J Clin Periodontol 2008; 35: 547–554 doi: 10.1111/j.1600-051X.2008.01230.x

Journal of Clinical Periodontology

Surfactive and antibacterial activity of cetylpyridinium chloride formulations in vitro and in vivo

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Busscher HJ, White DJ, Atema-Smit J, Geertsema-Doornbusch G, de Vries J, van der Mei HC. Surfactive and antibacterial activity of cetylpyridinium chloride formulations in vitro and in vivo. J Clin Periodontol 2008; 35: 547–554. doi: 10.1111/j.1600-051X.2008.01230.x.

Abstract

Aim: To compare effects of three cetylpyridinium chloride (CPC) formulations with and without alcohol and Tween80 on physico-chemical properties of salivary pellicles, bacterial detachment in vitro and bacterial killing in vivo.

Material and Methods: Adsorption of CPC to salivary pellicles in vitro was studied using X-ray photoelectron spectroscopy and water contact angle measurements. Adhesion and detachment of a co-adhering bacterial pair was determined in vitro using a flow chamber. Killing was evaluated after live/dead staining after acute single use in vivo on 24- and 72-h-old plaques after 2-week continuous use.

Results: The most pronounced effects on pellicle surface chemistry and hydrophobicity were observed after treatment with the alcohol-free formulation, while the pellicle thickness was not affected by any of the formulations. All CPC formulations detached up to 33% of the co-adhering pair from pellicle surfaces. Bacterial aggregate sizes during de novo deposition were enhanced after treatment with the alcohol-free formulation. Immediate and sustained killing in 24 and 72 h plaques after in vivo, acute single use as well as after 2-week continuous use were highest for the alcohol-free formulation.

Conclusions: CPC bioavailability in a formulation without alcohol and Tween80 could be demonstrated through measures of pellicle surface properties and bacterial interactions in vitro as well as bacteriocidal actions on oral biofilms in vivo.

Key words: antibacterial; bioavailability; biofilm; cetylpyridinium chloride; CLSM; mouthrinse; plaque; substantivity

Accepted for publication 19 February 2008

The application of cetylpyridinium chloride (CPC) in oral health care products has a long history of conflicting reports (Pader 1985, Wu & Savitt 2002). Cationic surfactants like CPC owe their antimicrobial properties to a strong tendency to adsorb to negatively charged bacterial cell surfaces (Chung et al. 2004), causing disruption of their membrane function (Helander et al. 2001). Monocationic CPC is readily soluble in alcohol or in surfactant-based aqueous solutions, and CPC exhibits antimicrobial activities in test tube assays against planktonic organisms similar to or better than most cationic surfactants, including even dicationic chlorhexidine (CHEX). Yet, CPC in early evaluations appeared to provide only a low or zero level of plaque inhibition in clinical trials (Moran et al. 2000). Hypotheses forwarded were that the oral retention of CPC was less than, for instance, CHEX or the antimicrobial activity of CPC was altered by salivary or formulation components when applied in vivo (Sheen & Addy 2003, Sheen et al. 2003).

Concurrent with their intrinsic microbial inhibition and bacteriocidal properties, the clinical efficacy of oral antimicrobials is hampered by the protection offered by the biofilm mode of growth of plaque organisms. Van der Mei et al. (2006) compared the antimicrobial bacteriocidal activity of a number of dentifrice slurries and CHEX. While killing of bacteria in a planktonic state was similar among different formulations, bacterial killing in 9-h-old plaques in vivo was already significantly less, with only CHEX showing substantive activity. Seventy-two-hour-old dental plaques were even less susceptible to antimicrobial effects than 9-h-old plaques. Evaluation was based

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

The project was funded in part by The Procter & Gamble Company, Mason, OH, USA and the University Medical Center Groningen, Groningen, the Netherlands.

on confocal laser scanning microscopic (CLSM) examination of dispersed and stained plaque that was formed and treated in vivo. CLSM is a powerful tool to study dead and live bacteria in dental plaque (Kuehn et al. 1998, Wood et al. 2000), but the microscopic nature of the technique makes the selection of regions of the dental plaque for analysis observer dependent. The use of dispersed plaques circumvents this problem and highly contributes to the statistical significance of the method. Moreover, dispersion of the plaque before staining also solves the problem of poor penetration of the required stains into the plaque and allows in vivo treatment followed by ex vivo examination. Thus, using quadrant sampling at different points in time after treatment, a powerful tool is obtained to study sustained antimicrobial effects under in vivo conditions (Van der Mei et al. 2006).

