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Evidence of the Efficacy of an Alcohol-Free Mouthwash Containing Cetylpyridinium Chloride

On the Cover: The cover image demonstrates a representative five-day multispecies biofilm in the Glass-bottom Microplate (GM) biofilm system, with the red cells demonstrating bacteria damaged after treatment with the alcohol-free 0.075% CPC-containing mouthwash, and the mostly green and some yellow cells demonstrating bacteria partially damaged after treatment with the placebo mouthwash.

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Contents

The Antibacterial and Antiplaque Effectiveness of Mouthwashes Containing Cetylpyridinium Chloride With and Without Alcohol in Improving Gingival Health

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Overview

This article briefly discusses the antibacterial action of cetylpyridinium chloride (CPC) and its efficacy in the removal of bacterial plaque as an adjunct to the mechanical cleaning of tooth surfaces. It reviews new studies on the effectiveness of mouthwash formulations containing CPC against two common oral bacteria species and in disrupting plaque biofilms. Finally, this article reviews three clinical studies which support that the daily use of mouthwashes containing 0.075% CPC, with and without alcohol, represents a valuable complement to daily mechanical plaque control.

Introduction

Bacteria accumulation on teeth results in the formation of dental plaque. Left untreated, dental plaque can lead to gingivitis, which is characterized by redness, edema, and bleeding on probing.[1,2] Gingivitis is the early and reversible form of periodontal disease with no permanent gum damage when treated. However, if not treated, gingivitis can lead to the development of periodontitis, which results in irreversible damage to the gums and underlying support tissues.[3-6]

The focus of any attempt to prevent and control periodontal disease is the maintenance of an effective level of plaque control by the individual through his or her daily oral hygiene.[4] The most important part of oral healthcare takes place at home. Mechanical cleaning by tooth brushing and flossing has been the cornerstone of oral hygiene and health. However, these mechanical routines, for various reasons, do not appear to be enough for the majority of people, as the incidence and prevalence of gum problems are high in both the developed[7-10] and developing world.[11-15] Many patients find it difficult to comply with this daily regimen. Insufficient and/or inadequate brushing and flossing, due to the lack of manipulative skills, can lead to plaque build-up. As these methods may be insufficient to achieve optimum results, a common strategy is to supplement mechanical plaque removal with a chemotherapeutic agent.[16,17]

To aid in the control of plaque build-up and gingivitis, oral care manufacturers have developed mouthwash formulations containing various chemotherapeutic agents, such as chlorhexidine, triclosan, essential oils, and cetylpyridinium chloride for use as adjuncts to mechanical cleaning.

Cetylpyridinium chloride (CPC) in mouthrinses has been studied extensively in clinical trials for its ability to control plaque and gingivitis.[18,19] It is one of only two antimicrobial mouthrinse ingredients that has received a Category I recommendation from a United States Food and Drug Administration (FDA) advisory panel for safety and effectiveness in reducing supragingival plaque and gingivitis.[20]

Antimicrobial Activity of CPC

CPC is a quaternary ammonium compound with broad spectrum antibacterial activity.[21-25] It is a cationic surface active agent (surfactant) which adsorbs readily to oral surfaces.[25,26] The molecule has both hydrophilic and hydrophobic groups, providing the possibility for ionic, as well as hydrophobic interactions. The positively charged hydrophilic region of the CPC molecule plays a major role in its antimicrobial activity, imparting a high binding affinity for bacterial cells whose outermost surface carries a net negative charge. The strong positive charge and hydrophobic region of CPC enable the compound to interact with the microbial cell surface and integrate into the cytoplasmic membrane. As a result of this interaction, there is disruption of membrane integrity resulting in leakage of cytoplasmic components, interference with cellular metabolism, inhibition of cell growth, and cell death.[23,27,28] CPC has also been shown to inhibit the co-aggregation of bacteria[29] thus interfering with plaque maturation, inhibit the synthesis of insoluble glucan by Streptococcal glucosyltransferase,[30] adsorb to pellicle-covered enamel and inhibit co-adhesion of bacteria,[25] and bind *Streptococcus mutans* biofilms.[31] The ability of CPC to adsorb to pellicle-covered enamel imparts substantivity to the molecule, that is, retention in the mouth and continued antimicrobial activity for a period of time after rinsing. The antibacterial activity of formulations containing CPC has been well-documented.

Studies have investigated the reduction in numbers of salivary bacteria following a single and multiple rinsing with mouthwashes containing CPC. These short-term studies can provide an assessment of the product's potential antiplaque effectiveness, as well as an indication of the persistence of action, or substantivity, of a formulation.[21,32] In a randomized, double-blind, parallel study, a mouthwash containing 0.05% CPC and 0.05% sodium fluoride was compared to a control mouthwash containing 0.05% sodium fluoride for its ability to control supragingival plaque bacterial counts for 12 hours after a single treatment, and 12 hours after 14 days' use.[33] The CPC mouthwash significantly reduced supragingival plaque bacteria counts by 35.3% and 70.9% compared to the control fluoride mouthwash 12 hours after a single use and after 14 days of use, respectively. Additional studies have shown significant reductions in salivary aerobic and/or anaerobic bacterial counts for up to seven hours following a single rinse with a CPC-containing product.[21,32,34] These studies

support that mouthwashes containing CPC are effective in reducing the levels of bacteria in plaque and saliva, and thus will have an effect on plaque formation.

Efficacy on Plaque and Gingivitis

A number of short-term clinical studies using mouthwashes containing 0.05% to 0.1% of CPC have demonstrated a significant reduction in plaque ranging from 25% to 39%.[24,35-42] In a six-week study by de Silva and co-workers, a mouthwash containing 0.05% CPC and 0.05% sodium fluoride was shown to significantly reduce plaque by 27.9% and gingivitis by 25.0% compared to a control fluoride mouthwash without CPC. This study clearly showed an effect on gingival health.[37] However, in other short-term studies, the effects of CPC mouthwashes on gingivitis have been mixed, and likely due to the treatment duration and CPC availability.[44,45]

Studies in which CPC mouthwashes were used for extended periods have shown that these mouthwashes can provide proven effectiveness against gingivitis as well as plaque. Three randomized controlled trials of six months duration have assessed the effectiveness of rinsing with a CPC mouthwash on plaque and gingivitis when used as an adjunct to tooth brushing with a fluoride toothpaste.[46-48] The studies varied in the concentration of CPC and the time spent brushing and rinsing; all included a placebo mouthwash. In the study by Allen and associates, in which subjects rinsed with 15 mL of a 0.05% CPC mouthwash for 60 seconds, the CPC group had 28% less plaque, a 63% reduction in plaque severity, 24% less gingivitis, and 67% less gingival bleeding when compared with the placebo group.[46] In the study by Mankodi and associates, in which subjects rinsed for 30 seconds with 20 mL of an alcohol-free 0.07% CPC rinse, the CPC group had 16% less plaque and 33% less gingival bleeding when compared with the control group.[47] Finally, Stookey, *et al*. compared two alcohol-free mouthwashes, one containing 0.075% CPC and the other 0.1% CPC.[48] Subjects rinsed with 20 mL for 30 seconds. The respective percentage reductions in plaque and gingivitis were 17% and 23% for the 0.075% CPC group and 19% and 20% for the 0.1% CPC group. Although differing in details, these studies showed the respective CPC formulations to have significant antiplaque and antigingivitis effectiveness.

Development and Validation of an Improved Alcohol-Free Mouthwash Formula with 0.075% CPC

Since the antimicrobial activity of CPC is dependent upon the positively charged hydrophilic region of the molecule, the clinical activity of CPC-containing mouthwashes is dependent upon the way in which the product is formulated; many ingredients are negatively charged and have the ability to form a complex with CPC, and thus deactivating it. In order to be effective against plaque and gingivitis, CPC-containing mouthwash formulations must provide a sufficient level of biologically active CPC. For this reason, formulations must not contain ingredients that could inactivate the molecule by interacting with the positively-charged region, or otherwise interfere with CPC activity. Formulations containing 0.05% CPC in the presence and absence of low levels (6%) of ethanol were developed and marketed globally by the Colgate-Palmolive Company. The ability of these mouthwashes

to inhibit bacteria[33] and to reduce supragingival plaque,[35-37] gingivitis,[37] and volatile sulphur compounds associated with bad breath[37] has been reported. Recently, these products have been reformulated to increase the level of CPC to 0.075% to boost activity.

To improve the efficacy of CPC-containing mouthwashes, a new formulation containing 0.075% CPC and 0.05% sodium fluoride in a base without alcohol was developed. The efficacy of the formulation has been assessed using *in vitro* methods and was found to demonstrate antimicrobial activity. Additionally, clinical studies have been conducted to demonstrate the long-lasting antibacterial efficacy of the formulation and its ability to prevent plaque formation, gingivitis, and gum bleeding. These studies are presented in this Special Issue.

In the first article, Schaeffer and co-workers report the *in vitro* antibacterial efficacy of new mouthwash formulations containing 0.075% CPC with and without alcohol against two common oral bacteria species, *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Streptococcus mutans* after a 30-second exposure.[49] Compared to a negative control mouthwash without CPC, the new mouthwash formulations containing 0.075% CPC statistically significantly (p < 0.05) reduced bacteria levels by > 99.9%.

In the second article, Rao and co-workers describe the development and use of two static model multispecies oral biofilm systems to compare the antibacterial activity of the new alcohol-free mouthwash containing 0.075% CPC and a negative control mouthwash without CPC.[50] The model systems were a 24-well glass-bottom microplate and a chamber slide system. Confocal Laser Scanning Microscopy (CLSM) and fluorometric analysis were used to assess the biofilms. CLSM demonstrated that the mouthwash containing 0.075% CPC resulted in the disruption of both biofilms. The disruption of the biofilm developed using the glass-bottom microplate was corroborated by fluorometric analysis. These models support the antibacterial effectiveness of the alcohol-free mouthwash containing 0.075% CPC.

In the third article, He and co-workers present results of a clinical study in which two mouthwash formulations containing 0.075% CPC, one in an alcohol-free base and the other containing 6% alcohol, were compared to a control mouthwash without CPC for their effect on bacteria in supragingival plaque 12 hours after a single use and 12 hours after 14 days of use.[51] Both mouthwashes statistically significantly reduced plaque bacteria compared to the control mouthwash at each post-treatment time point. The CPC mouthwash in an alcohol-free base reduced bacteria by 35.3% and 70.9% compared to the control mouthwash 12 hours after a single use and after 14 days of use, respectively. The CPC mouthwash in the 6% alcohol base reduced bacteria by 35.3% and 73.8% compared to the control mouthwash 12 hours after a single use and after 14 days of use, respectively. There were no statistically significant (p > 0.05) differences between the two CPC-containing mouthwashes at either of the post-treatment time points.

In the fourth article, Barnes and co-workers[52] present results of a clinical study in which the antiplaque efficacy of the two mouthwashes containing 0.075% CPC, one in an alcohol-free base and another in a 6% alcohol base, was evaluated using the

Modified Gingival Margin Plaque Index (MGMPI) method.[53] The CPC-containing mouthwashes were compared to a negative control mouthwash. In the study, participants rinsed twice within a 24-hour period, with the final reading 12 hours after the second rinsing. There was no statistically significant difference ($p > 0.05$) between the CPC-containing mouthwashes for their ability to reduce plaque re-growth. Both mouthwashes were statistically significantly ($p < 0.05$) more effective than the control in inhibiting plaque re-growth. Plaque re-growth was reduced by 35.1% and 27.4% for the CPC mouthwash in the alcohol-free and 6% alcohol base, respectively, compared to the control mouthwash.

In the fifth article, Ayad and co-workers present the results of a clinical study in which the alcohol-free mouthwash containing 0.075% CPC was compared to a control mouthwash without CPC on controlling established plaque and gingivitis after three and six months of use.[54] The alcohol-free mouthwash with 0.075% CPC was statistically significantly ($p < 0.05$) better than the control mouthwash in reducing established plaque and gingivitis after three and six months. After three and six months, the alcohol-free mouthwash with 0.075% CPC provided statistically significant ($p < 0.05$) reductions in gingival, gingival interproximal, gingival severity, plaque, interproximal plaque, and plaque severity index scores of 25.0%, 22.3%, 38.9%, 26.1%, 22.4%, and 75.0%, respectively, as compared to the control mouthwash. After six months of product use, the alcohol-free mouthwash with 0.075% CPC provided reductions in gingival, gingival interproximal, gingival severity, plaque, interproximal plaque, and plaque severity index scores of 38.1%, 37.1%, 63.6%, 36.5%, 33.2%, and 78.5%, respectively, as compared to the control mouthwash.

In conclusion, the laboratory and clinical research presented in this Special Issue provide scientific evidence that a new alcohol-free mouthwash formulation containing 0.075% CPC provides effective antibacterial, antiplaque, and antigingivitis efficacy. The CPC in the mouthwash is highly bio-available. The mouthwash effectively kills representative oral bacteria *in vitro* in the planktonic state by > 99.9%, reduces bacteria *in vivo* after a single use and after 14 days of continuous use, and reduces plaque build-up, gum inflammation, and bleeding. The research, therefore, supports that daily use of mouthwashes with 0.075% CPC, with and without alcohol, represents a valuable complement to daily mechanical plaque control.

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In Vitro Antibacterial Efficacy of Cetylpyridinium Chloride-Containing Mouthwashes

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Abstract

- **Objective:** The objective of this study was to examine the ability of three CPC-containing mouthwashes to kill planktonic bacteria in an *in vitro* short-exposure assay.

- **Methods:** This blind study was conducted on two common oral bacterial species: *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Streptococcus mutans.* The following mouthwashes were tested: two containing 0.075% CPC and 0.05% NaF in an alcohol-free base, and one containing 0.075% CPC and 0.05% NaF plus 6% alcohol. Additionally, a 0.05% NaF-only mouthwash was included as a negative control. Bacteria were exposed to one of the test mouthwashes for 30 seconds and then washed thoroughly, serially diluted, and plated on appropriate media to determine viable bacterial counts. Viable counts were converted to a log reduction in colony forming units (CFUs) relative to the negative control.

- **Results:** All three test mouthwashes included in this study gave a statistically significant reduction of > 3 log CFUs relative to samples treated with the negative control.

- **Conclusion:** All three experimental 0.075% CPC mouthwash formulas gave a > 99.9% reduction in viable bacteria of both species following 30 seconds of treatment.

Introduction

Mouthwashes are typically used as adjunctive therapies to tooth brushing regimens. Often these mouthwashes contain an antibacterial agent to aid in the prevention of oral diseases. Cetylpyridinium chloride (CPC) is a cationic quaternary ammonium compound that is commonly found as an active ingredient in mouthwashes intended to treat and prevent plaque and gingivitis. The clinical efficacy of CPC mouthwashes has been studied extensively. These studies show a significant inhibition of supragingival plaque and gingivitis following use of CPC mouthwashes.[1] Following longer, regular use of these mouthwashes (three to six months), significant reductions in plaque and gingivitis have been observed.[2]

A variety of methods are available to demonstrate the *in vitro* efficacy of mouthwash formulations. One preferred method is to examine the ability of a formula to kill representative bacterial species during treatment times that are consistent with the recommended use of the products being tested, typically 30 seconds.

Fine and co-workers have previously developed a method of examining the ability of a mouthwash formulation to kill planktonic oral bacteria.[3] Using this method, they demonstrated that a number of test mouthwashes were able to kill more than 99.99% of two strains of *Aggreggatibacter (Actinobacillus) actinomycetemcomitans* bacteria in a sample relative to a phosphate buffered saline (PBS) control. Additionally, they demonstrated more than 90% killing of biofilm bacteria of the same species. Because the results on biofilms were less consistent, we used the methodology for testing efficacy against planktonic bacteria to examine three CPC-containing mouthwashes, which differ in excipients and/or flavor.

Mouthwashes are complex mixtures and, in addition to the active ingredient, can contain a variety of excipient ingredients able to exert some effects on bacterial viability. These include surfactants and preservatives. However, the majority of *in vitro* studies of mouthwashes compare the efficacy of the test product to a relatively inert control, such as PBS. In order to better understand the true role of the active ingredient CPC in these mouthwashes, we used a negative control mouthwash containing the key mouthwash ingredients, except for CPC, as the basis of comparison for this study.

Materials and Methods

Test Mouthwashes

A total of three test mouthwashes and one negative control were included in this study. Table I summarizes the mouthwashes.

Table I
Summary of Mouthwashes Used in this Study

Mouthwash Description	CPC Level	NaF Level	Alcohol Level
Negative Control	0%	0.05%	0%
Test Mouthwash 1	0.075%	0.05%	0%
Test Mouthwash 2	0.075%	0.05%	6%
Test Mouthwash 3	0.075%	0.05%	0%

Bacterial Strains and Growth Conditions

Two bacterial strains were used in this *in vitro* study: *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (ATCC #43717) and *Streptococcus mutans* (ATCC #25175). *A. actinomycetemcomitans* was grown overnight at 37°C from a first generation

frozen glycerol stock in Brain Heart Infusion (BHI) broth supplemented with 40 µg/mL of sodium bicarbonate; *S. mutans* was grown overnight at 37°C from a first generation frozen glycerol stock in Trypticase Soy Broth (TSB).

