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Effects of low dose chlorhexidine mouthrinses on oral bacteria and salivary microflora including those producing hydrogen sulfide

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Background/aims: Clinical studies have demonstrated the considerable effects of chlorhexidine on dental plaque and oral microbiota as well as improvements in indices of oral health. This investigation examined the efficacy of lower concentrations of chlorhexidine.
Methods: Mouthrinses with 0.03%, 0.06%, 0.12% chlorhexidine and a control rinse without chlorhexidine were examined. Alamar blue, an oxidation-reduction dye with fluorescent end-points proportional to bacterial viability, was used to determine bacterial viability. Further clinical studies examined the effects of these rinses on salivary bacteria and on bacteria producing hydrogen sulfide (H_2S) and implicated in halitosis.
Results: In laboratory tests, a significant dose-dependent effect was observed with *Actinomyces viscosus* as a model system using the Alamar blue procedure ($P < 0.05$). Clinical studies examined the effects 1.5 h and 3 h post-treatment on salivary bacteria and bacteria producing H_2S. The first study compared the control rinse with the 0.03% and 0.06% chlorhexidine rinses; a second study compared the effects of the control rinse and the 0.06% and 0.12% chlorhexidine mouthrinses. In both studies, chlorhexidine rinses demonstrated significant dose-dependent effects post-treatment on salivary bacteria vs. the control rinse ($P < 0.05$). Significant decreases in H_2S-producing bacteria were noted with these chlorhexidine rinses vs. the control rinse ($P < 0.05$).
Conclusion: The results highlight the dose-dependent relationships noted in laboratory and clinical tests which have potential implications for the use of lower doses of chlorhexidine to inhibit oral bacteria, including those implicated in halitosis.

Key words: Alamar blue; chlorhexidine; clinical studies; hydrogen sulfide; malodor; mouthrinse; salivary bacteria

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Chlorhexidine (CHX), a biguanide antimicrobial with broad-spectrum antimicrobial properties, is notable for its applications in many health care settings (25, 26). In addition to its present uses, recent investigations with CHX have reported reductions in catheter-related infections (22), a decrease in the incidence of nosocomial infections among surgery patients with concomitant reductions in the use of prophylactic antibiotics (5), and use as a skin cleanser in pediatric dermatology (11).

Formulations with CHX as an active ingredient are widely utilized for applications in the human mouth (17). Laboratory investigations indicate a broad spectrum of activity on a range of oral bacteria (1, 7) with recent studies comparing CHX on isolated oral microorganisms in the planktonic and biofilm mode of growth (6). Given its well-known activity on microorganisms, recent research has examined the use of CHX for the control of bacteria in dental water lines (8).

A number of clinical trials have established the efficacy of CHX formulations in the human mouth (23, 24). Most of these studies have examined CHX mouthrinses, with additional reports examining CHX varnishes, as prophylactic treatments prior to surgery, for subgingival irrigation and for several other indications (17, 18, 26). CHX formulations demonstrate significant effects on dental plaque and salivary bacteria (15, 23) and oral malodor (24). Whilst CHX is considered the gold standard for antiplaque and gingivitis agents, the side-effects of CHX such as toothstaining and poor taste are well known (17). Therefore, investigations have examined approaches to reduce the side-effects of CHX formulations (3) and other avenues to improve formulations (13).

This investigation examined the effects of different concentrations of CHX in mouthrinses on oral bacteria in laboratory and clinical trials. Towards this end, rinses with different levels of CHX (0%, 0.03%, 0.06%, and 0.12%) were prepared and initially tested for laboratory antimicrobial efficacy. The procedure utilized a recently developed method with Alamar blue, a redox dye that rapidly estimates the viability of oral bacteria in laboratory and clinical studies (29). Clinical studies then determined the effects of these rinses on salivary bacteria at several post-treatment time points. An additional parameter in these clinical studies utilized a recent microbiological procedure to quantify effects on the microflora producing hydrogen sulfide and implicated in oral malodor (19).

Material and methods

Mouthrinse formulations

The formulations tested were a commercially available CHX mouthrinse with 0.12% chlorhexidine and additional mouthrinses formulated with 0.06%, 0.03% CHX and a control rinse without CHX. All rinses were identical in composition with the exception of CHX concentrations.