Recently, clinical evidence has become available on the efficacy of CPC in reducing dental plaque formation and gingivitis when applied in an alcohol- and surfactant-free (Tween80) formulation (Witt et al. 2005, 2006). This contrasts with marketed rinses also containing CPC, which are supported for breath and antibacterial activity without proven therapeutic effects.

Therefore, the aim of the present work was to combine in vitro and in vivo evaluations of different CPC-containing formulations in order to reveal the working mechanisms of the formulations under in vivo conditions. Three commercial formulations of CPC were evaluated for their effects on pellicle surface chemistry, thickness, and hydrophobicity in vitro. Adhesion and detachment of a co-adhering bacterial pair was determined in vitro using a parallel plate flow chamber. Bacteriocidal effects of the formulations were evaluated in vivo after acute single use of a formulation on dental plaques grown for 24 or 72 h as well as in a longitudinal study, during 2-week continuous use of a formulation in combination with tooth brushing.

Material and Methods

CPC formulations and saliva

Three commercially available CPC-containing formulations were evaluated in this study. Products differed in the level of CPC, the presence of alcohol and surfactant systems, as shown in Table 1.

Human whole saliva from 20 healthy volunteers, equally divided over both sexes (average age 30 ± 8 years) was collected into ice-chilled cups after stimulation of salivary flow by chewing Parafilm® (Alcan Packaging, Neenah, WI). The medical ethical committee approved collection of human saliva and patients gave their informed consent. After the saliva was pooled and centrifuged at 12,000 *g* for 15 min. at 4°C, phenylmethylsulphonylfluoride was added to a final concentration of 1 mM as a protease inhibitor. The solution was again centrifuged, dialysed for 48 h at 4°C against demineralized water and freeze dried for storage in order to provide for a stock. Finally, a lyophilized stock was prepared by mixing freeze-dried material originating from a total of 21 of saliva. Reconstituted, human whole saliva was prepared from the lyophilized stock by dissolution of 1.5 mg/ml in adhesion buffer (2 mM potassium phosphate, 1 mM $CaCl_2$, 50 mM KCl, pH 6.8). Note that recently it has been shown that freeze-thawing does not alter a saliva that has been stored at -80°C for a period of 6 months (Schipper et al. 2007).

Physico-chemical surface characterization of mouthrinse-treated salivary pellicles in vitro

Bovine enamel blocks were polished to a 0.3 μm mirror finish. In order to obtain clean enamel surfaces, without contamination from polishing, the final polishing step is performed in a slurry of Al_2O_3 particles (0.05 μm) in water, after which they were sonicated in demineralized water in order to remove the Al_2O_3 particles. X-ray photoelectron spectroscopy (XPS) demonstrated no residual aluminium on such polished surfaces, nor an inexplicably high carbon content, while water contact angles on thus-prepared enamel surfaces were in line with literature (Perdok et al. 1989). Salivary conditioning films were applied to all enamel blocks by 16 h immersion in reconstituted saliva at room temperature. Pellicle-coated enamel surfaces were treated by a CPC-containing formulation for 30 s and twice water washed by dipping. Subsequently, half of the blocks were exposed again to reconstituted saliva for 3 h. Contact angles with water on the pellicle-coated enamel surfaces were measured after air drying to a so-called ''plateau level'' of constant surface dryness (Van Oss & Gillman 1972), as monitored by water contact angle measurements over time. Contact angles were measured in triplicate with a camera goniometer system.

The chemical compositions of the salivary pellicle surfaces before and after treatments with mouthrinses were measured using XPS (S-Probe spectrometer, Surface Science Instruments, Mountain View, CA, USA) equipped with an aluminium anode (10 kV, 22 mA) and a quartz monochromator. XPS was conducted at a photoelectron collection angle of 55° with the sample with an electron flood gun setting of 10 eV. Elemental compositions were calculated from overall scans in the binding energy range of 1–1100 eV with a 1000×250 μm spot and a pass energy of 150 eV accounting for instrumental sensitivity factors. The thickness of the salivary conditioning films was estimated using an overlayer model based on the attenuation of the Ca_{2p} signal (Busscher et al. 2002). The XPS analyses were performed on three separately prepared enamel blocks per treatment.