Bacterial Kill Assay

After 24 hours, the absorbance of each culture was measured at 610 nm (OD_{610}) and cultures were adjusted to an $OD_{610} \sim 0.8$. For *A. actinomycetemcomitans*, this was found to correspond to $\sim 10^{10}$ CFU/mL. For *S. mutans*, this was equivalent to $\sim 9.5 \times 10^9$ CFU/mL.

One mL aliquots of the adjusted cultures were transferred to sterile microcentrifuge tubes, and samples were pelleted by centrifuging for 10 minutes at $15,000 \times g$. Cell pellets were treated with either sterile PBS, the negative control, or one of the test mouthwashes and dispersed by trituration. After 30 seconds of exposure, the samples were pelleted for 30 seconds at $15,000 \times g$. Supernatants were aspirated and samples washed three times with sterile PBS.

Washed pellets were resuspended in 100 µL of sterile PBS. Ten-fold serial dilutions were performed in sterile PBS, and 100 µL of appropriate dilutions were plated on agar plates. *A. actinomycetemcomitans* samples were plated on BHI agar containing 10% defibrinated sheep's blood. Plates were incubated for 48–72 hours in a 5% CO_2 atmosphere prior to counting colonies. *S. mutans* samples were plated on TSB agar plates containing 5% defibrinated sheep's blood, and were incubated under anaerobic conditions for 48–72 hours prior to counting.

Colony counts were used to determine the numbers of viable bacteria per mL sample (CFU/mL), and this value was used to determine the log reduction in CFUs relative to the negative control-treated samples.

For blinding purposes, samples were labeled only with numeric codes. The treatment step was completed by a separate scientist from the subsequent plating and data collection.

Statistical Analysis

Colony counts were log-transformed, and the log reduction in CFU/mL relative to the negative control mouthwash was calculated. Treatments were performed in duplicate and results represent the average of four independent experiments. Results were analyzed by analysis of variance (ANOVA). A Tukey's multiple comparison test was used to assess pairwise differences.

Results

Treatment of samples with the negative control mouthwash without CPC resulted in a limited reduction in CFUs/mL for both species tested. Overall, negative control treatment reduced *A. actinomycetemcomitans* counts by 4.26 log (Figure 1) and *S. mutans* counts by 0.45 log (Figure 2). This higher sensitivity of the *A. actinomycetemcomitans* strain to mouthwash ingredients in general was observed consistently throughout this study. Due to this differing sensitivity, the performance of the test mouthwashes containing CPC was compared to the colony counts obtained both with treatment with PBS (Figures 1 and 2), as well as after treatment with the negative control mouthwash (Figures 3 and 4).

Figure 1. *Reduction in CFUs following treatment of* A. actinomycetemcomitans *with test or commercial CPC mouthwashes. Data are reported as a log reduction in CFUs relative to a sample treated with sterile PBS.*

Figure 2. *Reduction in CFUs following treatment of* S. mutans *with test or commercial CPC mouthwashes. Data are reported as a log reduction in CFUs relative to a sample treated with sterile PBS.*

Figure 3. *Reduction in CFUs following treatment of* A. actinomycetemcomitans *with test or commercial CPC mouthwashes. Data are reported as a log reduction in CFUs relative to a negative control mouthwash lacking CPC.*

When *A. actinomycetemcomitans* was exposed to test mouthwashes, Test Mouthwash 1 gave a 9.28 log reduction in CFUs versus PBS treatment (Figure 1), and a 5.03 log reduction in CFUs relative to the negative control mouthwash (Figure 3).

Test Mouthwash 2 gave a 7.24 log reduction in CFUs versus PBS (Figure 1), and a 3.15 log reduction in CFUs versus the negative control (Figure 3).
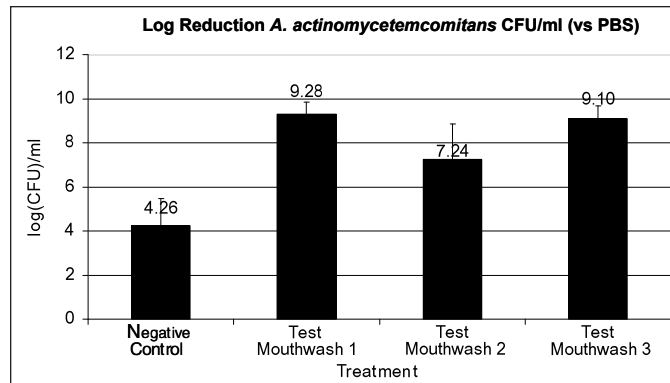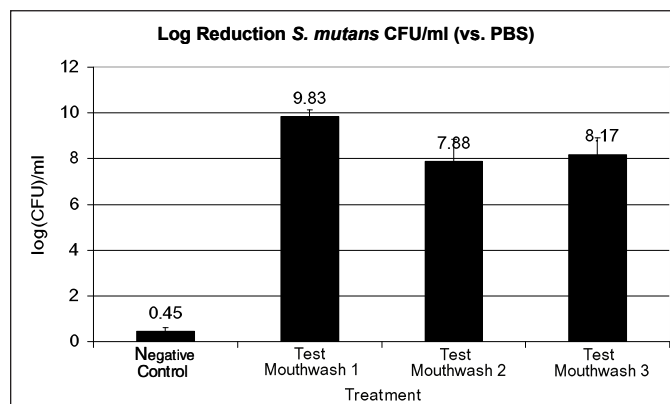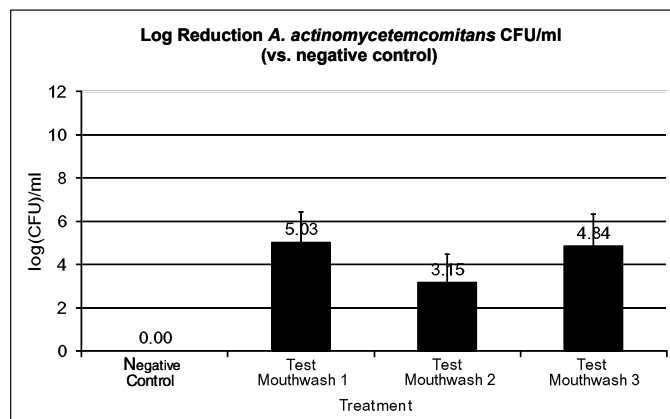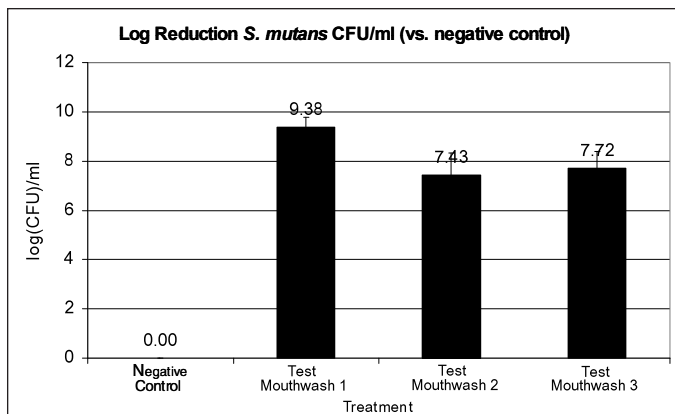
Figure 4. *Reduction in CFUs following treatment of* S. mutans *with test or commercial CPC mouthwashes. Data are reported as a log reduction in CFUs relative to a negative control mouthwash lacking CPC.*

Test Mouthwash 3 gave 9.10 (Figure 1) and 4.84 (Figure 3) log reductions in CFUs vs. PBS and the negative control mouthwash, respectively.

All three test mouthwashes reduced planktonic *A. actinomycetemcomitans* by greater than 99.9% over a negative control formula (Table II). All mouthwashes containing 0.075% CPC were statistically significantly better than the negative control mouthwash in reducing *A. actinomycetemcomitans* viability (p < 0.05). No differences were observed among the 0.075% CPC-containing mouthwashes.

Planktonic *S. mutans* were also exposed to the test mouthwashes. Test Mouthwash 1 reduced *S. mutans* counts by 9.83 log CFU over treatment with PBS (Figure 2), and 9.38 log CFU over the negative control mouthwash (Figure 4).

Test Mouthwash 2, the only formula containing alcohol, reduced CFU/mL by 7.88 log over treatment with PBS alone (Figure 2), and 7.43 log over the negative control mouthwash (Figure 4).

Test Mouthwash 3 gave a reduction of 8.17 log CFU/mL over PBS alone (Figure 2), and 7.72 log CFU over the negative control mouthwash (Figure 4).

For planktonic *S. mutans,* the test mouthwashes all gave a greater than 99.99% reduction in viable planktonic bacteria (Table II). There was no statistical difference among the three mouthwashes containing 0.075% CPC, and all three mouthwashes performed statistically better than the negative control.

Table II
Reductions in CFUs Following Treatment of *A. actinomycetemcomitans* and *S. mutans* with Test CPC Mouthwashes

| Mouthwash | *A. actinomycetemcomitans* | | *S. mutans* | |
	Log cfu/mL Reduction (vs. Control)	Percent Reduction (vs. Control)	Log cfu/mL Reduction (vs. Control)	Percent Reduction (vs. Control)
Test mouthwash 1	5.03	> 99.99	9.38	> 99.99
Test mouthwash 2	3.15	> 99.9	7.43	> 99.99
Test mouthwash 3	4.84	> 99.99	7.72	> 99.99

Data are reported as a log reduction in CFUs relative to the negative control mouthwash with no CPC/

Discussion

This study was based on the work of Fine, *et al.*, which demonstrated the ability of mouthwashes to kill planktonic oral bacteria to a high degree *in vitro*.[3] In that study, the efficacy of three antibacterial mouthwashes was tested against planktonic and biofilm forms of *A. actinomycetemcomitans.* Although all mouthwashes in their study were able to kill more than 99.99% of planktonic bacteria, antibacterial efficacy on biofilm bacteria was less consistent than for the planktonic.[3] Therefore, we conducted our study only on planktonic bacteria.

The oral cavity is a complex environment with an estimated 700 distinct species of bacteria.[4-6] Modeling such a complex environment in an *in vitro* system is difficult. However, to broaden the scope of this study, we chose to include a Gram-positive aerobic oral bacterium involved in the development of dental caries, *S. mutans*. This would provide a good contrast to the Gram-negative, capnophilic periodontal pathogen *A. actinomycetemcomitans*. Therefore, the inclusion of a second species of bacterium provided a broader demonstration of the efficacy of the mouthwashes studied here.

Although not as complex as toothpastes, mouthwashes contain a variety of inactive ingredients, such as surfactants and salts, which could impact the *in vitro* viability of planktonic bacteria. As can be seen in Figures 1 and 2, a negative control mouthwash, which contained the key ingredients but no CPC, did impact the viability of the two species tested. Therefore, it was important to compare the viability of treated organisms to those treated with this negative control mouthwash, as well as to PBS-treated controls. Using this comparison, the true effects of the available CPC in each formula, independent of any mitigating ingredients from the mouthwash base, could be discerned.

All test mouthwashes included in this study contained 0.05% NaF, in addition to CPC. Other methodologies used to study CPC mouthwashes have suggested that the presence of anions, in general, will limit the amount of free, bioavailable CPC.[7] This statement implies that the presence of NaF in a formula could have a detrimental effect on CPC activity. This study, however, shows that the tested CPC mouthwashes possess a high level of antibacterial activity with the inclusion of NaF.

Conclusions

All three mouthwashes tested in this study were able to kill more than 99.9% of two representative species of planktonic bacteria of dental relevance (*A. actinomycetemcomitans* and *S. mutans*) when compared to a negative control mouthwash. Additionally, the two representative species chosen are thought to be causative agents of periodontitis and caries, respectively, so these studies represent the potential of these mouthwashes to prevent the two major oral diseases.

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Efficacy of an Alcohol-Free CPC-Containing Mouthwash Against Oral Multispecies Biofilms

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Abstract

- **Objective:** The aim of this work was to develop two static-model multispecies oral biofilm systems to compare the efficacy of a placebo mouthwash to an alcohol-free mouthwash containing 0.075% CPC.

- **Methods:** Two model biofilm systems were used: a 24-well glass-bottom microplate (GM) system and a chamber slide (CS) system. These were inoculated with Schaedler media containing pooled, unfiltered saliva. During incubation at 37°C in 5% CO_2, Schaedler media was replaced every 24 hours. Five-day and 10-day multispecies biofilms in the GM and CS systems were then exposed to phosphate buffered saline, the placebo mouthwash, or the alcohol-free 0.075% CPC-containing mouthwash. Biofilms were visualized in three-dimensions by Confocal Laser Scanning Microscopy (CLSM), and fluorometric analyses were performed on biofilms in the GM system.

- **Results:** CLSM demonstrated that regardless of the model system used, the alcohol-free 0.075% CPC-containing mouthwash solution increased the number of damaged biofilm cells. The efficacy of CPC was inversely related to the age of the biofilm. A contrariety between the two biofilm systems was that the CS system indicated that alcohol-free 0.075% CPC-containing mouthwash partially disrupted biofilms. Fluorometric analysis of GM biofilms also demonstrated that the alcohol-free 0.075% CPC-containing mouthwash damaged biofilm cells.

- **Conclusion:** Two static oral multispecies model biofilms systems demonstrated that an alcohol-free 0.075% CPC-containing mouthwash had greater antimicrobial efficacy than a placebo mouthwash. The alcohol-free 0.075% CPC-containing formulation is effective against multispecies oral biofilms.

Introduction

Dental plaque is a multispecies oral biofilm that develops on tooth surfaces.[1] Development is proposed to involve orchestrated cell-cell interactions that promote the sequential integration of species.[2,3] If not adequately controlled, later species can integrate, including those that contribute to periodontal disease.[4] Attempts at controlling oral biofilm development are typically through regular mechanical oral cleaning regimens which help prevent periodontal disease.[5-7] However, mechanical regimens such as brushing and flossing are potentially not enough to prevent the development of dental plaque biofilms and the progression to oral disease.[8] This is probably due to the fact that certain areas of the oral cavity are difficult to effectively clean with mechanical tools, and the successful use of such tools may vary between individuals.[9,10] Therefore, either mechanical regimens need to be improved (such as through the use of newly designed toothbrushes or floss) and/or efficacious adjuncts (such as dental water jets or mouthwash) need to be applied to aid in the control of oral biofilms.[6,11-13] Consistent with problems associated with maintaining oral health, periodontal disease is one of the most common chronic infections in adults.[14] Pihlstrom, *et al.* suggests

that up to 90% of the world's population has or will suffer from periodontal disease.[15]

One approach to improving oral hygiene is the routine use of mouthwashes formulated with antimicrobial agents. Mouthwashes contain antimicrobials and additives that may individually or collectively have one or more modes of action against dental plaque biofilms and their constituent cells.[16] Mouthwashes can include various types of antimicrobials, such as metal ions, essential oil formulations, chlorhexidine, or the quaternary ammonium compounds (QACs).[12,16-20] QACs are surface-active agents that have an important role in the fields of medical, dental, and general disinfection. QACs have well-established antimicrobial properties, and these were indicated to have both antifungal and antibacterial activities in the early 1900s.[21-23] Cetylpyridinium chloride (CPC) is a QAC that has been increasingly used in a variety of mouthwashes over the past decades. Typically, the concentration (W/V) of CPC is 0.05%, although slightly higher concentrations (≥ 0.07) have been used.[20,24-26] Like other QACs, CPC is an antimicrobial that damages cells by interacting with bacterial membranes.[27] Interaction results in the solubilization of regions of the membranes to release "blebs"

(micelle-like vesicles released from the outer or cytoplasmic membranes) and cause leakage of bacterial intracellular contents.[28-30] Such damage is believed to be the main cause of cell death.

Dental plaque is composed of more than 500 species of bacteria.[31-33] This is an important consideration when attempting to study the efficacy of antimicrobials because different species possess different intrinsic resistances to antimicrobials such as CPC. For example, Roberts and Addy demonstrated that Gram-negative bacteria are generally less susceptible to CPC than Gram-positive species.[34] Furthermore, because CPC is a bacterial membrane-active antimicrobial, it is possibly subject to membrane-mediated reaction-diffusion-limitation through biofilms.[35] Contact time and species composition would conceivably affect penetration.[29] Thus, to begin to obtain a complete picture of the efficacy of CPC on biofilms, *in vivo* studies or environmentally *germane in vitro* experiments need to be conducted.