Chemicals and media

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Phosphate-buffered saline (PBS), pH 7.4, was obtained from Gibco-BRL (Grand Island, NY). The Alamar blue dye was obtained from Biosource International (Camarillo, CA) and was stored at 4°C as recommended by the manufacturer. Oral bacteria were routinely maintained on trypticase soy broth and agar obtained from Becton-Dickinson (Sparks, MD) that were prepared in accordance with manufacturer's recommendations.

Salivary oral bacteria in clinical studies were enumerated on enriched media (trypticase soy agar with 5% sheep's blood, Becton-Dickinson, Sparks, MD). The preparation and application of the oral hydrogen sulfide (OHO) medium to selectively quantify oral bacteria producing hydrogen sulfide (H_2S) has been previously described (19, 28).

Preparation of bacteria for laboratory efficacy tests with Alamar blue

Actinomyces viscosus, a gram-positive bacterium, was obtained from American Type Culture Collection (Manassas, VA) and routinely grown in trypticase soy broth with glucose or on corresponding agar at 37°C. When required for tests, overnight broth cultures were diluted in fresh broth to an optical density of 0.83 ± 0.03 at 610 nm. The procedures with the Alamar blue dye, an oxidation-reduction dye with fluorescent end-points to examine the viability of nonoral bacteria (20) and the antimicrobial efficacy of oral care formulations on oral bacteria have been described (29). In brief, cultures (5 ml) were treated with test formulations (0.5 ml) for 2 min prior to the addition of Alamar blue. Viable bacteria reduce Alamar blue, resulting in a fluorescent end-point. A significant correlation between the viability of common oral bacteria and Alamar fluorescence has been previously reported. All CHX mouthrinse formulations were tested with the control rinse (0% CHX) and untreated bacteria as controls. Additional controls examined fluorescence of sterile media and all formulations incubated in the presence and the absence of Alamar blue. All tests were conducted in triplicate with fluorescence from each replicate determined in triplicate.

Procedures for clinical studies

Clinical studies were conducted in accordance with procedures widely accepted for human trials (17). Volunteers for the studies were informed of study procedures and recruited on the basis of on their informed consent and willingness to comply with study protocols. Adult volunteers (age 24–65 years) in good medical and dental health from Piscataway, New Jersey, were included. Selected subjects underwent a 7-day washout phase with a commercially available fluoride dentifrice and discontinued the use of all other oral hygiene formulations, including chewing gums and mints, for the duration of the study. The subjects arrived on the day of the test prior to undertaking oral hygiene procedures and rinsed their mouth with 10 ml of commercially available potable water (Poland Spring, Poland Spring, ME) for 10 s. This rinse was collected for baseline microbial analysis. Treatments for the cross-over design studies were randomized and subjects rinsed with 15 ml of the assigned rinse for 30 s and abstained from food, drink or oral hygiene for the next 3 h. Microbial samples were collected from subjects after 1.5 h and 3 h for analysis (obtained by rinsing their mouth with 10 ml of commercially available water for 10 s). A washout phase of 1 week was included between treatments. Two clinical studies were conducted. Twenty-one subjects participated in the first study, which compared the control rinse to the 0.03% and 0.06% CHX rinses. An additional 20 subjects participated in the second study of treatments with the control rinse and rinses with 0.06% and 0.12% CHX. The study population was based on a previous study demonstrating significant effects of CHX rinses in a population of 14 subjects (29).

Microbiological procedures for clinical studies

Microbial samples collected from clinical studies were vortexed well and immediately diluted in PBS with 10-fold dilutions plated in duplicate on 5% sheep blood agar and OHO agar. The plates were incubated under anaerobic conditions in accordance with established procedures (19, 27). After 7 days of incubation, the number of colony forming units per ml (CFU/ml) of bacteria from duplicate plates was determined. The duplicate results from each time point were transformed to $\log_{10}$ and averaged for statistical analysis.

Statistical analysis

Analyses were conducted with the JMP Software (SAS Institute, Cary, NC). Triplicate results from laboratory tests with Alamar blue were averaged prior to analysis of variance (ANOVA) with subsequent *post hoc* analysis by Tukey tests to determine differences between treatments.