Bacterial strains and culture conditions

Actinomyces naeslundii T14V-J1 (in Schaedler's broth with 0.01 g/l hemin in an anaerobic cabinet at 37°C) and *Streptococcus oralis* J22 (in Todd Hewitt broth in ambient air at 37°C) were inoculated from blood agar plates

Table 1. Abbreviations, manufacturers and major active and inactive ingredients of the three cetylpyridinium chloride (CPC)-containing formulations evaluated in this study

Name	Abbreviation	Manufacturer	% CPC	Additional excipients
Scope	SM	Procter & Gamble	0.045	Ethyl alcohol (14.3 wt%), Tween 80
Viadent	VD	Colgate Oral Pharmaceuticals Inc.	0.052	Ethyl alcohol (5.5% w/w), PEG-40 sorbitan diisostearate
Crest Pro Health	CPH	Procter & Gamble	0.07	Poloxamer 407

and grown for 24 h in 10 ml of the respective media. These strains constitute a co-adhering pair of initial colonizers of pellicle surfaces in the oral cavity, which has previously been extensively studied with regard to co-aggregation (Cisar 1982, Kolenbrander 1988), co-adhesion (Bos et al. 1998) and in oral cleansing models (Yang et al. 2001). The resulting 10 ml culture was used to inoculate a second culture of 200 ml, which was grown for 16 h. Subsequently, streptococci were harvested by centrifugation at $5000 \times g$ and actinomyces at $10{,}000 \times g$ (5 min., 10°C), washed once and resuspended in adhesion buffer. To break up bacterial aggregates, the bacteria were sonicated intermittently (50% duty cycle) while cooling in an ice/water bath for 35 s at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA), which was found not to cause cell lysis in any strain nor did the supernatant of pelleted sonicated cells cause co-aggregation of the partner cells.

Bacterial detachment and de novo deposition in vitro

Actinomyces were suspended in adhesion buffer to a concentration of 1×10^8 bacteria/ml, while streptococci were suspended in adhesion buffer supplemented with 1.5 mg/ml lyophilized human whole saliva to a concentration of 3×10^8 bacteria/ml. Bacterial adhesion was observed on the bottom glass plate of a parallel plate flow chamber (17.5×1.7 cm) with a channel height of 0.075 cm. Before the experiments, the glass was cleaned by sonication in a non-adsorbing 2% surfactant solution, named RBS 35 (Fluka Chemie, Buchs, Switzerland), followed by alternately rinsing with methanol and demineralized water. This cleaning yielded full spreading of water on the glass surface. A salivary pellicle was applied by immersing the glass in saliva for 16 h at room temperature in the flow chamber.

Observation was carried out with a CCD-MXR camera (High Technology, Eindhoven, the Netherlands) mounted on a phase contrast microscope (Olympus BH-2, Olympus Nederland BV, Zoeterwoude, the Netherlands) equipped with a $\times 40$ ultra long working distance objective (Olympus ULWD-CD Plan 40 PL). The camera was connected to an image analyser (TEA, Difa, Breda, the Netherlands). Live images were stored on disc for enumeration.

Before each experiment, all tubes and the flow chamber were filled with buffer, and 10 min. perfusion with buffer was applied to remove remnants of saliva from the flow chamber. Subsequently, the actinomyces suspension was flowed first through the system until a surface coverage of 1×10^6 bacteria/cm^2 was reached as enumerated by the image analysis system. Thereafter, flow was switched again to buffer to remove unattached bacteria from the flow chamber and the tubes for 15 min. (This step was separately shown not to remove any attached actinomyces.) Co-adhesion was initiated by switching the flow to the streptococcal suspension in saliva for 2 h. Solutions were circulated through the system by means of hydrostatic pressure at a wall shear rate of 10/s, which corresponds to physiological conditions of low shear (Dawes et al. 1989), and yields a laminar flow (Reynolds number 0.6). Hereafter, flow was again switched to buffer to remove unattached bacteria from the flow chamber and tubes for 15 min.

Before perfusing the flow chamber with a CPC-containing formulation, five images were taken from different areas on the pellicle surface after which the flow chamber was perfused with 4.6 ml of a CPC formulation. Again, five images from different areas on the pellicle surfaces were taken, the flow chamber was flushed with 120 ml of adhesion buffer to remove formulation remnants and filled again with a fresh streptococcal suspension in saliva and de novo deposition was initiated for another 2 h.

