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Comparative analysis of the antibacterial effects of combined mouthrinses on *Streptococcus mutans*

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Background/aims: Chlorhexidine has been proposed as a potent chemotherapeutic agent against oral bacteria. However, there are some inconsistent results regarding the usefulness of chlorhexidine mouthrinse as an antimicrobial for *Streptococcus mutans*. The purpose of this study was to investigate the effectiveness of combining oral rinses to reduce *S. mutans* levels in human saliva.
Methods: Sixteen healthy adult subjects were randomly assigned to one of four rinse groups using a 4-cell crossover design. The groups rinsed twice a day for 7 days with one of the following: 0.12% chlorhexidine (PerioGard®), 1.5% hydrogen peroxide (Peroxyl®), a combined chlorhexidine + hydrogen peroxide, or water (control). Every 5 weeks, each group initiated a different rinse. Saline wash samples were collected on days 7 and 21 for assessment of *S. mutans* and total streptococci.
Results: No significant differences were seen in *S. mutans* levels among the groups; however, the levels of total streptococci on day 7 samples were significantly lower in the chlorhexidine and chlorhexidine + hydrogen peroxide groups than in the hydrogen peroxide and control groups. There was no additional decrease seen in *S. mutans* or total streptococci levels in the group receiving chlorhexidine + hydrogen peroxide compared to chlorhexidine alone.
Conclusions: Sample variation was high throughout the study, with a significant trend toward lower counts as the study progressed. Adding hydrogen peroxide to the chlorhexidine mouthrinse did not result in a further decrease in *S. mutans* levels.

Key words: chlorohexidine; hydrogen peroxide; *Streptococcus mutans*

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The use of antimicrobial mouthrinses has been proposed as a means of reducing the levels of oral bacteria, specifically *Streptococcus mutans*. Chlorhexidine is a broad-spectrum antimicrobial agent (9). One application of chlorhexidine in dentistry has been to reduce the level of oral mutans streptococci, and it has been incorporated into several mouthwashes, dental gels, and varnishes. Chlorhexidine has been shown to inhibit plaque formation, thereby reducing gingival inflammation and preventing dental caries (3, 9). However, studies aimed at reducing the levels of *S. mutans* in the oral cavity with chlorhexidine have reported large variations, inconsistencies, and an inability to ablate *S. mutans* (7, 11).

A study by Dona et al. (1) found that using a combination chlorhexidine and hydrogen peroxide rinse produced a reduction in plaque scores. Their results demonstrated a more effective inhibition of short-term plaque growth when patients rinsed with chlorhexidine + hydrogen peroxide than with chlorhexidine alone. Steinberg et al. (10) reported a synergistic, antibacterial effect when combinations of hydrogen peroxide and chlorhexidine were used in an *in vitro* study. They observed that chlorhexidine and hydrogen peroxide killed *Streptococcus faecalis* and *Streptococcus sobrinus* at concentrations lower than the minimal inhibitory concentration of each agent alone. A proposed mechanism for this synergistic bactericidal effect is that chlorhexidine alters the cell surface, allowing hydrogen peroxide to penetrate more effectively to damage and interact

with the intercellular organelles of the bacteria (1). The purpose of this study was to investigate the ability of a combination chlorhexidine and hydrogen peroxide oral rinse to reproducibly reduce *S. mutans* levels in saliva of adult subjects compared to rinses with chlorhexidine or hydrogen peroxide alone.

Material and methods

Clinical procedures

During the initial recruitment, 28 subjects were evaluated for eligibility at the University of Alabama at Birmingham School of Dentistry. To be eligible for the study, subjects had to meet the following requirements:

- presence of *S. mutans*;
- at least 24 teeth without obvious active dental caries or periodontal disease;
- no fixed or removable orthodontic appliances or removable prostheses;
- no history of antibiotic therapy within the previous 3 months.

Of the 28 subjects, a saline mouthwash sample was collected from 18 to screen for the presence of oral *S. mutans* and total streptococci. Sixteen healthy subjects (age 26–55 years) were then recruited for the study. Group size was estimated by power calculations (80%) based on previous results in our laboratory (8). A visual dental examination was performed to assess general oral health and number of existing teeth.

At the screening appointment, participants had a medical history review and, after explaining the study process to each subject, informed consent was obtained. The randomized, 4-cell crossover design of this study was approved by the Institutional Review Board at the University of Alabama at Birmingham. Each subject participated in each rinse group according to randomly generated assignments and was unaware of their group assignment. Initially, the subjects were assigned to one of four rinse groups consisting of:

- chlorhexidine only (0.12% PerioGard®, Colgate-Palmolive Company, Canton, MA) – chlorhexidine group;
- hydrogen peroxide only (1.5% Peroxyl®, Colgate-Palmolive Company) – hydrogen peroxide group;
- chlorhexidine followed by hydrogen peroxide – chlorhexidine + hydrogen peroxide group;
- placebo group (colored water with blue #4 food color, one drop per liter of water) – control group.