Bacterial counts from clinical tests on both microbiological media (5% trypticase soy agar for total salivary bacteria and H_2S-producing bacteria on the OHO

medium) were enumerated as CFU/ml and transformed to $\log_{10}$. The results from duplicate plates at each analysis point were averaged and differences between baseline and each post-treatment time point determined. These were analyzed by ANOVA with *post hoc* Tukey HSD tests to examine differences between each treatment. For all tests, statistical significance was set at $P < 0.05$.

Results

The laboratory efficacy of CHX rinses

The Alamar blue method was used to examine the antimicrobial effects of CHX rinses on *A. viscosus*. Bacteria treated with the control rinse (0% CHX) demonstrated no significant decrease in Alamar blue fluorescence compared with untreated bacteria (Figs 1 and 2). In contrast, treatment with the CHX formulations resulted in a significant decrease in bacterial fluorescence vs. the control rinse (0% CHX) and untreated bacteria ($P < 0.05$). Additionally, a significant dose–response effect was noted in the two studies that compared the 0.03% with the 0.06% CHX rinse (Fig. 1) and the 0.06% with the 0.12% CHX rinse (Fig. 2) ($P < 0.05$).

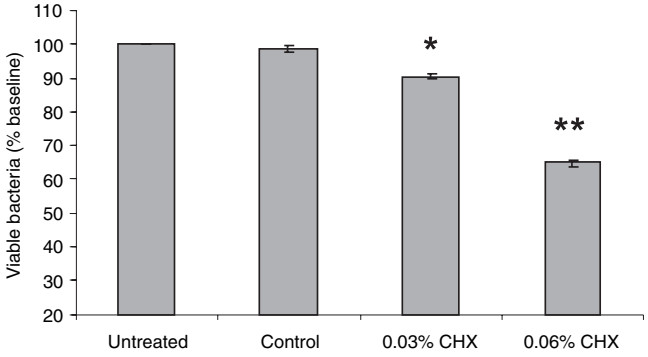

Fig. 1. The effects of a control and CHX rinses (0.03% and 0.06%) on *A. viscosus* by the Alamar blue method. Average numbers of bacteria recovered are shown as a percentage of the baseline or initial inoculum following each treatment ± the standard error of the mean. * and ** indicate statistically significant differences (by ANOVA and *post hoc* Tukey HSD analysis, respectively) compared with all other treatments ($P < 0.05$).

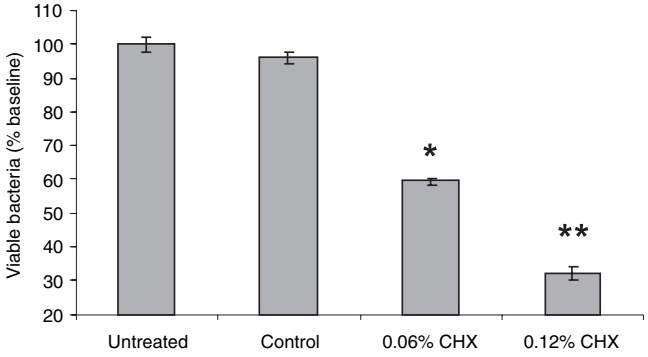

Fig. 2. The effects of a control and CHX rinses (0.06% and 0.12%) on *A. viscosus* by the Alamar blue method. Average numbers of bacteria recovered are shown as a percentage of the baseline or initial inoculum following each treatment ± the standard error of the mean. * and ** indicate statistically significant differences (by ANOVA and *post hoc* Tukey HSD analysis, respectively) compared with all other treatments ($P < 0.05$).

Effect of CHX rinses in clinical studies on salivary bacteria and those producing H_2S

Effects of 0.03% and 0.06% CHX rinses

A cross-over design clinical study was conducted with 21 subjects to compare the 0.03% and 0.06% CHX rinse with a control rinse without CHX. The results (Fig. 3) indicate significant reductions in salivary bacteria with both CHX rinses (0.03% and 0.06%) at all post-treatment time-points compared with the control rinse ($P < 0.05$). A statistically significant dose response was noted between the CHX rinses at 1.5 h and 3 h post-treatment ($P < 0.05$).