Analysis of the images included determination of the number of adhering organisms per square centimeter and the percentage distribution of the organisms in aggregates of different size. All data presented represent averages of triplicate runs with separately cultured bacteria.

Effects of CPC on the viability of established in vivo plaques

Acute single use study

Five healthy volunteers participated in this study, as approved by the Medical Ethics Committee of the University Medical Center Groningen, Groningen, the Netherlands. Volunteers were requested to brush their teeth according to their habitual routine with a sodium fluoride-containing dentifrice (Crest® Regular, Procter & Gamble, Cincinnati, OH, USA) for the duration of 1 week before treatment days. After this week, the volunteers were first asked to come to the laboratory on treatment days, 24 h after their last brushing, while in a second round of experiments the volunteers had refrained from brushing and all other oral hygiene for 72 h. On treatment days, plaque was removed from the buccal side of the tooth and molar surfaces down to the gingival margin from the left-upper quadrant of the dentition with sterile cotton swab sticks. A dental explorer was employed to remove the interproximal plaque from the buccal sides. The plaque collected was suspended in 1.5 ml demineralized water, dispersed by vortexing and immediately analysed after live/dead staining (BacLight™, Molecular Probes Europe BV, Leiden, the Netherlands) in the CLSM (base line). Subsequently, volunteers were requested to flush their mouth for 30 s with 15 ml of a CPC-containing formulation. After flushing, plaque was collected from the left-lower quadrant and subsequently after 1 and 6 h from the right-upper and right-lower quadrants, respectively, and suspended as described above. Possible differences in viability of the plaques collected from the different quadrants before application of the rinses were found negligible in a previous study (Van der Mei et al. 2006). Each volunteer participated on two separate occasions for the evaluation of each of the three CPC formulations included in the study against 24-h-old plaque, all with a wash-out period of 7 days in between. Evaluations against 72-h-old plaque were in single fold per volunteer for each formulation, as imposed by medical ethical considerations.

Thirty microlitres of each dispersed plaque was put on a microscope glass slide and stained for 30 min. in the dark with the live/dead stain. Confocal images were collected using a Leica TCS-SP2 confocal scanning laser microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with beam path settings for fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate-like labels. Two images ($375\ \mu m \times 375\ \mu m$) were randomly taken from each plaque sample immediately after focussing, the number of dead and live bacteria in each image was determined and averaged for the assessed effect per sample.

In order to standardize comparisons and to minimize the number of volunteers required to draw statistically

significant conclusions, the variation from day to day per volunteer and within different volunteers were excluded by expressing the results as %bacteria killed, relative to the internal control of each volunteer in a group according to

$$\%\text{Killed at time ``}t\text{''} = \left(1 - \frac{\%\ \text{Viable at time ``}t\text{''}}{\%\text{Viable at ``baseline''}}\right) \times 100 \quad (1)$$

Note that expression with respect to the percentage of viable organisms in each plaque sample makes this expression independent on the amount of plaque removed by each participant.

Two-week continuous longitudinal use study

In this study, volunteers were requested to brush their teeth twice a day according to their habitual routine with a conventional sodium fluoride-containing dentifrice (Crest® Regular, Procter & Gamble) for 2 weeks. After this control period, volunteers were asked to brush their dentition with the dentifrice in combination with the use of a CPC formulation (15 ml for 30 s per treatment after brushing) for another 2 weeks and to come to the laboratory on the evaluation day without brushing and rinsing, i.e. on average 24 h after the last brushing. Volunteers subsequently removed plaque from the entire dentition as described above and the amount and viability of the plaque was measured and expressed relative to the plaque collected during the use of the dentifrice only. Analogue to calculations in the acute single use study, %bacteria killed was calculated, now relative to the %viability observed during use of Crest® Regular only

$$\%\text{Killed at dentifrice + rinse} = \left(1 - \frac{\%\text{Viable at dentifrice + rinse}}{\%\text{Viable at dentifrice}}\right) \times 100 \quad (2)$$

Each volunteer participated two times for each CPC formulation in this longitudinal use study. Note again that expression with respect to the percentage of viable organisms in each plaque sample makes this expression independent of the amount of plaque removed by each participant.