Fourteen (28 in the case of the combined rinse group) coded but unlabeled tubes were provided to each individual for them to rinse twice a day for 7 days. Both verbal and written instructions were provided. Subjects were to rinse twice a day with 15 ml of the solution for 1 min and expectorate. In the combined rinse group, instructions were to rinse 1 min with the first tube (color coded; chlorhexidine) expectorate and then immediately rinse with the second solution (hydrogen peroxide). A diary sheet was provided for participants to record comments about compliance, as well as the time of rinsing. Participants were also told not to change their normal dietary and oral hygiene practices during the study. Subjects were requested to return the empty tubes as well as the diary sheet at the end of the treatment week to help evaluate compliance. Five weeks after the beginning of the first rinse regimen, the subjects were placed into a different rinse group based on their initial random assignments. This procedure was repeated two more times so that each subject randomly participated in each of the four rinse groups.

Microbiologic processing

After completing 7 days of rinsing (experimental day 7), each subject was requested to rinse their mouth with saline (10 ml) for 30 s and then to expectorate the wash into a sterile container for microbiological analysis. A second sample was collected 2 weeks later (day 21). Diluted (1 : 10) or undiluted saline rinse samples were plated directly onto Mitis Salivarius supplemented with tellurite (Becton Dickinson, Cockeysville, MD) for total streptococci levels and onto Mitis Salivarius plates supplemented with bacitracin (Sigma-Aldrich Co., St. Louis, MO) and tellurite (i.e. Gold's media) (5) for *S. mutans* levels using a Spiralplater (Spiral System, Inc., Cincinnati, OH). The plates were incubated at 37°C in an anaerobic chamber for 48 h. The total number of colony-forming units (CFU) per ml of saline rinse on mitis salivarius represented the total streptococci counts. *S. mutans* were identified on Gold's plate based on colony morphology (4). Further confirmation that colonies were *S. mutans* was obtained using a biochemical assimilation test (Minitek, Becton Dickinson), as described by the manufacturer.

Statistical analysis

Results from the saline rinse samples collected on days 7 and 21 were initially tabulated for *S. mutans*, total streptococci, and *S. mutans*/total streptococci levels. The values then were log transformed to control for variance. For values that were 0 (below the detectable levels), the common logarithm of 1 was used. Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure on SAS. Statistical significance was determined at the $P < 0.05$ level. The results were expressed as the geometric mean and asymptotic standard error (ASE).

Results

Of the 18 eligible participants, 17 had detectable *S. mutans* in their screening sample. The 16 subjects finally selected were those with the highest *S. mutans* levels (range 2.3×10^3 to 4.6×10^5 CFU/ml saline rinse). Total streptococci levels ranged from 4.7×10^5 to 3.5×10^6 CFU/ml saline rinse from the baseline samples.

Fourteen of the original 16 subjects successfully completed all four series of rinses. The remaining two subjects only completed three rinse regimens. One of these latter participants did not follow one set of rinsing instructions, and the other subject moved prior to the last rinse regimen. No adverse effects were reported by any of the subjects during or after any of the rinse regimens.

Data from the four randomized rinse schedules (i.e. cross-over design) were combined to assess the antimicrobial effects of the rinses. There were large

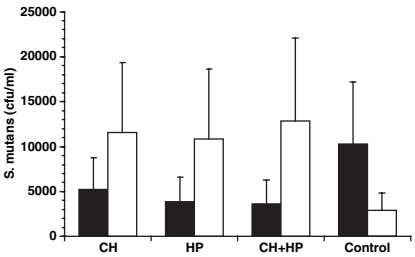

Fig. 1. S. mutans levels in saline rinse samples from 16 subjects following four random rinse solution schedules of antimicrobial or control mouthrinses. Saline rinse samples were collected on days 7 (■) and 21 (□) after each rinse regimen. Subjects rinsed for 7 days, twice a day, with chlorhexidine, hydrogen peroxide, chlorhexidine + hydrogen peroxide, or water (control). Values are the geometric mean and asymptotic standard error (ASE) of the combined results for the four rinse regimens (see Material and methods). No significant differences were found in the levels when compared to the control group or between day 7 and day 21 results ($P > 0.05$).

variations in the levels of *S. mutans* within the different groups after rinsing (Fig. 1). The levels of *S. mutans* were reduced, but not significantly, by the three experimental rinses on day 7 compared to the control group. By day 21, the *S. mutans* CFU counts in the experimental groups increased to the level in the control group on day 7. Surprisingly, the mean *S. mutans* counts decreased between day 7 and day 21 in the control group. No significant differences were seen in the levels of *S. mutans* between experimental or control groups on day 21.