The aim of this study was to examine the efficacy of an alcohol-free mouthwash formulation that contained 0.075% CPC on multispecies oral biofilms. In order to achieve this, two model systems were used to develop oral multispecies biofilms from inoculums of pooled saliva. Five-day and 10-day old biofilms were studied. The visualization of different cellular morphotypes, and by association the inference of different bacterial species in the biofilms was determined by epifluorescence microscopy. Untreated multispecies biofilms (exposed only to phosphate buffered saline), those treated with a placebo mouthwash (lacking CPC but containing 0.05% NaF), and those treated with an alcohol-free mouthwash containing 0.075% CPC and 0.05% NaF were then exposed to a vital stain and studied using epifluorescence and Confocal Laser Scanning Microscopy (CLSM). Biofilm architecture was studied to examine the disruptive effect of CPC, and fluorometric analysis in a microplate reader allowed for the relative amounts of cell damage to be inferred.

Materials and Methods

Test Mouthwash
The test solutions used in this study were comprised of an alcohol-free 0.075% cetylpyridinium chloride (CPC)-containing mouthwash with 0.05% NaF (Colgate-Palmolive Company, New York, NY, USA), a placebo mouthwash that lacked CPC but contained 0.05% NaF (Colgate-Palmolive Company, New York, NY, USA), and sterile phosphate buffered saline (PBS) which served as a negative treatment control. Each test solution was provided to the investigator in a container labeled with a unique numeric code.

Saliva Collection and Preparation
Saliva was collected from up to five healthy adults who had not consumed food for two hours prior to donation, and had only drunk water during that time. These individuals were non-smokers and had not taken antibiotics for at least three months prior to donation. Pooled saliva was treated in two ways depending upon use: cell-free saliva (CFS) was used to condition poly-L-lysine-coated surfaces in model biofilm systems, while bacterial cell-containing saliva (CCS) was used as an inoculum

for biofilm studies. In order to prepare CFS, saliva was pooled and treated with 2.5 mmol L^{-1} dithiothreitol (Sigma, St. Louis, MO, USA) for 10 minutes. This step was performed to reduce salivary protein aggregation. Subsequently, the reduced saliva was centrifuged at 17,000x g for 45 minutes, and the supernatant was diluted with distilled water to yield 25% saliva. Diluted saliva was frozen at $-20°C$ until required, whereupon aliquots were thawed and filter-sterilized through a 0.22-µm-pore size polyethersulfone membrane filter unit. In order to prepare CCS, saliva was pooled and stored in 50% glycerol until required, whereupon it was thawed and used.

Chamber Slide Biofilm System
Eight-well chamber slides (Lab-Tek, Rochester, NY, USA) were used to create the chamber slide (CS) biofilm system. Wells were first pre-treated with 0.05% poly-L-lysine (Electron Microscopy Sciences, Hatfield, PA, USA) in CFS. After four hours, the solution was removed and 50 µL inoculums of CCS were added to each of the wells with 200 µL of Schaedler medium (Difco Laboratories, Detroit, MI, USA). CS biofilm systems were incubated at 37°C with 5% CO_2, and the media was replaced every 24 hours. Following incubation for five or 10 days, media was discarded and the upper eight-well plastic chambers were removed according to the manufacture's instruction (Lab-Tek, Rochester, NY, USA). The slides with attached biofilm were then immersed in slide jars (Online Science Mall, Pinson, AL, USA) containing PBS (pH 7.4), placebo mouthwash, or an alcohol-free mouthwash containing 0.075% CPC. Slides were exposed for two minutes and then quickly transferred into fresh slide jars containing PBS (pH 7.4) for approximately 30 seconds. Subsequently, all the slides were placed in a slide jar containing LIVE/DEAD® stain (Invitrogen, Carlsbad, CA, USA). The final concentrations for each component of the LIVE/DEAD stain were 1.67 µM SYTO 9 and 10 µM propidium iodide. Following 20 minutes of staining, slides were washed in slide jars containing PBS (pH 7.4), and a cover slip was applied to the surface in preparation for microscopy. Biofilms were visualized using an upright Leica SP5 CLSM system (Leica, Exton PA, USA) equipped with a HCX PL APO 40×/1.25 oil immersion lens.

Glass-Bottom Biofilm System
Twenty-four well glass-bottom microplates (Greiner Bio-one, Frickenhausen, Germany) were used to create the glass-bottom microplate (GM) biofilm system. Wells were pretreated with 0.05% poly-L-lysine in CFS. After four hours, the solution was removed and 400 µL inoculums of CCS were added to the wells with 600 µL of Schaedler medium. CS biofilm systems were incubated at 37°C in 5% CO_2, with the media being replaced every 24 hours. Following incubation for five or 10 days, the media was removed. PBS (pH 7.4), placebo mouthwash, or an alcohol-free mouthwash containing 0.075% CPC was then transferred into the corresponding wells, and biofilms were exposed to these solutions for two minutes. This was followed by a washing step in PBS (pH 7.4) for 30 seconds, before being exposed to the LIVE/DEAD stain for 20 minutes. After staining, biofilms were washed in PBS (pH 7.4) before being visualized using an inverted

Leica SPE CLSM equipped with a HCX PL APO 40×/1.25 oil immersion lens. The relative proportion of live and dead cells was quantified using a fluorometric approach described below.

Fluorometric Analysis of Biofilms

The relative fluorescence of SYTO 9 and propidium iodide from LIVE/DEAD stained multispecies oral biofilms within GM biofilm systems was quantified. This was performed by measuring the amount of green (emission: 535/40 nm) and red (emission: 610/10 nm) fluorescence in a multilabel microplate reader (Victor X3 Multi-label Reader, Perkin Elmer, Waltham, MA, USA) during exposure to blue light (excitation: 485 nm). From the measured intensities of both stain components, the percentage of dead/damaged cells was approximated by tabulating the intensity of the red signal over the total intensity of both the red and green signals. Two-tailed t-tests were used to determine the statistical significance of the results. Values of $p < 0.05$ were considered significant.

Computational Rendering of Microscope Images

Multispecies biofilm stacks that were captured using CLSM were rendered using Imaris (Bitplane, Zurich, Switzerland) imaging software on an Intel® Core™ i5 (Intel, Santa Clara, CA, USA) computer equipped with an ATI (AMD, Sunnyvale, CA, USA) Radeon HD5850 graphics card. In order to discern live and dead cells as well as overall biofilm structure, stacks were constructed using the normal shading option in Imaris. The resulting rendered three-dimensional biofilm structures were also inspected and compared visually using the Easy 3D and Surpass options.

Results

Visualization of Different Cell Types in Biofilms

In order to establish whether the biofilms in the two model systems likely contained different species of bacteria, a microscopic inspection of five-day and 10-day biofilms was performed using an epifluorescence microscope. In both systems and at both time points, multiple cell shapes and arrangements were observed. These included cocci-shaped cells arranged in chains (Figure 1A), suggesting the presence of streptococci species. Cocci were also observed to be bound to long fusiform-like cells, suggesting that these corncob-like structures could be coaggregates of streptococci and *Fusobacterium nucleatum* (Figure 1B). Compared to other cells observed, large oval-shaped cells with the occasional associated thick cellular filaments were also observed, and these were likely yeast cells (Figure 1C). Rod-shaped cells were also often observed and found to be in a variety of arrangements including rosette-like structures (Figure 1D). These structures were observed in both the five-day and 10-day biofilms, although the relative proportions of each type in the different biofilm systems was difficult to compare.

Effect of an Alcohol-Free CPC-Containing Mouthwash on Multispecies Biofilms within the GM System

The GM system, while requiring a specialized microplate carriage in an inverted Leica SPE CLSM, was amenable to epifluorescence analysis (Figure 2), confocal analysis (Figure 3), and analyses in a fluorescent microplate reader (Table I). Visual
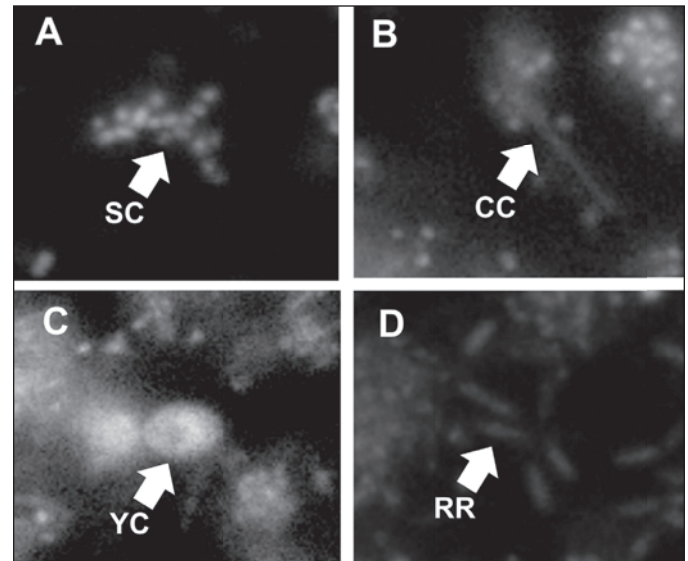
Figure 1. *Epifluorescence microscope images showing the presence of cell types that are typically observed in the model multispecies biofilms. Arrows in each image highlight the cells and structures being described. (A) Cocci-shaped cells arranged in chains, labeled as SC in the image, which are wrapped around each other. (B) A long fusiform-shaped cell with cocci-shaped cells attached that form corn-cob-like structures (labeled as CC in the image). (C) Large yeast-shaped cells, labeled as YC, embedded with masses of bacterial cells. (D) Rod-shaped cells, labeled as RR in the image, arranged in a rosette like structure.*
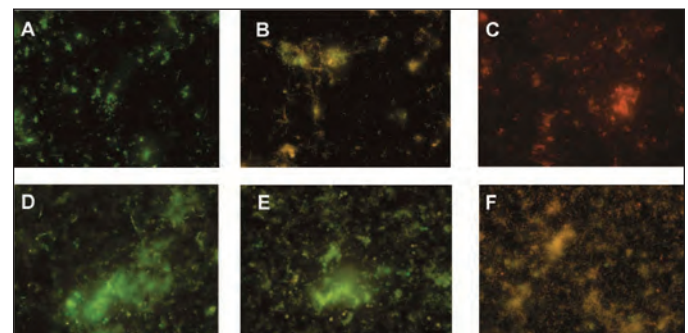
Figure 2. *Representative epifluorescence microscope images showing the effect of three different treatment regimens on five-day (A–C) and 10-day (D–F) multispecies biofilms in the GM system. Biofilms were stained with LIVE/DEAD stain. (A) Five-day multispecies biofilm following exposure to PBS. (B) Five-day multispecies biofilm following treatment with the placebo mouthwash. (C) Five-day multispecies biofilm following treatment with the alcohol-free 0.075% CPC-containing mouthwash. (D) Ten-day multispecies biofilm following exposure to PBS. (E) Ten-day multispecies biofilm following treatment with the placebo mouthwash. (F) Ten-day multispecies biofilm following treatment with the alcohol-free 0.075% CPC-containing mouthwash.*

Table I

Approximated Percentage of Dead/Damaged (Red) Cells Relative to Total Cells in Oral Multispecies Biofilms Developed in the GM System

Age of Biofilm	PBS Wash (Untreated)	Placebo Mouthwash	CPC Mouthwash
5 days	25.7 (2.7)	31.7 (1.7)	72.7 (5.1)
10 days	24.9 (2.0)	25.5 (3.5)	44.3 (10.1)

Values are the percentage of red signal relative to the total signal intensity for both red and green channels. The values within the brackets are the standard deviation of each mean percentage value.

Figure 3. *Oral multispecies biofilms that were developed in the GM biofilm system and exposed to PBS or treated with a placebo mouthwash or the alcohol-free 0.075% CPC-containing mouthwash. Two-dimensional projections and associated three-dimensional Z-dimension renderings of multispecies biofilms in z-dimensions. (A) A viable green five-day oral multispecies biofilm exposed to PBS. (B) A typical mostly green and some yellow viable five-day multispecies biofilm treated with the placebo mouthwash. (C) A representative five-day multispecies biofilm containing damaged bacteria (mostly red cells) that was treated with the alcohol-free 0.075% CPC-containing mouthwash. (D) A typical image of a viable (mostly green with some yellow cells) 10-day multispecies biofilm treated with the placebo mouthwash. (E) A typical mostly green and some yellow viable 10-day multispecies biofilm treated with the placebo mouthwash. (F) An image of a 10-day multispecies biofilm containing damaged and undamaged cells that was treated with the alcohol-free 0.075% CPC-containing mouthwash. Bar represents 30 µm.*

inspection by epifluorescence microscopy (Figure 2) and CLSM (Figure 3) demonstrated that five-day biofilms contained very different amounts of biofilm as compared to 10-day biofilms. Five-day biofilms typically contained much less biofilm (and by association, less cells) than 10-day biofilms. Compared to CLSM, this was less obvious using epifluorescence microscopy (Figure 2), because only one focal plane within the biofilms could be observed at one time. However, epifluorescence microscopy clearly demonstrated that LIVE/DEAD stained untreated five-day and 10-day multispecies biofilms (*i.e.,* those that were exposed to PBS) were predominantly green with a few yellow cells (Figure 2A and 2D). This indicated that the cells within the biofilms were undamaged and viable. Biofilms that were treated with the placebo mouthwash (that lacked CPC) were predominantly green, although a greater proportion of yellow cells was observed in five-day biofilms as compared to un-

treated biofilms (Figure 2B versus 2E). The presence of yellow cells suggests that while the cells were still viable, some membranes were possibly compromised by ingredients of the placebo mouthwash. When treated with the alcohol-free CPC-containing mouthwash, cell membranes within five-day multispecies biofilms were severely compromised, as indicated by the increase in the proportion of red cells (Figure 2C). Ten-day biofilms treated with the alcohol-free CPC-containing mouthwash contained comparatively far fewer red cells, and instead consisted of a mixture of mostly green and yellow cells (Figure 2F). By changing the focal plane within the biofilm, there was clearly an inverse relationship between biofilm depth and the numbers of red or yellow cells; most of the cells were green at the base of the biofilms (data not shown).