Results

Physico-chemical properties of pellicle surfaces after CPC treatment

The water contact angles measured on the pellicle surfaces together with their elemental surface compositions are presented in Table 2. Treatment with CPC-containing formulations increases the water contact angles, while contact angles further increased during the additional 3 h pellicle formation on treated pellicles. The alcohol-free CPC formulation produced the largest increase in water contact angle, indicating highest CPC adsorption. The pellicle film thickness was relatively unchanged directly following rinse treatments, suggesting exchange of pellicle components by bioavailable CPC, especially in view of the changes observed in hydrophobicity. Pellicle thickness increased following 3 h of additional pellicle formation, as also evidenced by increased amounts of nitrogen [almost doubling for alcohol-free CPC formulation, Crest Pro Health (CPH, Procter & Gamble)].

Bacterial detachment and de novo deposition

Table 3 summarizes the total number of adhering actinomyces and streptococci before initiating bacterial detachment $(2.0 \pm 0.5) \times 10^6/\text{cm}^2$, while the percentage aggregate size distribution is shown in Fig. 1. As can be seen, a sizeable number of organisms are involved in adhering aggregates containing more than 10 organisms. All CPC-containing formulations stimulated small levels of bacterial detachment above the buffer control and Viadent (VD, Colgate Oral Pharmaceuticals Inc., Canton, MA, USA) in particular appeared more effective in breaking up large bacterial aggregates. Following perfusion of the flow chamber with a CPC-containing formulation and subsequent de novo deposition, similar numbers of (co-)adhering bacteria were found as before perfusion for all formulations, while perfusion with the buffer control yielded increased numbers by about 20%. This is not withstanding the fact that pair-wise analysis per formulation demonstrated that the actual number of de novo-deposited streptococci appeared higher (from 1.6 to $2.4 \times 10^6/\text{cm}^2$) after CPH than after VD or Scope Mouthrinse (SM) (increase amounting only $0.5 \times 10^6/\text{cm}^2$), concurrent with a higher percentage of large aggregates formed during de novo deposition after CPH. Both effects point to changes brought about by bioavailable CPC to the pellicle and bacterial cell surfaces involved.

Antibacterial efficacy in vivo

The viability of 24-h-old plaques amounted on average $52 \pm 13\%$, with

Table 2. Water contact angles [θ_W (°)], elemental surface compositions and layer thicknesses [L_t (nm)] in vacuo of 16 h pellicles on enamel blocks treated with CPC-containing formulations and after 3 h additional pellicle formation

Treatment*	θ_W (°)	% Carbon	% Oxygen	% Calcium	% Nitrogen	% Phosphorus	L_t (nm)
16-h pellicle	36	68.5	20.7	2.0	7.2	1.5	3.2
After treatment with CPC formulations							
+SM	56	68.9	21.5	1.6	6.4	1.1	3.5
+VD	45	64.2	24.9	2.2	7.4	1.3	2.9
+CPH	60	69.2	19.9	2.4	6.3	2.2	3.0
After treatment with CPC formulations and subsequent pellicle formation							
16+3-h pellicle	44	77.8	13.2	1.5	5.9	1.7	3.8
+SM+3-h pellicle	65	70.9	18.0	1.1	8.9	1.2	4.0
+VD+3-h pellicle	58	66.5	20.9	1.3	10.2	1.2	3.7
+CPH+3-h pellicle	79	64.4	21.9	1.1	10.8	1.0	3.9

Standard deviation over three separately prepared samples amounts on average 1.0% in elemental composition and 0.3 nm in thickness.
*CPC, cetylpyridinium chloride; CPH, Crest Pro Health; SM, Scope Mouthrinse; VD, Viadent.

Table 3. The number of bacteria adhering before perfusion of the flow chamber with a cetylpyridinium chloride (CPC)-containing formulation, the percentage detachment by the formulation (calculated relative to the number of bacteria adhering before perfusion with the formulation in the same experiment) and the number of organisms adhering after de novo deposition of streptococci

Formulation*	Before formulation (10^6/cm^2)	After formulation		After de novo deposition (10^6/cm^2)
		(10^6/cm^2)	detachment (%)	
Buffer	2.0 ± 0.7^{a}	2.1 ± 0.8^{a}	-6 ± 19^{a}	2.6 ± 0.5^{a}
SM	1.7 ± 0.1^{a}	1.4 ± 0.3^{a}	$16 \pm 9^{a,b}$	2.0 ± 0.3^{a}
VD	2.1 ± 0.6^{a}	1.5 ± 0.2^{a}	33 ± 15^{b}	1.9 ± 0.2^{a}
CPH	2.3 ± 0.9^{a}	1.6 ± 0.4^{a}	$28 \pm 11^{a,b}$	2.4 ± 0.8^{a}

a≠b at $p < 0.05$ (paired Student's *t*-test).
*CPH, Crest Pro Health; SM, Scope Mouthrinse; VD, Viadent.