The additional microbial assessment examined effects on odorigenic (bad-breath) bacteria producing H_2S (Fig. 4). As seen with salivary bacteria, significantly lower numbers of H_2S bacteria were noted post-treatment with both 0.03% and 0.06% CHX rinses vs. the control rinse ($P < 0.05$). *Post hoc* analysis indicates dose-dependent effects with significantly higher effects by the 0.06% CHX rinse than the 0.03% CHX rinse at 1.5 h post-treatment ($P < 0.05$).

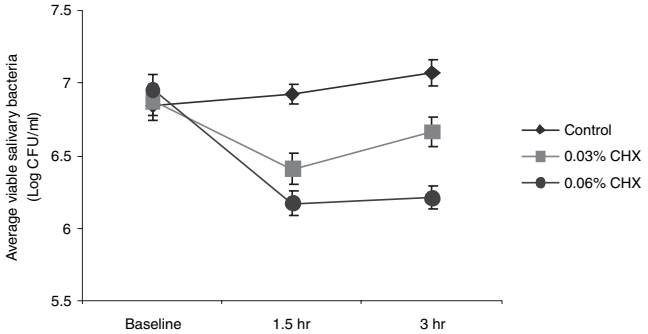

Fig. 3. The clinical effects of a control and CHX rinses (0.03% and 0.06%) on salivary bacteria at pretreatment and post-treatment time points. Average numbers of viable bacteria at each sampling time point ± the standard error of the mean are shown.

Effects of 0.06% CHX and 0.12% CHX rinses

The second cross-over design clinical study with 20 subjects compared the effects of the 0.06% CHX and 0.12% CHX rinses with the effect of the control rinse (Fig. 5). Both CHX rinses demonstrated a statistically significant decrease of salivary bacteria at 1.5 h and 3 h post-treatment compared with the control ($P < 0.05$). A dose-dependent effect was noted in this study, with significantly higher effects using the 0.12% CHX rinse than the 0.06% CHX rinse at both post-use time-points ($P < 0.05$). Both CHX rinses had significant effects on H_2S bacteria (Fig. 6) compared with the control at 1.5 h and 3 h post-treatment ($P < 0.05$);

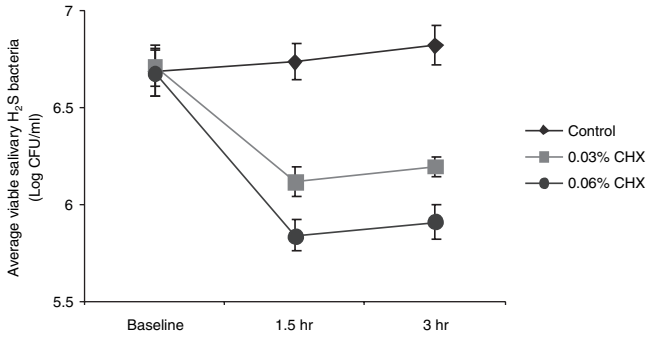

Fig. 4. The clinical effects of a control and CHX rinses (0.03% and 0.06%) on salivary bacteria producing H_2S at pretreatment and post-treatment time points. Average numbers of viable bacteria at each sampling time point ± the standard error of the mean are shown.

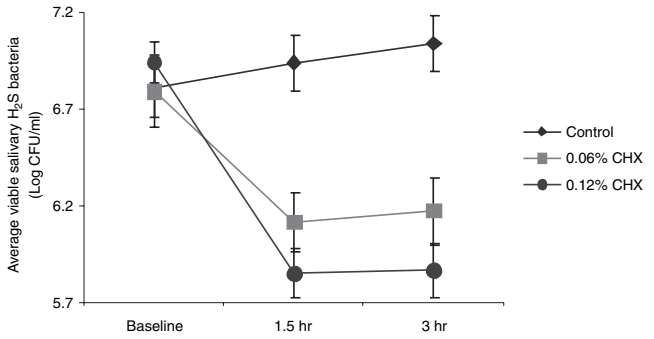

Fig. 5. The clinical effects of a control and CHX rinses (0.06% and 0.12%) on salivary bacteria at pretreatment and post-treatment time points. Average numbers of viable bacteria at each sampling time point ± the standard error of the mean are shown.