Microplate-based fluorescence analysis of multispecies biofilms within the GM biofilm systems confirmed the visual findings
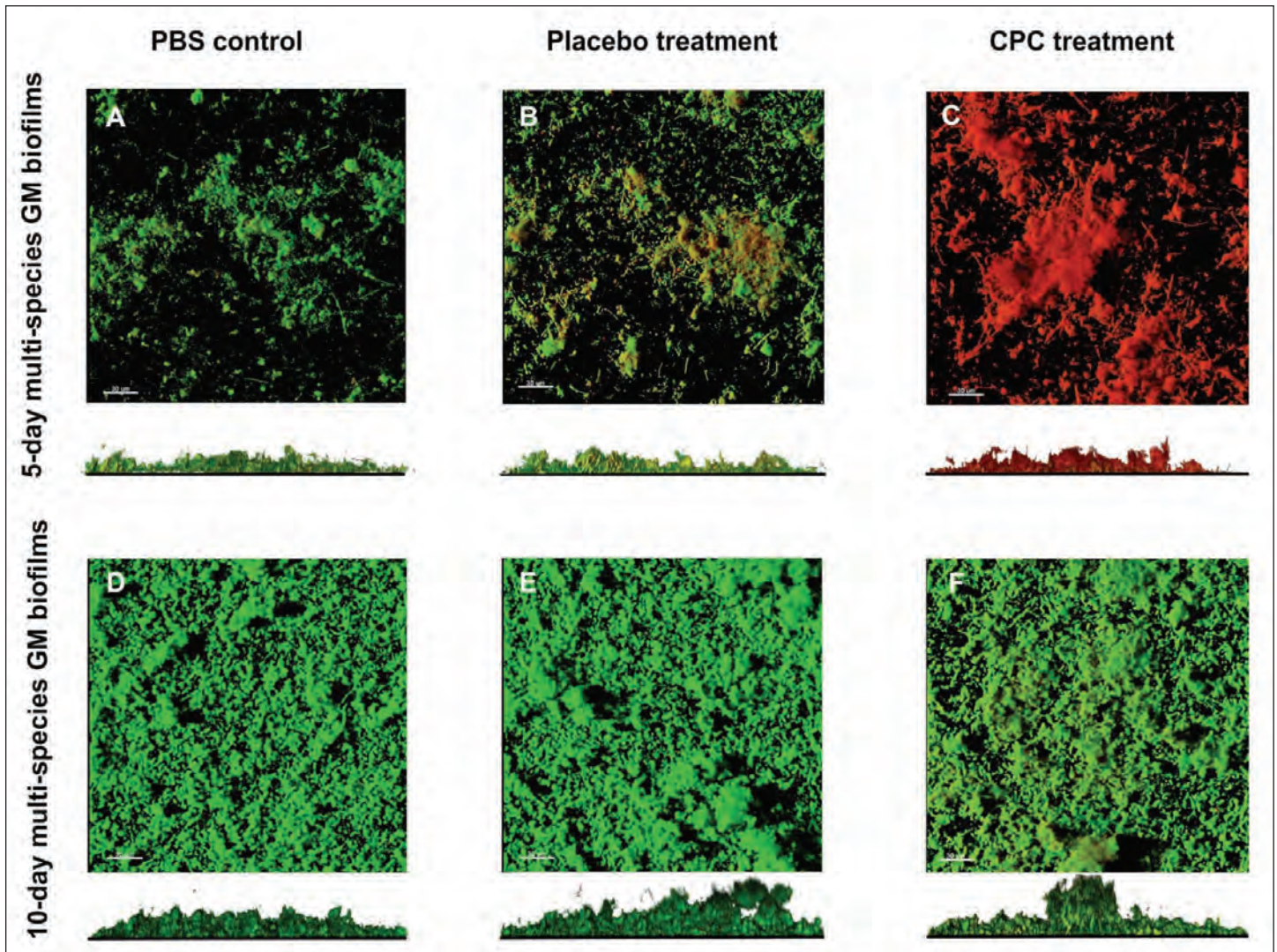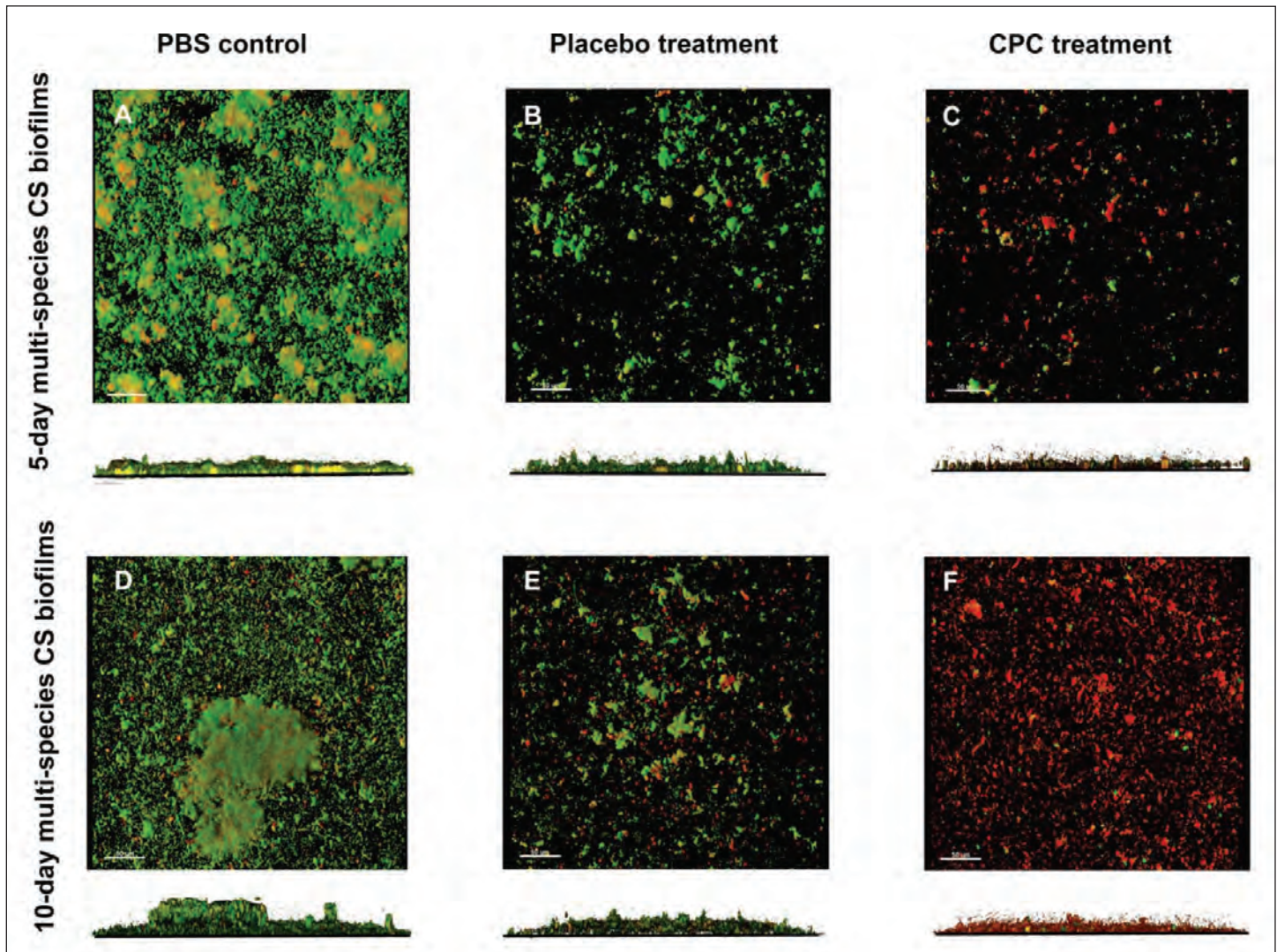
Figure 4. *Oral multispecies biofilms that were developed in the CS biofilm system and exposed to PBS or treated with a placebo mouthwash or the alcohol-free 0.075% CPC-containing mouthwash. Two-dimensional projections and associated three-dimensional Z-dimension renderings of multispecies biofilms in z-dimensions. (A) A viable green and yellow five-day oral multispecies biofilm exposed to PBS. (B) A typical mostly green and yellow viable five-day multispecies biofilm treated with the placebo mouthwash. (C) A representative five-day multispecies biofilm containing damaged bacteria (mostly red cells) that was treated with the alcohol-free 0.075% CPC-containing mouthwash. (D) A typical image of a viable (mostly green with few yellow and even less red cells) 10-day multispecies biofilm treated with the placebo mouthwash. (E) A typical mostly green and some yellow viable 10-day multispecies biofilm treated with the placebo mouthwash. (F) An image of a 10-day multispecies biofilm containing damaged bacteria (mostly red cells) that were treated with the alcohol-free 0.075% CPC-containing mouthwash. Different degrees of cellular deadhesion can be observed in the three-dimensional Z-dimension renderings. Bar represents 50 μm.*

of epifluorescence microscopy (Table I), and allowed for comparison of the percentage amounts of red signal from each of the multispecies biofilms. Specifically, the least amount of red fluorescent signal was detected in the PBS five-day and 10-day biofilms, while the most red fluorescence signal was detected in the biofilms treated with the alcohol-free CPC-containing mouthwash. The percentage amount of red fluorescence was significantly greater in the five-day biofilms treated with the alcohol-free CPC-containing mouthwash than with PBS or the placebo mouthwash ($p < 0.001$), suggesting the greatest proportion of dead/damaged cells were in these multispecies biofilms treated with the alcohol-free CPC-containing mouthwash. For 10-day biofilms, the amount of red fluorescent signal in the biofilm treated with the alcohol-free CPC-containing mouthwash, although much lower than five-day biofilms, was still significantly greater than the biofilm treated with PBS or the placebo mouthwash ($p < 0.05$).

CLSM allowed for three-dimensional analyses of multispecies biofilms developed in the GM system. Renderings from three-dimensional stacks allowed acceptable visual inspection of biofilm structure. Overall, CLSM clearly showed that regardless of treatment, five-day biofilms possessed relatively thin finger-like projections extruding from the glass surface, whereas 10-day biofilms not only covered the glass surface but also produced thick towers and mounds (Figures 3A, B, C versus Figures 3D, E, F). No clear disruption to biofilm structure was observed to occur when multispecies biofilms were treated with the placebo mouthwash or the alcohol-free CPC-containing mouthwash. Compared to epifluorescence imaging, a similar outcome of increased numbers of yellow or red cells was observed in the placebo or alcohol-free CPC-containing mouthwash. However, the biofilms tended to look greener in color and this may be an artifact of the rendering and overlaying colors. An advantage to

the CLSM approach, though, was that it allowed for the position of stained cells within the multispecies biofilms to be examined in the Z-dimension (Figures 3A–3F). This revealed that within untreated multispecies biofilms, there was a mixture of predominantly green and occasional yellow cells through the biofilms, regardless of whether the biofilm was five days or 10 days old (Figures 3 and 3D). In placebo-treated multispecies biofilms, yellow and occasionally red-colored cells were present at the tip of finger-like projections and within the troughs of the five-day biofilms (Figure 3B), while in the 10-day biofilms the few yellow cells present were typically observed on the uppermost surfaces of the biofilm towers (Figure 3E). In the alcohol-free CPC-containing mouthwash-treated five-day biofilm, the vast majority of the biofilm cells were red, with only the underlying, deeply situated biofilm cells being yellow (Figure 3C). In comparison, the 10-day alcohol-free CPC-containing mouthwash-treated biofilms contained yellow- and red-colored cells that were predominantly located on the surface of the thick multispecies biofilms, and the vast majority of cells within the inner layers of the biofilms were green (Figure 3F).

Effect of Alcohol-Free CPC-Containing Mouthwash on Multispecies Biofilms within the CS System

The CS biofilm system required the use of a different upright CLSM system (Leica SP5) to that used for the analysis of biofilms in the GM biofilm system (Leica SPE). A similar trend to the GM system was observed for the response of the multispecies biofilms to PBS (no treatment), the placebo mouthwash, and the alcohol-free CPC-containing mouthwash. Specifically, five-day biofilms consistently contained multispecies biofilms that were less substantial and had less structural heterogeneity than 10-day biofilms (Figures 4A, B and C versus Figures 4D, E, F). These five-day and 10-day biofilms, however, often yield much thinner and less structurally heterogeneous multispecies biofilms than the respective biofilms developed in the GM system for the same periods (Figure 4 versus Figure 3). The biofilms in the CS biofilm system yielded similar proportions of green, yellow, and red cells for placebo mouthwash treatments, whereas the alcohol-free CPC-containing mouthwash-treated multispecies biofilms were notably different. The alcohol-free CPC-containing mouthwash-treated multispecies five-day and 10-day biofilms contained mixtures of predominantly red, but also some yellow and green cells. Unfortunately, the relative amounts of red damaged cells could not be quantified within the wells of this system due to bleeding of signal between wells. Three-dimensional rendering and visualization of the Z-sections also indicated that more biofilm cells were dispersed in the planktonic suspension of the alcohol-free CPC-containing mouthwash-treated biofilms than in the PBS exposed or placebo mouthwash-treated biofilms. While these cells could be visualized, a quantitative comparison was not possible because of blurring due to their movement.

Discussion

The work presented here demonstrates that an alcohol-free mouthwash containing 0.075% CPC was effective at treating *in vitro* multispecies oral biofilms in two laboratory model systems. Few *in vitro* oral model biofilm systems have been developed

that contain multispecies biofilm communities. The two model systems described here are likely to contain species representative of natural dental plaque, and respond in a similar manner to natural dental plaque biofilms. Arguably, the development of representative *in vitro* model biofilms that contain species that are found in human dental plaque is important to better predict the *in vivo* efficacy of antimicrobial-containing mouthwashes.

Using pooled human saliva as an inoculum, developed biofilms were observed to contain a mixture of cell types and cellular arrangements (Figure 1). This observation is indicative of the multispecies nature of the developed oral biofilm. In addition, the cell morphologies and cellular arrangements indicated that both model multispecies biofilms contained species typically found in natural dental plaque biofilms. Dental plaque contains more than 500 species of bacteria, with many possessing very distinct cell shapes and abilities to form intraspecies and interspecies aggregations with complex cellular arrangements.[31,32] Streptococci normally dominate in healthy dental plaque and typically represent more than 50% of the healthy dental plaque species.[36] Within the two model oral multispecies biofilm systems described here, long interwoven chains of streptococci were often observed (Figure 1A), as well as large clusters of chains (data not shown). Not only were cocci observed in chains, but cocci-shaped cells were also observed attached to long fusiform-shaped cells (Figure 1B). Similar structures have been observed in dental plaque,[37] which are known to be composed of streptococci and a single *Fusobacterium nucleatum* cell at the core of the "corncob" structure.[38] Other intricate cell arrangements were observed, including rod-shaped cells that were observed to be associated with cocci in rosette-like radial arrangements (Figure 1D). The presence of such arrangements has also been observed in natural dental plaque.[39] Evidence also indicates that these structures can form as a consequence of coaggregation.[40] Coaggregation is the specific recognition and adhesion of different species of bacteria,[41] and is proposed to be integral to biofilm development.[3] In addition to bacteria, yeast-like cells were observed within the multispecies biofilms that were developed in both model systems. The most common yeast in the oral cavity is *Candida albicans*.[42] Depending upon the environment and bacterial species composition of the biofilm, the amount of yeast cells varies. Not only is *Candida albicans* associated with diseases, such as oral candidiasis, but it is also known to be able to coaggregate.[43,44] Thus, compared to dental plaque, similar cell types, structures, and possible interactions between similar species were observed in the model multispecies biofilm systems. Collectively, these observations indicate that the two model systems used in this study are at least partially representative of the composition of normal dental plaque, making them suitable for the testing of mouthwashes.

There are many single-species oral biofilm model systems reported,[45-49] but the use of model systems to develop oral multispecies biofilms are less common.[18,50,51] It is conceivable that every model system generates conditions that will alter biofilm composition and properties, and this would inevitably alter the outcome of most studies. Such a possibility is supported by the observations reported here, as the structure and biomass of the multispecies biofilms were dependent upon the use of either the

GM or CS biofilm model system. As a consequence, the efficacy of the alcohol-free CPC-containing mouthwash differed between the model systems.

From a mechanistic standpoint, the most notable effects of the alcohol-free CPC-containing mouthwash were the much greater efficacy against five-day biofilms than against 10-day biofilms in the GM biofilm system, and the echelon-like effect of killing (as inferred by the LIVE/DEAD stain) through biofilms, where outer cells were more likely to be damaged than their deeper-lying counterparts. While the degree of CPC-induced damage to biofilm cells varies between studies in the literature,[18,45,51] at least one supports our observation that cells in the outer extremities of biofilms tend to have a greater propensity to be damaged by CPC than deeper-lying cells.[45] Such a phenomenon likely relates to the cationic nature of CPC,[29] and the likely cell surface-mediated retardation of access of CPC to the core of biofilms by a process broadly described as reaction-diffusion-limitation.[35,52]

The CS biofilm system, but not the GM system, indicated that the alcohol-free CPC-containing mouthwash caused dispersion, or more appropriately, "deadhesion"[27] of a proportion of the biofilm cells. The CS system is more disruptive in biofilm treatment and preparation for microscopic analysis, as compared to the more clement GM biofilm system. Because the oral cavity contains exposed and unexposed supragingival surfaces and unexposed subgingival crevices, either model may represent the *in vivo* effect of the alcohol-free CPC-containing mouthwash. Supporting and conflicting evidence to either of our model systems can be found in the literature. In support of our observations of deadhesion in the CS biofilm system, a recent paper by Busscher, *et al.* has indicated that CPC induces dispersion of more than 33% of coadhering oral coaggregating bacteria in model biofilms.[50] In this study, however, it should be noted that the biofilms were relatively immature, with little or no growth of the biofilm cells prior to testing. It is possible that deadhesion of cells from biofilms is linked to either changes in cell-substratum or cell-cell interactions. Of relevance to this, a paper by Smith and coworkers demonstrated that CPC inhibits coaggregation between many Gram-positive and Gram-negative oral bacterial species.[53] Conceivably, this may occur in the alcohol-free CPC-containing mouthwash-treated CS model system to promote deadhesion. In addition, Neu wrote a very informative review of the effect of QACs, and suggested that this family of molecules, which includes CPC, promotes deadhesion of bacteria from both hydrophobic and hydrophilic surfaces.[27] Interestingly, when comparing these findings to those that have been observed in the clinic, Kozak and coworkers have shown that use of a mouthwash that contained 0.07% CPC resulted in biofilms that were more susceptible to debridement as compared to mouthwash treatments that did not contain CPC.[54] Similar to our findings with the GM system but in contrast to the findings of the CS biofilm system, there are also research papers that indicate that CPC does not induce deadhesion. These include a recent work by Pan and coworkers, who used two model biofilm systems to study the effect of a variety of mouthwash formulations on multispecies biofilms.[18] Using a recirculating biofilm system and a chambered cover-slip system (as opposed to our cover-slide CS

system), the researchers suggest that CPC has a marginal effect on multispecies oral biofilms, but no data were presented to indicate deadhesion. Collectively, results from our two model biofilm systems and research presented by other research groups that use model biofilm systems, demonstrate that CPC may have a multifaceted effect on oral multispecies biofilms. This may be relevant to the mode of action of CPC in natural dental plaque biofilms. Clearly, a contrariety exists between the two biofilm systems used in this study and with findings from other groups. This suggests that a combination of environmentally relevant model systems need to be used when testing the responsiveness of biofilms to oral mouthwashes.