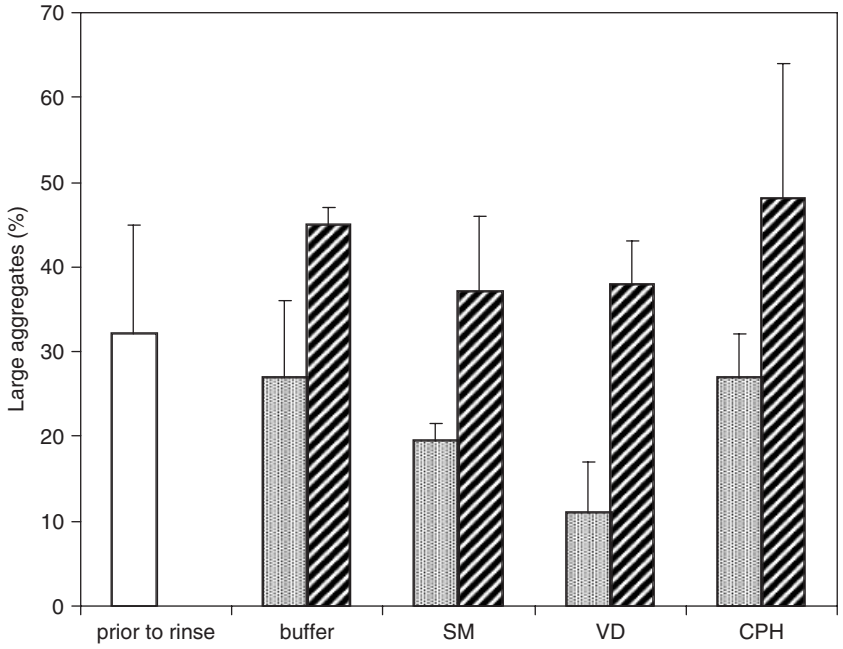

Fig. 1. The percentage of large (comprising more than 10 co-adhering *Actinomyces naeslundii* T14V-J1 and *Streptococcus oralis* J22) aggregates on salivary pellicle surfaces before and after perfusion of the flow chamber with buffer or cetylpyridinium chloride-containing formulations (dotted bar) and subsequent redeposition of streptococci (dashed bars). SM, Scope Mouthrinse; VD, Viadent; and CPH, Crest Pro Health.

an average number of $(7.5 \pm 2.8) \times 10^8$ organisms harvested from one quadrant. Seventy-two-hour plaques yielded more organisms $(14.5 \pm 3.4) \times 10^8$, while the viability was slightly lower ($46 \pm 14\%$) than of 24-h-old plaques.

The immediate kills achieved in 24- and 72-h-old plaques by SM and VD in vivo were not statistically different (see Table 4), but the bioavailable CPC formulation (CPH) yielded a higher bacteriocidal activity than the other two formulations, which was significantly different from the effects of VD on 24 h plaque. The sustained bacteriocidal effects by CPH were also higher than of the other two CPC-containing rinses. Six hours post-treatment a complete rebound in average plaque viability had already occurred after the use of VD (72-h-old plaques) and SM (24- and 72-h-old plaques), while CPH still yielded a 12% and 35% kill on 24- and 72-h-old plaques, respectively.

Table 5 shows that 2-week continuous longitudinal use of CPH produced statistically significant increases in the percentage of dead bacteria in sampled plaques as compared with brushing only. Especially after longitudinal use of CPH in combination with brushing, the percentage of dead bacteria is statistically higher than after the use of VD, and directionally higher than after the use of SM. To assess a %killing ratio comparable in the acute single use study, a %killing was calculated with respect to brushing with a dentifrice only. Because this involves a control in a different period (as opposed to the control in acute single use studies), there is considerable variability in the %killing after longitudinal use. Nevertheless, %killing achieved during 2-week continuous longitudinal use of a CPC-containing formulation in combination with brushing yielded a similar ranking of the bacteriocidal efficacies of the formulations as obtained in the acute single use study.