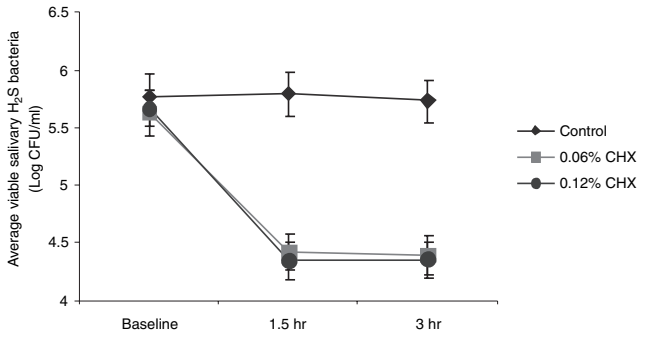

Fig. 6. The clinical effects of a control and CHX rinses (0.06% and 0.12%) on salivary bacteria producing H_2S at pretreatment and post-treatment time points. Average numbers of viable bacteria at each sampling time point ± the standard error of the mean are shown.

however, a statistically significant dose–response effect was not noted.

Discussion

The considerable potency of CHX on a variety of oral bacteria including gram-positive and gram-negative bacteria has been extensively described (1, 7, 30). Recent investigations with CHX have examined the effects on the mediators of inflammation such as matrix metalloproteases, which are produced in response to gingival inflammation (12). The clinical effects of CHX formulations on dental plaque and significant improvements CHX has made on other indices of oral health, gingivitis and bleeding on probing, have been comprehensively documented (12, 26). Most of these investigations have examined the effects of mouthrinses with 0.2% CHX, with other clinical studies examining the effects of lower concentrations of CHX over an extended period (6 months) on oral bacteria (21) and on reducing plaque and gingivitis (10, 15). However, there are few studies on the dose-dependent effects of CHX. Initial studies on doses of CHX are available as an abstract (2). In a 4-day clinical study with different doses of CHX and no other oral hygiene procedures, a dose-dependent reduction in dental plaque formation was reported (16). However, the immediate effects of different CHX doses on oral bacteria, including oral bacteria implicated in halitosis, remain unexplored. This investigation examined a range of CHX concentrations with a few rinses formulated below 0.2% forming the focus of these studies.

In laboratory tests with oral bacteria and in clinical tests, the Alamar blue method discriminates between formulations with commonly used antimicrobial agents (29). The present investigation sought to further characterize this method and examined the dose–response effects of CHX. Using *A. viscosus* as the model system, a significant dose-dependent effect was noted, with increasing concentrations of CHX resulting in increasing antimicrobial effects. Although all formulations were not tested simultaneously in one test, it is interesting to note that in separate trials, the 0.06% CHX rinse demonstrated similar reductions in bacteria compared with the control rinse. The results are comparable to published reports that indicate effects of CHX formulations on a range of oral bacteria following a short incubation period (14).

While laboratory tests on bacterial strains may be useful for initial screening, the limitations of laboratory methods are well-known (9). Clinical studies with appropriate controls are required to confirm the utility of formulations. Although several clinical end-points may be used to demonstrate efficacy, the effect on salivary microflora has been used extensively. For instance, the effects of different 0.12% CHX formulations have been recently reported (14) with additional reports comparing CHX rinses to other active agents such as cetylpyridinium chloride (23). In the present investigation, a cross-over study design was employed with all the subjects rinsing with each test formulation to minimize individual variations in the results. Larger ($\sim$ 20) groups of subjects were enrolled to distinguish differences between formulations and a recently standardized clinical design was also included that used a microbiological approach to selectively quantify oral H_2S bacteria. The studies were based on reports describing the significant effects of CHX formulations on halitosis in clinical studies (24).

Significantly, in both clinical studies, the effects of the CHX rinses on salivary and H_2S-producing bacteria showed similar trends, with substantial inhibitions noted at all post-treatment points. These results are comparable to earlier studies that demonstrate the residual effects of CHX on oral bacteria (14, 24). Statistically significant effects were also noted on the oral H_2S bacteria in the clinical studies comparing the various CHX rinses with the control rinse. These results may enable additional studies to examine the effects of these lower dose CHX rinses in other clinical trials.

The utility of the Alamar blue procedure as a micrbiological screening procedure to examine different doses of CHX is demonstrated. Further, the relationship between the laboratory and clinical trials and the dose-dependent effects in clinical studies and on oral H_2S bacteria represent microbiological approaches for future investigations.

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