In conclusion, the work presented here demonstrates that an alcohol-free mouthwash that contains 0.075% CPC was effective at damaging cells within two model oral multispecies biofilm systems. Both model oral multispecies biofilm systems contained cell shapes and cellular arrangements observed in natural dental plaque. The extent of cell damage was dependent upon the model used; one model system indicated that CPC may promote deadhesion of cells from biofilms. In order to confirm that the deadhesion effects of CPC are not an artifact of the model system and are environmentally relevant, further *in vitro* and ultimately *in vivo* studies need to be conducted.

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A Clinical Study to Assess the 12-Hour Antimicrobial Effects of Cetylpyridinium Chloride Mouthwashes on Supragingival Plaque Bacteria

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Abstract

- **Objective:** This randomized double-blind clinical study evaluated the antimicrobial efficacy of two mouthwashes containing 1) 0.075% cetylpyridinium chloride (CPC) + 0.05% sodium fluoride (NaF) in an alcohol-free base and 2) 0.075% CPC + 0.05% NaF in a 6% alcohol base, versus a negative control mouthwash containing 0.05% NaF in an alcohol-free base on numbers of bacteria in supragingival plaque 12 hours after a single use and 12 hours after 14 days' use.

- **Methods:** Enrolled subjects completed a one-week washout phase prior to providing baseline samples of supragingival plaque that were analyzed for numbers of anaerobic microorganisms. Subjects were randomized to a treatment group and instructed to rinse with 20 mL of the assigned mouthwash for 30 seconds. Post-treatment microbiological analyses were conducted on plaque samples collected 12 hours after the first use of each assigned mouthwash and after completing 14 days of twice-daily use of each assigned mouthwash. Oral examinations were completed by a dentist at each sample collection to assess soft and hard tissue oral health over the course of the study.

- **Results:** The study enrolled 188 adults (mean age 45.78 years; age range 23–69). Subjects rinsing with the CPC-containing mouthwash realized a statistically significant ($p < 0.05$) reduction in numbers of supragingival anaerobic bacteria at the 12-hour evaluation after a single use. In comparison to the control mouthwash, use of the CPC mouthwash in an alcohol base resulted in a 35.3% reduction in numbers of anaerobic plaque bacteria, while the CPC mouthwash in an alcohol-free base demonstrated a 34.5% reduction. Further, the analysis after twice-daily use for 14 days indicated that the CPC mouthwash in an alcohol base demonstrated a 73.8% reduction in anaerobic plaque bacteria, while the CPC mouthwash in an alcohol-free base demonstrated a 70.9% reduction in anaerobic plaque bacteria versus the control mouthwash.

- **Conclusion:** The CPC mouthwash in an alcohol-free base reduced supragingival plaque bacteria by 34.5% and 70.9% compared to the control mouthwash 12 hours after a single use and after 14 days of use, respectively. In addition, the CPC mouthwash in an alcohol base reduced supragingival bacteria by 35.3% and 73.8% compared to the control mouthwash 12 hours after a single use and after 14 days of use, respectively. There were no statistically significant differences between the CPC-containing mouthwashes at either of the post-treatment time points.

Introduction

Large densities of diverse microorganisms are commonly recovered from the human mouth. Physiological conditions of the mouth include periodic food intake, along with stability in temperature and moisture, resulting in an optimal environment for the growth and proliferation of these organisms.[1] From a microbiological perspective, supragingival plaque on the surface of the exposed dentition represents one of the most widely investigated natural polymicrobial biofilms, and may comprise 1,000 different types of organisms.[1] Physical and chemical properties of plaque bacteria facilitate binding to oral surfaces of the tooth enamel to form biofilms.[2]

Clinical studies have evaluated the role of dental plaque bacteria in the initiation and progression of common oral conditions. These studies have examined the relationships between organisms of dental plaque in health and diseases such as caries and gingivitis, along with conditions such as oral malodor. Based on these studies, it is now widely accepted that accumulations of microorganisms play a primary role in the initiation and progression of gingivitis and other oral diseases,[1] and an array of microbiological techniques have been utilized to identify them.[3,4] On the basis of these analyses, it is now widely recognized that the enumeration of anaerobic bacteria in dental plaque provides an estimate of the total cultivable microflora.

Effective oral hygiene represents a patient-directed means to control the microbial populations of dental plaque.[1,2,5] However, studies demonstrate that poor oral hygiene is widespread, with about 60% of the plaque found on the surfaces of the teeth after brushing. An inability to maintain optimal oral hygiene is reflected in epidemiological surveys that demonstrate the

world-wide prevalence of gingivitis and other oral conditions.[6,7]

The use of dentifrices and mouthwashes formulated with anti-microbial agents is one approach to augment routine oral hygiene.[7] Antimicrobials commonly formulated in mouthrinses include chlorhexidine gluconate, essential oils, and cetylpyridinium chloride (CPC). CPC, an amphiphilic quaternary compound, represents an ingredient with a significant history of use in oral hygiene formulations.[7-11] Properties of CPC include solubility in water and alcohol, along with detergent-like attributes useful for preparing effective formulations.[8-10] Laboratory studies have demonstrated the antimicrobial effects of CPC on oral organisms and yeast found in dental plaque.[11-13] Furthermore, clinical studies have demonstrated the clinical efficacy of CPC mouthwash formulations on supragingival plaque and gingivitis.[14-16] A recent meta-analysis indicates the role of CPC mouthwashes as adjuncts for oral hygiene.[17]

Whereas several studies have evaluated the clinical effects of CPC mouthwashes, few investigations have determined their effects on plaque bacteria. This single-center, double-blind clinical study compared the effects of two 0.075% CPC mouthwashes, one formulated with and one without alcohol, in comparison to a control mouthwash without CPC on anaerobic dental plaque bacteria. Microbiological evaluations of dental plaque bacteria were conducted 12 hours after one use and after 14 days of twice-daily use.

Materials and Methods

Subjects

The study protocol was approved by the institutional review board of the State Key Laboratory of Oral Diseases (Sichuan University, Chengdu, China). Prospective subjects (18–70 years old) were recruited from the local area. Individuals completing informed consent were scheduled for a clinical evaluation to determine study eligibility. Adults in good oral and general health with at least 20 natural teeth and without dental prostheses were eligible for study. Subjects with no serious medical conditions or chronic disease, AIDS, pregnancy, or on prescription medications were enrolled. Exclusion criteria for female subjects included pregnancy or breast feeding. Individuals who had undergone dental prophylaxis in the month prior to study enrollment were excluded. Subjects with average whole mouth dental plaque scores of > 1.5 by the Turesky Modification of the Quigley-Hein Index,[18] and an average score of > 1.0 by the Löe-Silness Index[19] were enrolled. Subjects were provided a commercially available fluoride toothpaste and a soft-bristled toothbrush for oral hygiene during the one-week washout period prior to the start of the study. Following study enrollment, subjects were instructed to discontinue use of all other oral hygiene formulations for the duration of the study.

Study Procedures

The study design was similar to one reported previously.[20] Briefly, subjects were instructed to perform twice-daily oral hygiene during the washout phase, and directed to brush before going to bed on day 7. On day 8, subjects refrained from oral hygiene and arrived at the dental clinic between 8:00 a.m. and 10:00 a.m. to provide baseline samples of dental plaque. Plaque

samples for baseline and all post-treatment evaluations were randomly collected from either the right or the left quadrants of the upper arch (teeth numbers 2–8 or 9–15, respectively). For each sampling, a dentist utilized a sterile Columbia 13/14 scaler (Hu-Friedy, Chicago, IL, USA) to collect all plaque from the buccal surfaces of identified teeth. Plaque samples collected at each evaluation were transferred to a tube containing one mL of sterile phosphate buffered saline (PBS) for microbiological analysis.

After collection of the baseline samples, subjects were randomly assigned a mouthwash for twice-daily use beginning that evening. Treatments included 1) 0.075% CPC + 0.05% sodium fluoride (NaF) in an alcohol-free base (CPC Mouthwash in an Alcohol-free Base, Colgate-Palmolive Co., New York, NY, USA) and 2) 0.075% CPC + 0.05% NaF in a 6% alcohol base (CPC Mouthwash in an Alcohol Base, Colgate-Palmolive Co., New York, NY, USA), in addition to a negative control mouthwash containing 0.05% NaF in an alcohol-free base (Control Mouthwash, Colgate-Palmolive Co., New York, NY, USA). All treatments for this double-blind clinical study were supplied in original containers, overwrapped, and provided a unique code. Subject allocations to each treatment group were conducted in an area separate from all other study personnel to ensure blinding. Subjects were provided their test mouthwashes and instructed not to discuss their treatment with other study personnel. Subjects were also provided a commercially available fluoride toothpaste and directed to brush their teeth for one minute and rinse with tap water for 10 seconds twice daily, after which subjects were to rinse with 20 mL of the assigned test mouthwash for 30 seconds. This was to be performed 12 hours prior to their next appointment. Subjects were instructed to refrain from oral hygiene before their arrival at the dental clinic the next day (day 9). A dentist collected samples of dental plaque from the quadrant not sampled during the baseline visit. After collecting this sample, subjects were instructed to commence twice-daily hygiene with the dentifrice and assigned mouthwash for the next 14 days. Subjects arrived on day 23 at the dental clinic, 12 hours after their final use of the assigned mouthwash, to provide samples of dental plaque. Subjects underwent an oral examination prior to concluding their participation in the study.

Microbiological Procedures

The procedures for microbiological analysis of all samples were reported previously.[20] Briefly, samples of supragingival plaque collected from subjects were transferred into vials in 1 mL PBS for brief sonication. Samples were serially diluted in 10-fold dilutions in PBS, and aliquots plated on 5% sheep blood agar. Inoculated plates were incubated under anaerobic conditions at 37°C, with the number of viable colony-forming units (CFU/mL) reported for each sample.

Statistical Analysis

The number of viable organisms (CFU/mL) determined from each sample at each phase of the study was transformed to log10 for statistical analysis. Analysis of variance (ANOVA) compared the baseline microbiological results between the treatment groups. Intergroup comparisons between baseline and each post-treatment assessment were evaluated by t-tests. Analysis of

Table I

Subject Demographics for the Entire Population and for Each of Three Treatment Groups

Parameter	Entire Population			Control Mouthwash			CPC Mouthwash in an Alcohol Base			CPC Mouthwash in an Alcohol-free Base		
	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female
No. of Subjects	188	77	111	62	27	35	63	24	39	63	26	37
Mean Age	45.78	46.17	45.52	45.06	45.63	44.63	46.46	48.25	45.36	45.83	44.81	46.54
(SD)	(11.09)	(11.11)	(11.12)	(12.53)	(13.23)	(12.14)	(10.52)	(10.31)	(10.63)	(10.24)	(9.44)	(10.85)
Range	23–69	25–68	23–69	24–69	28–68	24-69	26–68	28–68	26–68	23–68	25–68	23–68

covariance (ANCOVA) compared the post-treatment microbial counts with the corresponding baseline as covariates. *Post hoc* Tukey multiple comparison tests determined the effects of the three mouthwashes. Comparisons between treatment groups at each post-treatment evaluation were conducted as described previously.[20]

Statistical analyses were conducted using Minitab statistical software (Minitab Inc, State College, PA, USA), with statistical significance reported at $p < 0.05$.

Results

A total of 188 subjects (77 males and 111 females; age range 23–69 years) who met the inclusion criteria were enrolled in study. Shown in Table I are demographics of enrolled subjects. Also shown in Table I are demographics of subjects in each treatment group. All subjects completed the study with no adverse events reported by the clinical investigator. At each post-treatment evaluation, subjects reported no taste alterations or concerns with the mouthwashes.

Randomized subject allocation to the treatment groups resulted in the assignment of 62 subjects (average age 45.06 years) to the Control Mouthwash, 63 subjects (average age 46.46 years) to the CPC Mouthwash in an Alcohol Base, and 63 subjects (average age 45.83 years) to the CPC Mouthwash in an Alcohol-free Base. Analyses by chi-square and t-tests indicate no differences in demographics among the three groups. A summary of baseline microbiological scores for the three treatment groups is shown in Table II. Analyses of the three groups at baseline indicate no statistical differences.

Shown in Table II are results from the analysis of plaque samples collected 12 hours after the first use of the mouthwashes. Results demonstrate significantly lower numbers of anaerobic bacteria among subjects assigned the CPC mouthwashes in comparison to the Control Mouthwash ($p < 0.05$). In comparison to the Control Mouthwash, the CPC Mouthwash in an Alcohol Base demonstrated a 35.3% reduction in plaque bacteria,

Table II

Effects of Mouthwashes on Supragingival Plaque Bacteria
(Log Colony-Forming Units/ml)

Treatment	# of Subjects	Baseline	12 Hr after Single Use	12 Hr after 14-Day Use
Control Mouthwash	62	7.62 ± 0.51	7.42 ± 0.46	7.29 ± 0.29
CPC Mouthwash in an Alcohol Base	63	7.63 ± 0.46	7.24 ± 0.47‡	6.71 ± 0.56‡
CPC Mouthwash in an Alcohol-free Base	63	7.48 ± 0.54	7.16 ± 0.55‡	6.71 ± 0.55‡

‡ Statistically significantly different from Control Mouthwash at corresponding evaluation ($p < 0.05$). At both post-treatment evaluations, there were no significant differences between the CPC mouthwash in an alcohol base and the CPC mouthwash in an alcohol-free base.

while the CPC Mouthwash in an Alcohol-free Base demonstrated a 34.5% reduction in anaerobic plaque bacteria (Table III). For each CPC mouthwash, additional analyses demonstrated significant reductions in anaerobic plaque organisms from baseline to the post-treatment evaluation ($p < 0.05$).

The effect of twice-daily oral hygiene for 14 days with the assigned mouthwash, with evaluations conducted 12 hours after final use, is shown in Table II. Results demonstrate significant reductions in anaerobic plaque bacteria in samples collected from subjects assigned the CPC mouthwashes in comparison to those provided the Control Mouthwash ($p < 0.05$). Reduction in plaque bacteria following use of the CPC Mouthwash in an Alcohol Base was 73.8%, and following use of the CPC Mouthwash in an Alcohol-free Base was 70.9%, as shown in Table III. Intergroup evaluations for each mouthwash demonstrate significant reductions from baseline to their post-treatment evaluation ($p < 0.05$).

Discussion

CPC, a cationic detergent, has antimicrobial effects on a range of organisms.[8] A number of studies are available in the literature that demonstrate the antimicrobial effects of CPC on oral

Table III

Comparisons Between the CPC Mouthwash and the Control Mouthwash Groups at Each 12-Hour Post-treatment Evaluation

	CPC Mouthwash in an Alcohol Base versus Control Mouthwash 12 Hours after Single Use	CPC Mouthwash in an Alcohol Base versus Control Mouthwash 12 Hours after 14 days' Use	CPC Mouthwash in an Alcohol-free Base versus Control Mouthwash 12 Hours after Single Use	CPC Mouthwash in an Alcohol-free Base versus Control Mouthwash 12 Hours after 14 days' Use
Difference in Means	− 0.189	− 0.581	− 0.184	− 0.536
Percent reduction	35.3%	73.8%	34.5%	70.9%
Significance (p value)	< 0.05	< 0.05	< 0.05	< 0.05

Results indicate differences in adjusted means (Log Colony-Forming Units/ml) between the CPC mouthwashes and the Control Mouthwash, calculated using the formula $(1-10^{diff}) \times 100$, where *diff* is the difference in means in $\log_{10}$ scale.

organisms, *Helicobacter pylori* and yeasts.[11-13] Laboratory evaluations demonstrate the ability of CPC to bind bacterial cells[21] with efficacy on planktonic cultures, adherent organisms along with synergistic activity with metal salts on microbial biofilms.[22] Other attributes of CPC include its ability to bind to hydroxyapatite, saliva, and extracellular matrix, as well as diffusion into microbial biofilms and inhibitory effects on insoluble glucan synthesis due to effects on streptococcal glucosyltransferase and fructosyltransferase. [23]

The present investigation evaluated the antimicrobial effects of 0.075% CPC mouthwashes formulated with or without alcohol on the numbers of anaerobic organisms in dental plaque. Specifically, this study determined the effects on dental plaque bacteria after a single use of each mouthwash, and following twice-daily use for 14 days. Microbiological effects of each mouthwash were evaluated 12 hours after use. Based on the clinical relationships between accumulating dental plaque and gingivitis, this study evaluated effects on the natural microbial biofilms that form on the surfaces of the exposed dentition.

Notable features of this investigation were procedures designed to reduce confounders associated with studies on clinical oral microbiology. Whereas the design of the current study was based on our previous research,[20] several areas deserve highlight. Adult subjects between the ages of 18 and 70 years, without predisposing health factors, were recruited. Those with gingival indices >1 and plaque scores >1.5 were enrolled as generally representative of adult oral health status. Enrolled subjects completed a one-week washout phase to institute a standardized regimen for oral hygiene prior to baseline evaluations. Further, subjects were directed to not change any dietary habits to reduce the influences of these variables on oral bacteria. Procedures for dental plaque collection were standardized, with plaque collections conducted from the buccal surfaces of the entire arch using a randomized scheme. This facilitated collections of sufficient dental biofilm for analysis, with no sites used more than twice during the entire study.