Discussion

Chemical plaque control does not solely involve antibacterial aspects of the formulations used, but is a complicated interplay of antibacterial efficacy in saliva, adsorption and desorption of actives from oral surfaces, and effects on both developing and established biofilms. Dentifrice components, for instance, have been demonstrated to adsorb to pellicle surfaces with a profound effect on subsequent adhesion of oral bacteria (Hannig & Joiner 2006). Usually, formulations that excel in one aspect fail with regard to another, and the net effect often is that differences between formulations that appeared promising in vitro disappear under the multifactorial in vivo conditions (Busscher et al. 2007). The development of clinically effective CPC-containing formulations is hampered by complex interactions of CPC with emulsifiers used to solubilize the active. This is particularly important in efforts to develop alcohol-free formulations.

This study was directed towards the development of a comprehensive concept on effects of different formulations on CPC delivery to surfaces and bacteriocidal effects on oral biofilms. The substratum involved in in vitro and in vivo evaluations throughout this study is a pellicle-coated surface, although in vitro pellicles are applied on bovine enamel and glass, while in vivo they develop on human enamel. Effects of the difference in substratum materials are generally considered negligible after pellicle coating, because bioadhesive properties of materials with different surface-free energies have in the past been demonstrated to converge to a single small band (Van Dijk et al.

Table 4. Sustained (immediate, 1- and 6-h post-treatment) in vivo bacterial killing by acute single use of a cetylpyridinium chloride (CPC)-containing formulation on 24- (two evaluations per person) or 72-h-old (one evaluation per person) plaques, expressed as %killed *versus* the base line plaque sampled in a group of five volunteers, using only Crest Regular

Rinse*	Immediately post-treatment	1-h post-treatment	6-h post-treatment
24-h-old plaques, %killing, single use			
SM	22 ± 26^{a}	23 ± 23^{a}	-11 ± 42^{a}
VD	23 ± 13^{a}	18 ± 23^{a}	4 ± 18^{a}
CPH	56 ± 15^{b}	34 ± 12^{a}	12 ± 17^{a}
72-h-old plaques, %killing, single use			
SM	5 ± 32^{a}	$1 \pm 45^{a,b}$	$-13 \pm 53^{a,b}$
VD	$31 \pm 23^{a,b}$	-4 ± 30^{a}	-28 ± 39^{a}
CPH	52 ± 18^{b}	39 ± 28^{b}	35 ± 14^{b}

$\pm$ denotes the SD over five volunteers.
a$\neq$b $p<0.05$ (paired Student's *t*-test).
*CPH, Crest Pro Health; SD, standard deviation; SM, Scope Mouthrinse; VD, Viadent.

Table 5. Amounts of plaque, plaque viability and bacterial killing relative to the use of Crest regular only after 2-week continuous longitudinal use of a cetylpyridinium chloride (CPC)-containing formulation in combination with brushing using Crest Regular (two evaluations per person)

Rinse†	#Bacteria retrieved $\times$ $1/10^{9*}$	%Bacteria dead
Bacterial retrieval and bacteriocidal activity		
Brushing only	1.08 ± 0.43	50 ± 13^{a}
SM	1.11 ± 0.40	$61 \pm 11^{a,b}$
VD	1.09 ± 0.31	59 ± 9^{a}
CPH	1.44 ± 0.95	70 ± 10^{b}
	%organisms	%killing
Bacterial retrieval and bacteriocidal activity with respect to brushing only		
SM	106 ± 34^{a}	6 ± 15^{a}
VD	102 ± 22^{a}	4 ± 14^{a}
CPH	129 ± 48^{a}	25 ± 13^{a}

$\pm$ denotes the SD for five volunteers.
*nsd in bacteria retrieved (paired Student's *t*-test, $p = 0.947$).
a$\neq$b $p<0.05$ (paired Student's *t*-test).
†CPH, Crest Pro Health; SD, standard deviation; SM, Scope Mouthrinse; VD, Viadent.

1988). The results show that CPC reacts strongly with pellicle surfaces, initially through exchange with pellicle components although exposure to saliva after treatment leads to rapid reestablishment of pellicle constituents. The various CPC formulations evaluated here differed in their interaction with salivary pellicles. The alcohol- and Tween80-containing formulations (SM and VM) caused smaller changes in surface hydrophobicity and decreased propensity to increase the pellicle thickness, probably as an effect of the emulsification systems used in these formulations.

