsion on biofilms

Since there was full growth of biofilm in group C, we decided to use this group to test the effect of CPC-containing nanoemulsion on the viability of an existing biofilm.

Group C was exposed to emulsion for 30 seconds. Following this, biofilm samples were collected from group C tooth slabs at 0 and 30 seconds, 1 min, 5 min, 10, 20, 30, 40, 50 and 60 min. The collected samples were transferred to sterile polypropylene tubes containing phosphate-buffered saline immediately following collection and were dispersed by sonication (20s) and vigorous vortex mixing (30s). Bacterial viability was assessed by serial dilution, inoculation into culture media, and incubation at 37°C and 5% CO₂ for 24–48 hours.

Transverse Microradiography and image analysis

Three tooth slices (~150 µm thick) were cut from each tooth block using a water-cooled diamond wire saw (Buehler, Switzerland). These slices were used to determine the Transverse Microradiographic (TMR) parameters (mineral loss (Δz) and lesion depth (LD)) of the caries lesion as follows. First, both sides of the slice were polished using Adhesive Back 6µm lapping film in a MultiPrep™ Precision Polishing machine (Allied High Tech, USA) to achieve planoparallel surfaces as well as to reduce the thickness of the slice to 80 µm (the appropriate thickness for TMR). Following this, the slices were microradiographed on type 1A high resolution glass X-ray plates (Microchrome Technology, CA, USA) using a Phillips x-ray generator system (Panalytical, Amsterdam) set up for this purpose. The plates were exposed for 10 minutes at an anode voltage of 20kV and a tube current of 10 mA, and then processed. Processing consisted of a 5 minute development in Kodak HR developer and 15 min fixation in Kodak Rapid-fixer before a final 30 minute wash period. After drying, the microradiographs were subjected to visualization with a Leica DMR optical microscope linked via a Sony model XC-75CE CCTV camera to a computer housing the image analysis program (TMR2006 version 3.0.0.6, Inspektor Research, Amsterdam). The enhanced image of the microradiographs were analyzed under standard conditions of light intensity and magnification and processed, along with data from the image of the step wedge, by the TMR program. The computer program calculated the parameter of integrated mineral loss (vol % .µm) and the lesion depth (µm) based on the work described by De Josselin de Jong *et al.* [15]. The integrated mineral loss was defined as the difference in volume percent of mineral between sound and demineralized tissue integrated over the lesion depth [16]. The lesion depth was assessed as the distance from the measured sound enamel surface to the location in the lesion at which the mineral content is greater than 95% of the mineral content in sound enamel. By this method, the parameters of integrated mineral loss (Δz , vol% .µm) and lesion depth (LD, µm) were quantified for each caries lesion.

Statistical procedures

Due to the small sample size of 13 teeth per treatment group, box plots were used to depict the distributions of the lesion depth and mineral loss data for treatment groups, for which the lack of symmetry made the assumption of normality inappropriate. As a result, non-parametric Kruskal-Wallis analyses of variance were performed for both measurements, and non-parametric Spearman rank correlations between lesion depth and mineral loss were performed for each treatment group and all treatment groups combined. If a Kruskal-Wallis test was significant, then Bonferroni-adjusted non-parametric Mann-Whitney U tests were performed comparing Control vs. Emulsion, Control vs. Chlorhexidine, and Emulsion vs. Chlorhexidine. For all statistical tests, $p < 0.05$ was considered significant. Stata 11.0 (StataCorp, College Station, TX) statistical software was used.

RESULTS

Effect of treatments on demineralization

Both Emulsion and Chlorhexidine treatments correlated with a reduction in lesion depth relative to Control, Fig. 3. Emulsion treatment appeared to reduce lesion depth more than did Chlorhexidine treatment. In an X-ray micrograph, loss of mineral structure is noted as a darkened area. As seen in Fig. 3, demineralization, beginning at the tooth surface and extending inward, occurred to a minimal extent in the Emulsion group, and to progressively greater extents in the Chlorhexidine and Control groups. It is difficult to note differences in mineral loss visually among the 3 groups of Fig. 3.

Statistical results

Box plots for lesion depth for the 3 groups are presented in Figure 4 and percent mineral loss are presented in Figure 5. The box plot for lesion depth revealed a lack of symmetry about the median for Emulsion and 3 low value outliers (represented as dots) for Chlorhexidine and Control, so parametric statistical analysis using the raw or log transformed data was not appropriate. The Kruskal-Wallis tests for lesion depth and mineral loss were both significant ($p < 0.001$), and subsequent Mann-Whitney U tests for both measures indicated that Emulsion was significantly lower than Control and Chlorhexidine ($p = 0.001$) and Chlorhexidine was significantly lower than Control ($p = 0.001$). The consistency in these results was reflected by the overall Spearman correlation of 0.937 with 95% confidence interval of (0.883, 0.967) between lesion depth and mineral loss; within treatment groups, the rank correlations were 0.928 with 95% c.i. of (0.773, 0.979) for Emulsion, 0.797 with 95% c.i. of (0.438, 0.937) for Chlorhexidine, and 0.556 with 95% c.i. of (0.008, 0.848) for Control.