Techniques for microbiological evaluations were based on widely accepted procedures utilized in clinical oral microbiology[3,4] that were also employed in our previous study.[20] These techniques enumerate viable organisms and are in contrast to molecular methods that enumerate both viable and nonviable organisms. Viable organisms represent those with functional metabolic processes, including production of virulence factors, ability to transmit and colonize different oral sites and between people.

A total of 188 adults completed the entire study resulting in the analysis of 564 samples for anaerobic organisms, providing a substantial dataset for statistical analysis. In comparison to their corresponding baselines, the CPC mouthwashes demonstrated significant reductions in the numbers of plaque bacteria ($p < 0.05$). On the other hand, there was no effect by the Control Mouthwash on the numbers of plaque bacteria as compared to baseline. Statistical evaluations by ANCOVA compared the effects of the three mouthwashes tested. Analyses demonstrate significantly better effects by both CPC mouthwashes on dental plaque bacteria 12 hours after a single use in comparison to the control mouthwash ($p < 0.05$). The CPC Mouthwash in an Alcohol Base demonstrated a 35.3% reduction in viable anaerobic

organisms after one use, while the CPC Mouthwash in an Alcohol-free base demonstrated a 34.5% reduction. Correspondingly, additional use of the CPC mouthwashes resulted in a further decrease in the numbers of anaerobic organisms observed during the day-14 evaluations conducted 12 hours after final use of each CPC mouthwash. In comparison to the Control Mouthwash, analysis of dental plaque samples from subjects assigned CPC mouthwashes formulated with or without alcohol resulted in a 73.8% and 70.9% reduction of viable organisms, respectively.

From the standpoint of clinical significance, supragingival plaque represents a natural biofilm with substantial numbers of organisms.[1,2,5,6] Results from this investigation demonstrate the antimicrobial effects of both CPC mouthwashes 12 hours after the first use. The subsequent evaluation conducted after twice daily use for 14 days with each application every 12 hours demonstrated substantially higher antimicrobial effects. It is highly likely that the sustained antimicrobial effects observed over the course of this clinical study provide rationale for the antiplaque and antigingivitis effects reported with use of a CPC mouthwash in an alcohol-free base and a CPC mouthwash in an alcohol base.

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Evaluation of the Antiplaque Efficacy of
Two Cetylpyridinium Chloride-Containing Mouthwashes

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Abstract

- **Objective:** The purpose of this clinical study was to evaluate the efficacy in reducing dental plaque regrowth of two mouthwashes containing 0.075% cetylpyridinium chloride (CPC), one with 6% alcohol and one alcohol-free, as compared to a negative control mouthwash without CPC, using the Modified Gingival Margin Plaque Index (MGMPI).

- **Methods:** The study was a double-blind, randomized, three-way crossover, controlled design. Following a washout period, subjects reported to the dental clinic where they were instructed to brush their teeth, used their assigned mouthwash, and were scored by the examining dentist for plaque using the MGMPI method. Subjects were instructed to refrain from all oral hygiene for the next 24 hours, except for rinsing with their assigned mouthwash 12 hours post-brushing. After this 24-hour period, subjects returned to the dental clinic and were once again scored for plaque. This sequence of washout followed by mouthwash use and plaque scoring was repeated until each subject had used all three mouthwashes. An ANOVA was conducted to assess between-group differences.

- **Results:** The two test mouthwashes significantly reduced plaque regrowth over a 24-hour period ($p < 0.05$) as compared to the negative control mouthwash. The difference between the CPC-containing mouthwashes was not significant ($p = 0.4868$).

- **Conclusion:** Two mouthwashes containing 0.075% CPC, one with 6% alcohol and the other alcohol-free, were found to be safe and effective in reducing plaque accumulation when compared a negative control mouthwash without CPC. In short-term studies, the MGMPI appears useful for evaluating the antiplaque efficacy of mouthwash products.

Introduction

It has been well established that dental plaque is the primary etiologic agent for the most common dental diseases, *i.e.*, dental caries and periodontal disease.[1-7] Traditional methods for the mechanical removal of dental plaque are tooth brushing and the use of dental floss and/or other interdental cleaning devices.

Among dental professionals, the use of fluoride is universally recognized for the prevention and control of dental caries.[8,9] Fluoride is available in home use products such as dentifrices, for professional application as fluoride treatments such as varnish applications, and within the community through local water supplies. Periodontal disease, however, does not have a universal solution for prevention and control as does dental caries. While the etiology of periodontal diseases can be highly complex, the primary control mechanism for the prevention and control of periodontal disease remains mechanical removal of dental plaque, traditionally accomplished with a toothbrush and dental floss or other interdental cleaning aids.[10] Therefore, the goal in treating periodontal disease from the first stages of gingivitis to the severe chronic forms, remains the removal of supragingival and subgingival plaque.[11]

While there are numerous toothbrush designs and interdental cleaning devices, and many formulations of toothpaste, these mechanical methods for the regular removal of dental plaque have not had the level of success in preventing and controlling periodontal disease that fluoride has had in preventing and controlling dental caries. The primary reason that mechanical plaque removal is relatively unsuccessful is a lack of patient proficiency.[12] Many patients feel as though they are highly compliant and believe they are thoroughly removing dental plaque simply because they faithfully use a toothbrush and interdental cleaning device. Unfortunately, patients may lack the patience or the ability to adequately remove dental plaque.[13,14] When dental plaque is not thoroughly removed, the bacteria and related toxins repeatedly insult the periodontal tissues and contribute to the destructive inflammatory process and immune response, resulting in a loss of the supporting structures of the teeth.[15,16]

One of the most successful adjuncts for patients who cannot perform adequate mechanical dental plaque control has been chemotherapeutic antiplaque, antigingivitis agents in the form of mouthwashes. Generally, chemotherapeutic antiplaque mouthwashes are effective for their mechanisms of action,[3,17] as they decrease new dental plaque growth, decrease or remove existing dental plaque, diminish the growth of pathogenic bacteria, and inhibit the production of virulence factors. Antibacterial ingredients in contemporary mouthwashes include chemotherapeutic agents such as chlorhexidine, essential oil mixtures, and cetylpyridinium chloride (CPC).[19,20]

Chlorhexidine gluconate (CHG) is considered the "gold standard" of mouthwashes due to its antiplaque and antigingivitis effects.[21-23] CHG, however, can produce staining and altered taste, and is formulated with 11.9% alcohol, making it contraindicated

for patients with a history of alcohol dependency or a history of xerostomia. Essential oil (EO) mouthwashes have been firmly established as effective antiplaque and antigingivitis agents but, unfortunately, they have the highest alcohol content among mouthwashes, up to 27.9%, and can also produce staining.[24]

CPC is a quaternary ammonium compound that is an antiseptic and can kill bacteria and other microorganisms.[25] Notably, this cationic surface-active agent has a broad antimicrobial spectrum and shares some bactericidal similarities to CHG. CPC has a rapid bactericidal effect on Gram-positive microorganisms and has a fungicidal effect on yeast.[26] CPC produces its bactericidal effect by disrupting the bacteria's membrane function, causing cytoplasmic material to leak and collapsing intracellular equilibrium.[17]

The US Food and Drug Administration's Plaque Subcommittee has classified CPC as safe and effective for treatment of plaque-induced gingivitis when formulated in a concentration range of 0.045–0.10%.[27] A meta-analysis of six-month studies of antigingivitis and antiplaque agents was conducted by Gunsolley,[28] and confirms the antigingivitis and antiplaque properties of CPC-containing mouthwashes. CPC is one of the most commonly used ingredients in over-the-counter mouthwashes.[29] It is important to note that the bioavailability of CPC is strongly related to product formulation. The majority of CPC-containing mouthwashes have a CPC concentration of 0.05%.[30,31] CPC-containing mouthwashes with high concentrations and high bioavailability of CPC demonstrate higher clinical efficacy. The CPC-containing mouthwashes with concentrations lower than 0.05% and lower bioavailability are considered to be cosmetic products for temporary halitosis control. Like CHG, CPC mouthwashes have greater efficacy with increased frequency of use.[17]

Numerous clinical studies have been conducted on CPC-containing mouthwashes in various concentrations, and have varied from short-term trials of three days to long-term trials of six-month duration. The purpose of this clinical study was to evaluate the efficacy in reducing dental plaque regrowth of two mouthwashes containing 0.075% CPC as compared to a mouthwash without CPC, via a 24-hour plaque regrowth methodology using a double-blind, randomized, three-way crossover, controlled design.

Materials and Methods

Adult male and female subjects were enrolled into the study based on the following criteria:
- Subjects had to be between 18 and 65 years of age, in good general health, and have signed an Informed Consent.
- Subjects had to be available for the duration of the study.
- Subjects needed to possess a minimum of 17 natural uncrowned teeth (excluding third molars).
- Subjects had to discontinue oral hygiene for 24 hours after the initial appointment
- Subjects had to have no known history of allergy to personal care/consumer products or their ingredients, as determined by the dental/medical professional monitoring the study.

Subjects were excluded from the study if they:
- had five or more decayed, untreated dental sites (cavities);
- had advanced periodontal disease or other diseases of the soft or hard oral tissues;
- had a medical condition that required pre-medication prior to dental visits/procedures;
- had abnormal salivary function or were using drugs that affect salivary flow;
- used antibiotics within one month prior to, or during the study. Used any over-the-counter medications, other than analgesics, which in the opinion of the investigator would affect the outcome of the study; or
- were pregnant or breastfeeding, or had participated in another oral care clinical study within one month prior to the start of the study.

Prospective subjects reported to the clinical facility for an oral examination and a review of their medical history. All subjects who met the study inclusion and exclusion criteria and were accepted into the trial received a prophylaxis, and began a washout period of one to two weeks using Colgate® Cavity Protection Toothpaste (Colgate-Palmolive Company, New York, NY, USA; Washout Dentifrice). Mechanical devices such as floss were permitted during the washout phase, but no other oral care products such as mouthwash or additional dentifrice were permitted. After the washout period, subjects returned to the dental clinic and were randomly assigned to one of three mouthwash treatments. The three mouthwashes used in this study were: Test Mouthwash 1—0.075% CPC, 0.05% sodium fluoride (NaF), 0% alcohol (Colgate-Palmolive Company, New York, NY, USA); Test Mouthwash 2—0.075% CPC, 0.05% NaF, 6% alcohol (Colgate-Palmolive Company, New York, NY, USA); and Negative Control Mouthwash—0% CPC, 0.05% NaF, 6% alcohol (Colgate-Palmolive Company, New York, NY, USA).

Treatment Period 1

After the washout period was complete, subjects reported to the dental clinic on their assigned morning and brushed with the Washout Dentifrice for one minute and rinsed with water. Subjects then rinsed with 20 mL of their assigned Test Mouthwash for 30 seconds, and were instructed not to rinse with water after the test treatment. The clinical examiner applied red disclosing solution (Butler Red-Cote®, J.O. Butler Company, Chicago, IL, USA) with a cotton swab to the subject's teeth. After the disclosing solution was applied, plaque was scored using the Modified Gingival Margin Plaque Index (MGMPI).[32] Subjects were instructed to refrain from tooth brushing for the next 24 hours. Subjects again rinsed with their assigned mouthwash 12 hours post-brushing. Twenty-four hours post-brushing, subjects returned to the dental clinic, rinsed with disclosing solution, and had their plaque scored using the same method. Upon completion of the scoring, the subjects received a prophylaxis and resumed normal oral hygiene (brushing twice a day) using the Washout Dentifrice for two weeks.

Treatment Periods 2 and 3

Two weeks after completing Treatment Period 1, the subjects returned to the dental clinic, received their second assigned mouthwash, and repeated the above procedure. The same procedure that was used for the first two assigned mouthwashes was repeated for the third mouthwash.

Statistical Analysis

The increase in the Modified Gingival Margin Plaque Index from baseline over the 24-hour post-treatment period was calculated for each subject for each mouthwash. A two-factor ANOVA, using the subject and mouthwash as factors, was used to detect if significant differences among mouthwashes existed. A difference between mouthwashes was significant if a 95% confidence level ($p < 0.05$) was achieved. Based on a standard deviation of 12 plaque score units, it was estimated that a 9.5-unit difference in mean plaque scores between the mouthwash groups, with a sample size of 15 subjects (80% power), could be detected.

Results

Of the 28 subjects entered into the study, three subjects were dropped due to missed appointments. Twenty-five subjects, 19 females and six males, completed all phases of the study and were included in the analysis. There were no adverse effects from product use reported or observed at any time in the study.

Mean scores at baseline and 24 hours and the change in these scores are shown in Table I. There were no statistically significant differences in baseline scores between the three groups ($p > 0.05$). A statistically significant increase in MGMPI scores ($p < 0.001$) was determined between baseline and 24 hours in all groups. The percent changes in the MGMPI were 31.8, 38.1, and 54.8 for Test Mouthwash 1, Test Mouthwash 2, and the Negative Control Mouthwash, respectively (Table I and Figure 1). All percent changes were statistically significant ($p < 0.05$). Increases in plaque were significantly less for Test Mouthwash 1

Table I

The Mean (± SD) MGMPI Scores at Baseline, at 24 Hours, and their Difference for Each of the Three Test Mouthwashes

Product	MGMPI Mean (SD)				% Change
	Baseline	24 Hours	delta	p-value	
Test Mouthwash 1	33.1 ± 14.9	43.7 ± 18.5	10.5 ± 9.8	< 0.001	31.8
Test Mouthwash 2	30.9 ± 15.2	42.7 ± 18.8	11.8 ± 9.2	< 0.001	38.1
Negative Control Mouthwash	29.5 ± 12.7	45.7 ± 17.7	16.2 ± 12.7	< 0.001	54.8

Figure 1. *Mean differences in Modified Gingival Margin Plaque Index scores.*

and Test Mouthwash 2 as compared to the Negative Control Mouthwash ($p = 0.0027$ and $p = 0.017$, respectively). The difference in MGMPI plaque scores between the two CPC-containing rinses was not statistically significant ($p = 0.4868$). There were no differences with respect to oral soft or hard tissue abnormalities noted during the study among the three mouthwashes.

Discussion

The MGMPI has been shown to accurately document the short-term efficacy of several toothpaste products and to predict their long-term antiplaque and antigingivitis efficacy.[32-37] In the study reported here, the MGMPI has been documented to demonstrate the efficacy of two antibacterial mouthwashes containing CPC, one with 6% alcohol and one alcohol-free, in reducing plaque regrowth as compared to a negative control.

Conclusions

Two mouthwashes containing 0.075% CPC, one with 6% alcohol and the other alcohol-free, were found safe and effective in reducing plaque accumulation when compared a negative control mouthwash without CPC. In short-term studies, the MGMPI appears useful for evaluating the antiplaque efficacy of mouthwash products.

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A Comparative Investigation to Evaluate the Clinical Efficacy of an Alcohol-Free CPC-Containing Mouthwash as Compared to a Control Mouthwash in Controlling Dental Plaque and Gingivitis: A Six-Month Clinical Study on Adults in San Jose, Costa Rica

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Abstract

- **Objective:** This study was designed to evaluate the clinical efficacy of an antiplaque alcohol-free mouthwash containing 0.075% cetylpyridinium chloride (CPC) and 0.05% sodium fluoride (NaF), as compared to a control mouthwash containing only 0.05% NaF, in controlling established dental plaque and gingivitis after three and six months of product use.

- **Methods:** This was a single-center, parallel-group, two-cell, double-blind, randomized clinical study. Prospective adult male and female subjects from San Jose, Costa Rica reported to the clinical facility having refrained from all oral hygiene procedures for 12 hours, and from eating, drinking, or smoking for four hours prior to their visit. Qualifying subjects who presented with Gingival Index scores (Löe and Silness Index) of at least 1.0 and Plaque Index scores (Turesky Modified Quigley-Hein Index) of at least 1.5 were allowed to participate in this study. Subjects were randomly assigned to one of two treatment groups according to their baseline gingival and plaque scores. In the first treatment group (Test), subjects used an alcohol-free mouthwash containing 0.075% CPC and 0.05% NaF, whereas in the second treatment group (Control), subjects used a mouthwash containing only 0.05% NaF. Gingivitis and plaque assessments, and examinations of oral hard and soft tissues were conducted after three months and six months of product use.