A separate set of experiments examined the effects of different CPC formulations on bacterial (co-)adhesion and detachment. The ability of the formulations to detach co-adhering bacteria in vitro is clearly less than that of dentifrice supernates (Busscher et al. 2007), detaching 30–49% of co-adhering streptococci and actinomyces. This supports that the primary action of rinse formulations is delivery of an active rather than direct cleansing, which requires stronger detergent action. Indeed, dentifrices achieving strong bacterial detachment, like Crest Regular, contain high concentrations of sodium lauryl sulphate as compared with rinse formulations. On the other hand, it might be considered that the cleansing actions of dentifrices act in opposition to delivery and retention of actives on pellicle surfaces, complicating their development as chemotherapeutics. De novo redeposition of streptococci in our co-adhesion model after treatment with any of the CPC formulations was also different than seen previously for dentifrices (Busscher et al. 2007), and the model revealed differences in formulations with respect to controlling the formation of large co-aggregates. Particularly, VM seemed effective in preventing the formation of large co-aggregates during de novo deposition after treatment.

In line with more pronounced effects on pellicle chemistry and hydrophobicity of the alcohol-free formulation, also in vivo results attest to the greater bioavailability of CPC in such formulations. The alcohol-free formulation produced elevated acute single use antibacterial effects compared with other CPC formulations on 24- and 72-h-old plaques as well as after 2-week continuous longitudinal use. Interestingly, the pellicle surface properties treated with any of the CPC-containing formulations become more hydrophobic, even after subsequent pellicle formation on top of formulation-treated pellicles. This suggests that the hydrophobicity of the underlying pellicle surface dictates selective adsorption processes and differences in protein conformations as adapted on the differently treated pellicles. Hydrophobic surfaces usually attract little plaque under fluctuating shear conditions, but are not non-adhesive as such (Roosjen et al. 2006). After longitudinal use of CPH in combination with a dentifrice, the total number of plaque organisms that could be removed by the volunteers was highest for CPH, which may imply weaker binding to the more hydrophobic pellicle surfaces, as arise after CPH treatment.

The protocol applied in this study for the evaluation of the viability of dental plaques formed and treated in vivo has recently been developed and described with respect to its advantages and disadvantages (Van der Mei et al. 2006). One of the main advantages of the protocol is the use of an internal control in each value for bacterial killing calculated, enabling statistically significant conclusions while using a relatively small group of volunteers. No comparable data are available for 24-h-old plaques, but previously published data (Quirynen et al. 2005) allow comparison of the effects of CHEX with bioavailable CPC killing in 72-h-old plaques. Immediate killing by CHEX was 81%, which is superior to bioavailable CPC, but surprisingly bioavailable CPC shows far stronger sustained killing (35%) than CHEX (8%). Although this study may be considered to have a limitation with respect to the number of subjects involved in the in vivo studies, it shows that CPC in a bioavailable formulation must be considered as an equally good rinse component as

CHEX, without suffering from the traditional drawbacks of CHEX, like discolouration of mucosal surfaces and loss of taste.

Conclusions

Bioavailability of CPC yields more hydrophobic pellicles and affects bacterial co-adhesion in vitro, yielding weak plaque-binding forces, possibly causing plaque to be removed more easily in vivo.

The CPC formulations evaluated detach less-adhering bacteria from salivary pellicles than dentifrice supernates.

Clinically, the bioavailable CPC formulation yields superior immediate and sustained bacterial killing compared with the other alcohol- and Tween80-containing formulations upon acute single use. After 2-week continuous combination with dentifrice brushing, antibacterial efficacy of the alcohol-free CPC-containing formulation was still highest.

Acknowledgements

This study is supported by the University Medical Centre Groningen, Groningen, the Netherlands and the Procter & Gamble Company, Cincinnati, OH, USA.

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Clinical Relevance

Scientific rationale for the study: CPC has a long-lasting history of conflicting reports on its clinical efficacy. The development of clinically effective CPC-containing formulations is hampered by complex interactions of CPC with emulsifiers used to solubilize the active. This is particularly important in efforts to develop alcohol-free formulations.
Principle findings: This paper demonstrates that CPC is only bioavailable in a formulation that is free of alcohol and Tween80, with pronounced effects on pellicle surface chemistry.
Practical implications: CPC in a bioavailable formulation shows greater clinical efficacy in killing plaque organisms than in a formulation containing alcohol and Tween80.