Effect of nanoemulsion on existing biofilms

Microbial growth was observed only on plates of samples collected at 0 and 30 seconds after exposure to CPC-containing nanoemulsion, Fig. 6. No growth was observed on plates of samples collected from 1 to 60 min after exposure, indicating the efficacy of the emulsion in disrupting existing biofilms.

DISCUSSION

It is now well established that caries is dependent on fermentable dietary carbohydrates. Cariogenic microorganisms, especially mutans streptococci, utilize dietary carbohydrate for their metabolic needs, creating organic acids as by-products that demineralize the tooth tissue. A relatively high proportion of mutans streptococci within dental plaque is required for the initiation of caries, while lactobacilli are associated with the progression of the carious lesion. The organisms also utilize sucrose to synthesize and store both extracellular polysaccharides and intracellular polysaccharides, leading to the production of biofilms[17].

The artificial mouth model used in this study provided a continuous or intermittent supply of nutrients to bacterial plaque growing within an environment that mimics the *in vivo* oral niches and habitats. The apparatus can be used to obtain information on the etiology, prevention and management of major oral diseases such as caries and possibly periodontal diseases. Caries-like lesions of enamel, such as those created in the artificial mouth, show all of the principal histological features of natural caries and have been successfully used to study the remineralization of enamel *in vitro*[18].

Numerous studies have shown the marked resistance of biofilms to antibiotics[19–22], for reasons that are not fully understood. One reason that biofilms are antibiotic resistant may be the heterogeneity within the biofilm, with gradients of pH, oxygen concentration, waste

products and nutrients[23–25]. Antibiotics that require a certain environment to act may be ineffective in certain areas of the biofilm. A second reason may be related to the reduced growth rate of bacteria at the interior of the biofilm. The most metabolically active organisms tend to be located on the periphery of the biofilm[26–28]. There is a general correlation between reduced growth rate and antibiotic resistance[28–30]. Bacteria in biofilms tend to undergo phenotypic changes, and transfer of transmissible genetic material occurs at an accelerated rate[4,5]. Such changes may increase the transfer of virulence and resistance factors to antibiotics. In addition, it is thought that the matrix surrounding the microcolonies serves as a protective barrier to the intrusion of large molecules such as antibiotics.

The nature of the biofilm helps explain why dental caries and periodontal diseases have been so difficult to prevent and treat. Systemic and locally delivered antimicrobials have not always proven successful, even when targeted at specific microorganisms[31]. Biofilms can be removed by mechanical means, but they immediately begin to reform.

Antimicrobial nanoemulsions are surfactant-containing oil-in-water emulsions (droplet size 100–300nm) which have been shown to be non-toxic to animals but very effective against many bacteria, viruses, fungi and spores in the free-floating or planktonic form[2,3,32–35]. The mechanism of action of nanoemulsions is not fully understood, but it has been proposed that the nanodroplets fuse with the outer membrane of the microorganism, destabilizing the organism's lipid envelope and initiating its disruption[36]. The nanodroplets acquire significant energy as they are formed by an extrusion process under high shear forces, and may pass this energy to the cell membrane upon contact. The nanoemulsions are apparently non-toxic to skin and mucosal surfaces because of the keratinized nature of the outer layers of those tissues. Remarkably, the mechanism of action of nanoemulsions is non-specific, unlike that of antibiotics, and is therefore very unlikely to stimulate resistance as occurs with antibiotics.

Emulsions are thermodynamically unstable and tend to revert to their unemulsified state. The emulsified state can be prolonged considerably by the addition of a suitable surfactant, which resides at the interface between the oil droplets and the continuous water phase. In our laboratory, we conducted minimum inhibitory concentration assays (MIC) every two weeks for one year using the CPC-containing nanoemulsion from the present pilot study and planktonic *S. mutans*. No change in MIC or in the appearance of the emulsion was noted over the one-year interval (our unpublished data).

The addition of a cationic halogen-containing compound such as cetylpyridinium chloride (CPC) places a positive surface charge on the nanodroplet by being incorporated as a co-surfactant[2]. Bacteria in dental plaque have a net negative surface charge[37,38]. In addition, exopolysaccharide chains, which vary in size from 103–108 kDa, are usually negatively charged, sometimes neutral or rarely positively charged[39]. Positively-charged nanodroplets should have increased potential to interact with the biofilm cells and matrix.