- **Results:** One-hundred and ten (110) subjects complied with the protocol and completed the six-month study. After six months of product use, the Test Mouthwash group exhibited statistically significant reductions from baseline with respect to Gingival (33.5%), Gingival Interproximal (34.5%), Gingival Severity (63.2%), Plaque (33.6%), Plaque Interproximal (30.0%), and Plaque Severity (73.6%) Index scores. After six months of product use, the Control Mouthwash group exhibited statistically significant increases from baseline with respect to Gingival (6.9%), Plaque Interproximal (7.2%), and Plaque Severity (32.7%) Index scores. Furthermore, after six months of product use, the Control Mouthwash group exhibited reductions from baseline with respect to Plaque (6.1%), Gingival Interproximal (3.6%), and Gingival Severity (1.1%) Index scores which were not statistically significant. After three months of product use, the Test Mouthwash group exhibited statistically significant reductions in Gingival (25.0%), Gingival Interproximal (22.3%), Gingival Severity (38.9%), Plaque (26.1%), Plaque Interproximal (22.4%), and Plaque Severity (75.0%) Index scores as compared to the Control Mouthwash group. After six months of product use, the Test Mouthwash group exhibited statistically significant reductions in Gingival (38.1%), Gingival Interproximal (37.1%), Gingival Severity (63.6%), Plaque (36.5%), Plaque Interproximal (33.2%), and Plaque Severity (78.5%) Index scores as compared to the Control Mouthwash group.

- **Conclusion:** The results of this double-blind clinical study support that 1) an alcohol-free mouthwash containing a combination of 0.075% CPC and 0.05% NaF produces statistically significant reductions in dental plaque and gingivitis after three and six months compared to baseline, and 2) the alcohol-free CPC mouthwash provides a statistically significantly greater level of efficacy in controlling established dental plaque and gingivitis after three and six months of product use as compared to the Control Mouthwash containing only NaF.

Introduction

Dental plaque has long been identified as an etiological factor in the development of gingival inflammation and, ultimately, chronic periodontitis.[1] Controlling the formation of supragingival plaque biofilm is an important step in maintaining oral health. While tooth brushing and flossing are the highly recommended methods for removing supragingival plaque, patient's efforts may be compromised by hard-to-reach areas in the mouth, as well as by inadequate brushing technique and a lack of motivation and/or compliance.[2] It has been reported that tooth brushing alone removes as much as 65% to 75% of the total plaque on or around the dentition.[3] Since mechanical methods of plaque control may not be sufficient to prevent diseases of the periodontium, the use of chemical agents may add relevant benefits[2] when used in addition to tooth brushing and flossing. For this reason, antibacterial agents are used widely in a variety of mouthwash preparations.

In mouthwashes, the ideal antibacterial agent is one that can be delivered to the hard and soft tissues to accomplish the desired result, substantivity and intrinsic efficacy in the control of plaque without producing any secondary negative effects, such as staining of tissues or leaving an unpleasant taste. It should also be cost effective, have low toxicity, and be compatible with a good delivery vehicle.[4] In addition, sodium fluoride (NaF) solutions may be added to commercially available mouthrinses for their caries preventive effect.

Chlorhexidine gluoconate (CHX) mouthwashes have been used as prescription antiseptics for their antiplaque and anti-inflammatory properties. Mouthwashes containing CHX target a broad-spectrum of gram-negative and gram-positive bacteria, and work by damaging bacterial cell walls and producing a sustained inhibition of bacterial colonization.[5] CHX mouthwashes have been shown to reduce plaque levels by 50.3% to 60.9%, and gingivitis by 30.5% to 42.5%.[6] However, side effects reported with the use of CHX include, but are not limited to brownish staining of the teeth and oral mucosa, increased formation of calculus, altered taste, and ulcerations.[7]

There is also strong evidence that when cetylpyridinium chloride (CPC) is added to a mouthwash, it becomes an effective antiplaque and antigingivitis product, and because of this positive benefit, it should be added to daily oral hygiene routines.[4,8-10] CPC is a cationic surface-active agent that targets a broad spectrum of microorganisms. It rapidly kills gram-positive pathogens and yeasts by disrupting the membrane function, causing leakage of cytoplasm material and, ultimately, collapse of intra-cellular equilibrium.[2] The US Food and Drug Administration has classified CPC as safe and effective for over-the-counter use for its antiplaque and antigingivitis efficacy.[11] Mouthwashes containing CPC have been studied in recent years, showing CPC's antiplaque and antigingivitis efficacy in different clinical trials.[2,4,8,10,12,13] The magnitude of the reductions in supragingival plaque and gingivitis reported in these studies support claims of efficacy for varying concentrations of CPC.

Haps, et al.,[2] in a meta-analysis, reviewed eight published studies that evaluated the efficacy of products containing CPC. Of these, the authors selected three studies that evaluated the antiplaque, antigingivitis efficacy of different CPC formulations (in concentrations of 0.05 to 0.1% CPC) after six months of product use. The authors concluded that formulations containing CPC produce a significant effect of limited magnitude. Based on this conclusion, formulations containing CPC may be a useful adjunct to mechanical plaque control.

Lotufo, et al.[8] demonstrated that a mouthwash containing 0.05% CPC provides greater effect in preventing the formation of dental plaque compared to a control mouthwash without 0.05% CPC. The mean plaque level for the CPC mouthwash group 12 hours after rinsing was reduced to 46.1% of the pre-prophylaxis plaque level, while the mean plaque level for the control mouthwash group was reduced to a less impressive 75.5% of the pre-prophylaxis plaque level. These results demonstrate a statistically significant reduction in plaque formation for the CPC mouthwash group that is 29.3% greater than the control group, and shows that the CPC mouthwash provides 12-hour protection against dental plaque build-up.

Hernandez, et al.[12] conducted a double-blind, seven-day clinical study to assess the efficacy of a mouthwash containing 0.05% CPC for controlling plaque compared to a control mouthwash without 0.05% CPC. After seven days of product use and 12 hours after the last rinsing, both the CPC mouthwash and control groups showed statistically significant reductions in plaque scores measured for the whole mouth (25.3% and 6.6%, respectively) and at interproximal sites (51.3% and 32.9%, respectively), and in plaque severity scores (43.5% and 25.4%, respectively). The overall results support the conclusion that a mouthwash containing 0.05% CPC provides greater efficacy for reducing plaque 12 hours after use than a control mouthwash without 0.05% CPC.

Silva, et al.[4] also assessed the efficacy of a mouthwash containing 0.05% CPC for controlling established dental plaque and gingivitis as compared to a control mouthwash without 0.05% CPC. For the 0.05% CPC mouthwash group, statistically significant reductions were measured in whole mouth gingivitis scores (19.8%) as well as interproximally (20.7%), and in gingivitis severity scores (35.5%) as compared to the control mouthwash after six weeks of product use. The authors concluded that after the six-week study, the mouthwash containing 0.05% CPC was efficacious for controlling established dental plaque and gingivitis.

Stookey, et al.,[10] using a randomized, single-center, parallel-group, double-blind, positive- and placebo-controlled clinical trial, evaluated the effects of two experimental mouthwashes containing 0.075% and 0.10% CPC on the development of gingivitis and plaque as compared to a placebo control over a period of six months. After six months, subjects rinsing with either 0.075% or 0.10% CPC had significantly (p < 0.0001) less gingivitis, gingival bleeding, and plaque, on average, than those using the placebo. The six-month mean reductions in gingivitis, gingival bleeding, and plaque for the 0.075% and 0.10% CPC mouthwash versus placebo were 23%, 30%, and 17%, and 20%, 27%, and 19%, respectively. There was no statistically significant difference in efficacy between the two CPC mouthwashes.

In a six-month double-blind clinical study with 111 subjects, Allen, et al.,[9] following the American Dental Association (ADA) guidelines, assessed the effectiveness of a mouthwash containing

0.05% CPC for the control of supragingival dental plaque and gingivitis. The CPC mouthwash group exhibited statistically significantly less supragingival plaque and gingivitis than did the control mouthwash group. At the six-month examination, the magnitude of these differences met or exceeded 24% for all four parameters measured (28.2% for plaque, 63.4% for plaque severity, 24.0% for gingivitis, and 66.9% for gingivitis severity). Hence, the authors concluded that mouthwashes containing 0.05% CPC provide a statistically significant, clinically relevant level of efficacy for the control of supragingival plaque and gingivitis, in accordance with the criteria provided by ADA guidelines.

Finally, Mankodi, *et al*.[13] evaluated the effects of a mouthwash containing 0.07% CPC on the development of gingivitis and plaque versus a placebo control over a six-month period. Participants were randomly assigned to the CPC or placebo mouthwash, and after six months participants who rinsed with the CPC mouthwash showed 15.4% less gingival inflammation, 33.3% less gingival bleeding, and 15.8% less plaque relative to the placebo group (p < 0.01).

The objective of the present, six-month, single-center, parallel-group, two-cell, double-blind, and randomized clinical study was to evaluate the clinical efficacy of an alcohol-free mouthwash containing a combination of 0.075% CPC and 0.05% NaF, as compared to a control mouthwash containing only 0.05% NaF, in controlling established dental plaque and gingivitis after three and six months of product use.

Materials and Methods

This clinical study employed a double-blind, randomized, two-treatment, parallel-group design. Adult male and female subjects from the San Jose, Costa Rica area were enrolled in the study if they satisfied the following inclusion criteria:

1. Subjects must be between the ages of 18 and 70 (inclusive) and in good health.
2. Subjects must be available for the six-month duration of the study.
3. Subjects must have at least 20 uncrowned permanent natural teeth (excluding third molars).
4. Subjects must have a mean Gingival Index score of at least 1.0 as determined by the Löe-Silness Gingival Index,[14] and a mean Plaque Index score of at least 1.5 as determined by the Turesky Modification[15] of the Quigley-Hein Plaque Index.

Prospective subjects were excluded from and not allowed to participate in the study if they:

1. had moderate to advanced periodontal disease, five or more decayed untreated dental sites at screening, or tumor(s) of the soft or hard oral tissues;
2. began taking medications that affected salivary flow, antibiotics, or antimicrobial drugs within one month prior to the start of the study, or if they started taking such medications during the course of the study;
3. received a dental prophylaxis in the past two weeks prior to the baseline examination;
4. were women who were pregnant or lactating;
5. were participating in any other clinical study or who had

participated in a study within one week prior to enrollment of this study; or
6. had a history of allergies to CPC products, allergies to oral care/personal care consumer products or their ingredients, or had existing medical conditions that prohibited them from eating and drinking for periods of up to four hours.

Prospective study subjects reported to the clinical facility having refrained from all oral hygiene procedures for 12 hours, and from eating, drinking, or smoking for four hours prior to their baseline visit. All prospective subjects who met the inclusion/exclusion criteria and signed an informed consent form received a baseline gingivitis and plaque evaluation, along with an oral soft and hard tissue assessment. The scores from these baseline evaluations were later compared to the scores of test group and control group subjects obtained in evaluations conducted after three and six months.

Qualifying subjects were randomized into two balanced groups based on their baseline Gingival and Plaque Index scores. The two treatments employed in this study were identified as follows:

Test Group: participating subjects used a mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base (Colgate-Palmolive Company, New York, NY, USA).

Control Group: participating subjects used a mouthwash containing 0.05% NaF in an alcohol-free base (Colgate-Palmolive Company, New York, NY, USA).

Both product formulations included 0.05% NaF to assure control for any potential confounding effects on plaque reduction by differential "usual practices" of the subject groups regarding fluoride rinses.

The two mouthwashes were over-wrapped to maintain the double-blind study design. Following treatment assignment, subjects were provided with a soft-bristle toothbrush, a tube of regular fluoride toothpaste, and their assigned mouthwash for home use. Subjects were instructed to brush their teeth for one minute, twice daily (morning and evening), to rinse with water following brushing, and then to rinse for thirty seconds with 20 mL of their assigned mouthwash. They were also instructed not to eat or drink for 30 minutes after the mouthwash rinse and to refrain from flossing or using interdental stimulators.

Subjects returned to the clinical facility for gingivitis and plaque examinations after three and six months of product use. Additionally, at each examination subjects received an evaluation of their oral soft tissue by the examining dentist, and were questioned about the occurrence of any adverse events.

Clinical Scoring Procedures

Oral Soft and Hard Tissue Assessment. The dental examiner visually examined the oral cavity and peri-oral area using a dental light and dental mirror. This examination included an evaluation of the soft and hard palate, gingival mucosa, buccal mucosa, mucogingival fold areas, tongue, sublingual and submandibular areas, salivary glands, and the tonsilar and pharyngeal areas.

Gingivitis Assessment. The degree of gingival inflammation was scored at six sites (disto-, mid-, mesio-buccal, and disto-, mid-, mesio-lingual) of each tooth according to the criteria of the

Gingival Index system (Löe and Silness[14]) as follows:

  0 = Absence of inflammation;
  1 = Mild inflammation—slight change in color and little change in texture;
  2 = Moderate inflammation—moderate glazing, redness, edema and hypertrophy;
  3 = Severe inflammation—marked redness and hypertrophy. Tendency for spontaneous bleeding.

Each subject's scores were calculated by summing all scores for all sites, and dividing by the total number of sites scored for that person.

Dental Plaque Assessment. The dentition was disclosed with disclosing solution and plaque was scored at the disto-, mid-, mesio-buccal, and disto-, mid-, mesio-lingual surfaces of each tooth according to the criteria of the Modified Quigley and Hein Index (Turesky, *et al.*[15] and Quigley and Hein[16]) as follows:

  0 = No plaque;
  1 = Separate flecks of plaque at the cervical margin;
  2 = A thin, continuous band of plaque (up to 1 mm) at the cervical margin;
  3 = A band of plaque wider than 1 mm, but covering less than 1/3 of the side of the tooth crown;
  4 = Plaque covering at least 1/3, but less than 2/3 of the side of the tooth crown;
  5 = Plaque covering 2/3 or more of the side of the tooth crown.

Each subject's scores were calculated by summing all scores for all sites, and dividing by the total number of sites scored for that person.

Severity Indices Assessment. In addition to the plaque and gingivitis indices discussed above, whole-mouth scores were also obtained with respect to the Plaque Severity Index and the Gingivitis Severity Index. These severity indices measure the proportion of the tooth surfaces in the mouth which have received high scores on the respective indices:

- The Plaque Severity Index indicates the proportion of scored tooth surfaces in the mouth which were assigned Turesky Modified Quigley-Hein Plaque Index scores of 3, 4, or 5.
- The Gingivitis Severity Index indicates the proportion of scored tooth surfaces in the mouth which were assigned Löe-Silness Gingival Index scores of 2 or 3 (*i.e.*, bleeding sites).
- The Gingivitis Interproximal Index scores are calculated by adding the mesial and distal (interproximal areas) scores of each tooth, and dividing the sum by the total number of interproximal areas scored using the Löe-Silness Index.
- The Plaque Interproximal Index scores are calculated by adding the mesial and distal (interproximal areas) scores of each tooth, and dividing the sum by the total number of interproximal areas scored using the Turesky Modification of the Quigley-Hein Index.

Adverse Events

Reports of adverse events that occurred were obtained by interviewing study subjects, and from the dental examinations conducted at three and six months by the investigators.

Statistical Methods

Statistical analyses were performed separately for the gingivitis assessments and dental plaque assessments. Comparisons of the treatment groups with respect to gender were performed using a Chi-Square analysis, and for age using an independent t-test. Comparisons of the treatment groups with respect to baseline Gingival Index scores and Plaque Index scores were performed using an independent t-test. Within-treatment comparisons of the baseline versus follow-up Gingival and Plaque Index scores were performed using paired t-tests. Comparisons of the treatment groups with respect to baseline-adjusted gingival and plaque scores at the follow-up examinations were performed using analyses of covariance (ANCOVA). All statistical tests of hypotheses were two-sided, and employed a level of significance of $\alpha = 0.05$.

Results

One-hundred and twenty (120) subjects entered this six-month plaque and gingivitis clinical study, and one-hundred and ten (110) subjects completed the study. Ten (10) subjects either did not comply with the study protocol, or encountered an event unrelated to product use.

A summary of the gender and age of the study population is presented in Table I. The treatment groups did not differ significantly ($p > 0.05$) with respect to either characteristic.

Table I
Summary of Age and Gender for Subjects
Who Completed the Six-Month Clinical Study

Treatment	Number of Subjects			Age[3]	
	Male	Female	Total[3]	Mean	Range
CPC Mouthwash[1]	22	32	54	33.1	19–79
Control Mouthwash[2]	23	33	56	33.6	19–61

[1] A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base (Colgate-Palmolive Company, New York, NY, USA)
[2] A mouthwash containing 0.05% NaF in an alcohol-free base (Colgate-Palmolive Company, New York, NY, USA)
[3] No statistically significant difference ($p > 0.05$) was indicated between the treatment groups with respect to either gender or age.

Throughout the study, no adverse events on the soft and hard tissues of the oral cavity were observed by the examiner or reported by the subjects when questioned, and there were no reported or stain-related adverse events.