In addition to the above proposed action, quaternary ammonium salts such as CPC have antimicrobial activity of their own, apparently through multiple mechanisms. One mechanism is thought to be due to disruption of intermolecular interactions, causing a dissociation of cellular membrane lipid bilayers, compromising cellular permeability controls, and inducing leakage of cellular contents[40]. Longer exposure times may result in additional breakdowns of intracellular material which are indicative of autolysis[40,41]. In addition, CPC has an inhibitory action against fructosyltransferases, extracellular enzymes which synthesize fructans from sucrose, which then play an important role in the progression of dental caries by serving as an extracellular nutrition reservoir for bacteria[42]

Attempts to reduce or inhibit microbial adherence are a viable means to control infection. The *in vitro* anti-adherence of *Candida albicans* to oral buccal mucosal cells by low concentrations of CPC has been described[43,44]. Peripheral coating of poly(ethylcyanoacrylate) nanoparticles has been shown to result in decreased adhesion of *C. albicans* to buccal epithelial cells *in vitro*[45]. The mechanism of anti-adherence appears to be both a disruption of the fungal membrane and also a steric interference of the approach of the microbial cell to the epithelial cell. Similar mechanisms may operate in the vicinity of the enamel surface.

In the present study, both Emulsion and Chlorhexidine treatments were effective in resulting in reduced lesion depth and mineral loss relative to controls, and Emulsion was more effective than Chlorhexidine. The separate mechanisms of action of both nanoemulsions and CPC may be operating to reduce biofilm formation. It is possible that the positively-charged emulsion remains attached to the biofilm for a longer time than does chlorhexidine, and is therefore able to prevent the formation of further biofilm.

In conclusion, CPC-containing nanoemulsions appear to present a feasible means of preventing the occurrence of early caries.

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Figure 1. Particle size analysis of a nanoemulsion composed of 25 vol.% soybean oil, 65 vol.% deionized water, 10 vol.% Triton X-100, 1 wt.% cetylpyridinium chloride. Mean droplet size is 168nm.

Figure 2. Schematic representation of the artificial mouth system and its components. A - Programmable circulating pump for broth; B - Programmable pump for broth and sucrose; C - Oral chamber for nanoemulsion; D - Oral chamber for chlorhexidine mouth rinse; E - Oral chamber for no treatment (Control); F- Todd Hewitt broth; G - Sucrose; H – Return flow-line; I -Tooth block; J - Broth and sucrose pumping tubes; K - Broth circulating tube.

Figure 3. Transverse microradiographic images from each experimental group. Lesion depth (μm), mineral loss ($\text{vol}\%\mu\text{m}$) – **a:** 21.1 μm , 250 $\text{vol}\%\mu\text{m}$, **b:** 46.0 μm , 650 $\text{vol}\%\mu\text{m}$, **c:** 70.8 μm , 1320 $\text{vol}\%\mu\text{m}$.

Figure 4. Statistical analysis of lesion depth of the three experimental groups. Emulsion was significantly lower than Control and Chlorhexidine ($p < 0.001$) and Chlorhexidine was significantly lower than Control ($p = 0.001$).

Figure 5. Statistical analysis of mineral loss of the three experimental groups. Emulsion was significantly lower than Control ($p<0.001$) and Chlorhexidine ($p=0.001$), and Chlorhexidine was significantly lower than Control ($p<0.001$).

Figure 6. Agar plates showing growth of organisms collected at 0 and 30 seconds after a 30 second exposure to emulsion. No growth was found on plates of samples collected from 1 to 60 minutes after exposure to CPC-containing nanoemulsion, indicating the ability of the emulsion to disrupt existing biofilms.

Table 1

Artificial mouth procedure.

	Emulsion	Chlorhexidine	Control
Day 1	1 hour inoculation of bacterial consortium	1 hour inoculation of bacterial consortium	1 hour inoculation of bacterial consortium
	30 second exposure to nanoemulsion	30 second exposure to chlorhexidine	
	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily
Day 2	1 hour inoculation of bacterial consortium	1 hour inoculation of bacterial consortium	1 hour inoculation of bacterial consortium
	30 second exposure to nanoemulsion	30 second exposure to chlorhexidine	
	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily
Day 3	30 second exposure to nanoemulsion	30 second exposure to chlorhexidine	
	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily
	Measurement of pH	Measurement of pH	Measurement of pH
Day 4	30 second exposure to nanoemulsion	30 second exposure to chlorhexidine	
	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily
Day 5	30 second exposure to nanoemulsion	30 second exposure to chlorhexidine	
	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily	6 minute exposure to 10% sucrose 3x daily
	Confocal microscopy	Confocal microscopy	Confocal microscopy