Baseline Data

Table II presents a summary of the Gingival, Gingival Interproximal, Gingival Severity, Plaque, Plaque Interproximal, and Plaque Severity Index score means, as measured at the baseline examination for those subjects who completed the six-month clinical study. On the Gingival Index, mean baseline scores were 2.03 for the CPC Test Mouthwash group and 2.04 for the Control Mouthwash group. For the Gingival Interproximal Index, mean baseline scores were 2.23 for the CPC Test Mouthwash group and 2.24 for the Control Mouthwash group. For the Gingival Severity Index, mean baseline scores were 0.87 for the CPC Test Mouthwash group and 0.87 for the Control Mouthwash group. For the Plaque Index, mean baseline scores were 2.65 for

Table II
Summary of the Baseline Gingival, Gingival Interproximal,
Gingival Severity Index Scores and Plaque, Plaque
Interproximal, Plaque Severity Index Scores for Subjects
Who Completed the Six-Month Clinical Study

Parameter	Treatment	n	Baseline Summary (Mean ± SD)
Gingival Index[3]	CPC Mouthwash[1]	54	2.03 ± 0.22
	Control Mouthwash[2]	56	2.04 ± 0.22
Gingival Interproximal Index[3]	CPC Mouthwash	54	2.23 ± 0.24
	Control Mouthwash	56	2.24 ± 0.23
Gingival Severity Index[3]	CPC Mouthwash	54	0.87 ± 0.07
	Control Mouthwash	56	0.87 ± 0.08
Plaque Index[3]	CPC Mouthwash	54	2.65 ± 0.31
	Control Mouthwash	56	2.61 ± 0.37
Plaque Interproximal Index[3]	CPC Mouthwash	54	2.70 ± 0.32
	Control Mouthwash	56	2.64 ± 0.38
Plaque Severity Index[3]	CPC Mouthwash	54	0.53 ± 0.20
	Control Mouthwash	56	0.49 ± 0.22

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]No statistically significant difference ($p > 0.05$) was indicated between the treatment groups with respect to either plaque or gingival indices.

the CPC Test Mouthwash group and 2.61 for the Control Mouthwash group. For the Plaque Interproximal Index, mean baseline scores were 2.70 for the CPC Test Mouthwash group and 2.64 for the Control Mouthwash group. For the Plaque Severity Index, mean baseline scores were 0.53 for the CPC Test Mouthwash group and 0.49 for the Control Mouthwash group. No statistically significant difference ($p > 0.05$) was indicated between the treatment groups with respect to Gingival, Gingival Interproximal, Gingival Severity, Plaque, Plaque Interproximal, or Plaque Severity Index scores at baseline.

Three-Month Data—Gingival
Table III presents a summary of the mean Gingival Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.
Comparisons vs. Baseline. The mean three-month Gingival Index scores were 1.59 for the CPC Test Mouthwash group and

2.12 for the Control Mouthwash group. The mean percent reductions from baseline were 21.7% for the CPC Test Mouthwash group and -3.9% for the Control Mouthwash group; both were statistically significant ($p < 0.05$).
Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 25% reduction ($p < 0.05$) in mean Gingival Index scores after three months of product use.

Three-Month Data—Plaque
Table III presents a summary of the mean Plaque Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.
Comparisons vs. Baseline. The mean three-month Plaque Index scores were 1.98 for the CPC Test Mouthwash group and 2.68 for the Control Mouthwash group. The mean percent reductions from baseline were 25.3% for the CPC Test Mouthwash group (statistically significant at $p < 0.05$) and -2.7% for the Control Mouthwash group (not statistically significant).
Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 26.1% reduction ($p < 0.05$) in mean Plaque Index scores after three months of product use.

Three-Month Data—Gingival Interproximal
Table IV presents a summary of the mean Gingival Interproximal Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.
Comparisons vs. Baseline. The mean three-month Gingival Interproximal Index scores were 1.78 for the CPC Test Mouthwash group and 2.29 for the Control Mouthwash group. The mean percent reductions from baseline were 20.2% for the CPC Test Mouthwash group (statistically significant at $p < 0.05$) and -2.2% for the Control Mouthwash group (not statistically significant).
Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 22.3% reduction ($p < 0.05$) in mean Gingival Interproximal Index scores after three months of product use.

Table III
Summary of the Three-Month Gingival and Plaque Index Scores for Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	n	Three-Month Summary (Mean ± SD)	Within-Treatment Analysis Percent Reduction[3]	Sig.[4]	Between-Treatment Comparison vs. Control Mouthwash Percent Difference[5]	Sig.[6]
Gingival	CPC Mouthwash[1]	54	1.59 ± 0.31	21.7%	p < 0.05	25.0%	p < 0.05
	Control Mouthwash[2]	56	2.12 ± 0.29	− 3.9%	p < 0.05		
Plaque	CPC Mouthwash[1]	54	1.98 ± 0.33	25.3%	p < 0.05	26.1%	p < 0.05
	Control Mouthwash[2]	56	2.68 ± 0.39	− 2.7%	NS		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the three-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the three-month examination.
[4]Significance of paired t-test comparing the baseline and three-month examinations.
[5]Difference between the three-month means expressed as a percentage of the three-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Three-Month Data—Plaque Interproximal

Table IV presents a summary of the mean Plaque Interproximal Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean three-month Plaque Interproximal Index scores were 2.11 for the CPC Test Mouthwash group and 2.72 for the Control Mouthwash group. The mean percent reductions from baseline were 21.9% for the CPC Test Mouthwash group (statistically significant at $p < 0.05$) and −3.0% for the Control Toothpaste group (not statistically significant).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 22.4% reduction ($p < 0.05$) in mean Plaque Interproximal Index scores after three months of product use.

Three-Month Data—Gingival Severity

Table V presents a summary of the mean Gingival Severity Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean three-month Gingival Severity Index scores were 0.55 for the CPC Test Mouthwash group and 0.90 for the Control Mouthwash group. The mean percent reductions from baseline were 36.8% for the CPC Test Mouthwash group and −3.4% for the Control Mouthwash group, both of which were statistically significant ($p < 0.05$).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 38.9% reduction ($p < 0.05$) in mean Gingival Severity Index scores after three months of product use.

Three-Month Data—Plaque Severity

Table V presents a summary of the mean Plaque Severity Index scores measured after three months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean three-month Plaque Severity Index scores were 0.14 for the CPC Test Mouthwash group and 0.56 for the Control Mouthwash group. The mean percent reductions from baseline were 73.6% for the CPC Test Mouthwash group and −14.3% for the Control Mouthwash group, both of which were statistically significant ($p < 0.05$).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 75.0% reduction ($p < 0.05$) in mean Plaque Severity Index scores after three months of product use.

Table IV

Summary of the Three-Month Gingival and Plaque Interproximal Index Scores for Subjects Who Completed the Six-Month Clinical Study

| Index | Treatment | n | Three-Month Summary (Mean ± SD) | Within-Treatment Analysis | | Between-Treatment Comparison vs. Control Mouthwash | |
				Percent Reduction[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival Interproximal	CPC Mouthwash[1]	54	1.78 ± 0.36	20.2%	$p < 0.05$	22.3%	$p < 0.05$
	Control Mouthwash[2]	56	2.29 ± 0.29	−2.2%	NS		
Plaque Interproximal	CPC Mouthwash[1]	54	2.11 ± 0.30	21.9%	$p < 0.05$	22.4%	$p < 0.05$
	Control Mouthwash[2]	56	2.72 ± 0.39	− 3.0%	NS		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the three-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the three-month examination.
[4]Significance of paired t-test comparing the baseline and three-month examinations.
[5]Difference between the three-month means expressed as a percentage of the three-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Table V

Summary of the Three-Month Gingival and Plaque Severity Index Scores for Subjects Who Completed the Six-Month Clinical Study

| Index | Treatment | n | Three-Month Summary (Mean ± SD) | Within-Treatment Analysis | | Between-Treatment Comparison vs. Control Mouthwash | |
				Percent Reduction[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival Severity	CPC Mouthwash[1]	54	0.55 ± 0.24	36.8%	$p < 0.05$	38.9%	$p < 0.05$
	Control Mouthwash[2]	56	0.90 ± 0.11	−3.4%	$p < 0.05$		
Plaque Severity	CPC Mouthwash[1]	54	0.14 ± 0.20	73.6%	$p < 0.05$	75.0%	$p < 0.05$
	Control Mouthwash[2]	56	0.56 ± 0.25	− 14.3%	$p < 0.05$		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the three-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the three-month examination.
[4]Significance of paired t-test comparing the baseline and three-month examinations.
[5]Difference between the three-month means expressed as a percentage of the three-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Six-Month Data—Gingival

Table VI presents a summary of the mean Gingival Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Gingival Index scores were 1.35 for the CPC Test Mouthwash group and 2.18 for the Control Mouthwash group. The mean percent reductions from baseline were 33.5% for the CPC Test Mouthwash group and −6.9% for the Control Mouthwash group, both of which were statistically significant ($p < 0.05$).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 38.1% reduction ($p < 0.05$) in mean Gingival Index scores after six months of product use.

Six-Month Data—Plaque

Table VI presents a summary of the mean Plaque Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Plaque Index scores were 1.76 for the CPC Test Mouthwash group and 2.77 for the Control Mouthwash group. The mean percent reductions from baseline were 33.6% for the CPC Test Mouthwash group

(statistically significant at $p < 0.05$) and −6.1% for the Control Mouthwash group (not statistically significant).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 36.5% reduction ($p < 0.05$) in mean Plaque Index scores after six months of product use.

Six-Month Data—Gingival Interproximal

Table VII presents a summary of the mean Gingival Interproximal Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Gingival Interproximal Index scores were 1.46 for the CPC Test Mouthwash group and 2.32 for the Control Mouthwash group. The mean percent reductions from baseline were 34.5% for the CPC Test Mouthwash group (statistically significant at $p < 0.05$) and −3.6% for the Control Mouthwash group (not statistically significant).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 37.1% reduction ($p < 0.05$) in mean Gingival Interproximal Index scores after six months of product use.

Table VI
Summary of the Six-Month Gingival and Plaque Index Scores for Subjects Who Completed the Six-Month Clinical Study

| Index | Treatment | n | Six-Month Summary (Mean ± SD) | Within-Treatment Analysis | | Between-Treatment Comparison vs. Control Mouthwash | |
				Percent Reduction[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival	CPC Mouthwash[1]	54	1.35 ± 0.36	33.5%	$p < 0.05$	38.1%	$p < 0.05$
	Control Mouthwash[2]	56	2.18 ± 0.43	−6.9%	$p < 0.05$		
Plaque	CPC Mouthwash[1]	54	1.76 ± 0.52	33.6%	$p < 0.05$	36.5%	$p < 0.05$
	Control Mouthwash[2]	56	2.77 ± 0.58	− 6.1%	NS		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the six-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the six-month examination.
[4]Significance of paired t-test comparing the baseline and six-month examinations.
[5]Difference between the six-month means expressed as a percentage of the six-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Table VII
Summary of the Six-Month Gingival and Plaque Interproximal Index Scores for Subjects Who Completed the Six-Month Clinical Study

| Index | Treatment | n | Six-Month Summary (Mean ± SD) | Within-Treatment Analysis | | Between-Treatment Comparison vs. Control Mouthwash | |
				Percent Reduction[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival Interproximal	CPC Mouthwash[1]	54	1.46 ± 0.44	34.5%	$p < 0.05$	37.1%	$p < 0.05$
	Control Mouthwash[2]	56	2.32 ± 0.46	−3.6%	NS		
Plaque Interproximal	CPC Mouthwash[1]	54	1.89 ± 0.53	30.0%	$p < 0.05$	33.2%	$p < 0.05$
	Control Mouthwash[2]	56	2.83 ± 0.58	− 7.2%	$p < 0.05$		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the six-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the six-month examination.
[4]Significance of paired t-test comparing the baseline and six-month examinations.
[5]Difference between the six-month means expressed as a percentage of the six-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Table VIII

Summary of the Six-Month Gingival and Plaque Severity Index Scores for Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	n	Six-Month Summary (Mean ± SD)	Within-Treatment Analysis		Between-Treatment Comparison vs. Control Mouthwash	
				Percent Reduction[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival Severity	CPC Mouthwash[1]	54	0.32 ± 0.31	63.2%	p < 0.05	63.6%	p < 0.05
	Control Mouthwash[2]	56	0.88 ± 0.24	−1.1%	NS		
Plaque Severity	CPC Mouthwash[1]	54	0.14 ± 0.24	73.6%	p < 0.05	78.5%	p < 0.05
	Control Mouthwash[2]	56	0.65 ± 0.30	−32.7%	p < 0.05		

[1]A mouthwash containing 0.075% CPC and 0.05% NaF in an alcohol-free base.
[2]A mouthwash containing 0.05% NaF in an alcohol-free base.
[3]Percent reduction exhibited by the six-month mean relative to the baseline mean. A negative value indicates a reduction in index scores at the six-month examination.
[4]Significance of paired t-test comparing the baseline and six-month examinations.
[5]Difference between the six-month means expressed as a percentage of the six-month mean for the Control Mouthwash. A negative value indicates a reduction in index scores for the CPC Mouthwash relative to the Control Mouthwash.
[6]Significance of ANCOVA comparison of baseline-adjusted means.

Six-Month Data—Plaque Interproximal

Table VII presents a summary of the mean Plaque Interproximal Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Plaque Interproximal Index scores were 1.89 for the CPC Test Mouthwash group and 2.83 for the Control Mouthwash group. The mean percent reductions from baseline were 30.0% for the CPC Test Mouthwash group and −7.2% for the Control Mouthwash group, both of which were statistically significant (p < 0.05).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 33.2% reduction (p < 0.05) in mean Plaque Interproximal Index scores after six months of product use.

Six-Month Data—Gingival Severity

Table VIII presents a summary of the mean Gingival Severity Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Gingival Severity Index scores were 0.32 for the CPC Test Mouthwash group and 0.88 for the Control Mouthwash group. The mean percent reductions from baseline were 63.2% for the CPC Test Mouthwash group (statistically significant at p < 0.05) and −1.1% for the Control Mouthwash group (not statistically significant).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 63.6% reduction (p < 0.05) in mean Gingival Severity Index scores after six months of product use.

Six-Month Data—Plaque Severity

Table VIII presents a summary of the mean Plaque Severity Index scores measured after six months of product use. A positive percentage indicates a reduction from baseline.

Comparisons vs. Baseline. The mean six-month Plaque Severity Index scores were 0.14 for the CPC Test Mouthwash group and 0.65 for the Control Mouthwash group. The mean percent reductions from baseline were 73.6% for the CPC Test Mouthwash group and −32.7% for the Control Mouthwash group, both of which were statistically significant (p < 0.05).

Comparison between Treatment Groups. Relative to the Control Mouthwash group, the CPC Test Mouthwash group exhibited a statistically significant 78.5% reduction (p < 0.05) in mean Plaque Severity Index scores after six months of product use.

Discussion and Conclusion

The present randomized clinical study was designed to evaluate the clinical efficacy of an alcohol-free mouthwash containing 0.075% CPC and 0.05% NaF, as compared to a control mouthwash containing only 0.05% NaF, to control established dental plaque and gingivitis after three and six months of product use. Based on the study's overall findings, the use of an alcohol-free mouthwash containing the CPC/NaF mixture was more effective in controlling dental plaque and gingivitis relative to the mouthwash containing only NaF.

To our knowledge, no previous study has addressed the efficacy of an alcohol-free mouthwash containing the mixture of CPC (0.075%) and (0.05%) NaF. Previous publications have clearly shown the efficacy of using different CPC concentrations in reducing supragingival plaque and gingivitis.[8-10,12,13,17] While study designs differ, the results of this study are in complete accordance with prior studies on CPC. Previous peer-reviewed publications that evaluated the effect of CPC in controlling plaque and gingivitis have employed different concentrations and formulations. In addition, there were variations in rinsing times, differences in the indices used to measure plaque and gingivitis outcomes,[13] different approaches to administering oral prophylaxis prior to product use[9,17] versus evaluating the effect of CPC on established plaque,[4,8,12] and different study durations. Despite these differences, the results obtained in the present study are in complete accordance with the studies reported in the peer-reviewed literature, including a systematic review[2] showing a statistically significant reduction in plaque and gingivitis when different CPC mouthwashes were compared to control groups.

Although the study was not designed or powered to detect a small antiplaque effect for NaF (within the control group), the findings of no statistical difference between the baseline and

six-months scores suggest, in this cohort, there is no detectable effect on plaque by NaF.

This double-blind clinical study supports the conclusions that an alcohol-free mouthwash containing a mixture of 0.075% CPC and 0.05% NaF provides a statistically significant reduction in dental plaque and gingivitis after three and six months of product use as compared to baseline, and the alcohol-free CPC mouthwash provides a greater level of efficacy in controlling established dental plaque and gingivitis after three and six months of product use as compared to the control mouthwash without CPC.

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