The levels of total streptococci were significantly reduced by chlorhexidine and the combined chlorhexidine + hydrogen peroxide mouthrinses on day 7 when compared to both the hydrogen peroxide and control groups on day 7 and to the levels on day 21 with the same rinse (Fig. 2). On day 21 the levels of total streptococci had increased and were similar in all four groups. No significant difference was seen in the ratio of *S. mutans* to total streptococci in subjects receiving the chlorhexidine, hydrogen peroxide, or chlorhexidine + hydrogen peroxide rinses when compared to the control group on days 7 or 21 (data not shown).

A time effect was observed during successive rinse protocols, and this confounding factor may have influenced the results of each rinse regimen. In this regard, the levels of *S. mutans* were reduced as the study progressed, and this reduction was statistically significant in the months of December and January as compared to October ($P = 0.05$). This finding is further illustrated by observing the individual rinse group results during each of the 4 months of the study (Fig. 3). The chlorhexidine and chlorhexidine + hydrogen peroxide groups showed decreased levels of *S. mutans* on day 7 compared to day 21 in 3 of the 4 months (the exception was November). Furthermore, there was an overall reduction in the *S. mutans* levels at day 7 or day 21 from month to month. However, the results from the control group were not expected. Although it was anticipated that no change in *S. mutans* levels would occur in the control group, a large decrease in *S. mutans* levels was observed from day 7 to day 21 in October and November (although not significant).

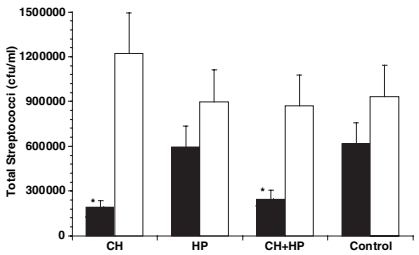

Fig. 2. Total streptococci levels in saline rinse samples from 16 subjects following four random rinse solution schedules of antimicrobial or control mouthrinses. Saline rinse samples were collected on days 7 (■) and 21 (□) after each rinse regimen. Subjects rinsed for 7 days, twice a day, with chlorhexidine, hydrogen peroxide, chlorhexidine + hydrogen peroxide, or water (control). Values are the geometric mean and ASE of the combined results for the four rinse regimens (see Material and methods). Levels of total streptococci were significantly reduced ($P < 0.01$) by chlorhexidine and chlorhexidine + hydrogen peroxide mouthrinse on day 7 compared with day 21, and when compared to the hydrogen peroxide and control group on day 7.

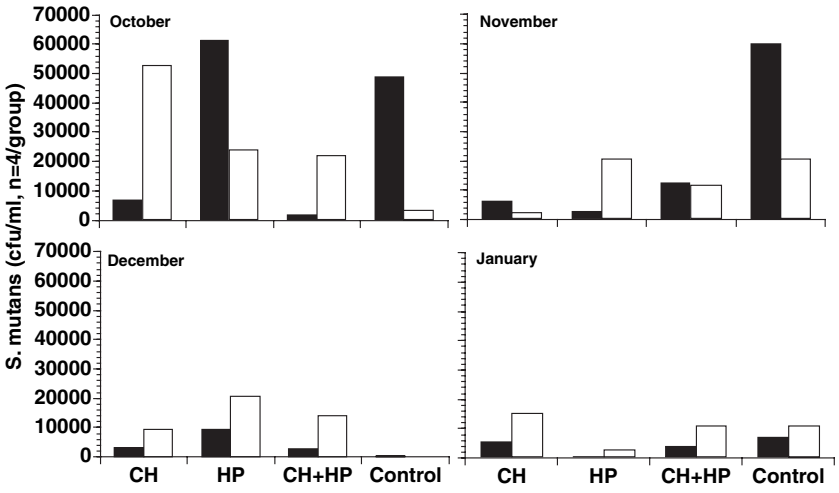

Fig. 3. Levels of *S. mutans* in saline rinse samples from individual groups per month. Sixteen subjects rinsed for 7 days (four subjects each rinsing with chlorhexidine, hydrogen peroxide, chlorhexidine + hydrogen peroxide, or water [control]) and samples collected on days 7 (■) and 21 (□) after rinsing began. Every participant was randomly assigned to a rinse group. Every rinse group (different rinsing solution) was initiated every 5 weeks. Overall, levels in the months of December and January were lower than those in October and November ($P < 0.05$).

The time effect observed with successive rinse protocols was not seen with total streptococci. Total streptococci results for each rinse group (Fig. 4) showed decreases each month in both the chlorhexidine and chlorhexidine + hydrogen peroxide groups on day 7 compared to day 21. Furthermore, the control groups showed expected results, with there being no statistical difference in the total streptococci levels from day 7 to day 21.

Discussion

Chlorhexidine has been successfully used in Europe as an antiplaque-antimicrobial agent for almost 30 years and, more recently, has been approved for use in the United States. Chlorhexidine is considered to be one of the most highly efficient and potent chemoprophylactic agents for oral use (9). In the US it is available as a 0.12% mouthrinse, which is a lower concentration than that used in Europe (0.2%). Numerous studies have demonstrated the effectiveness of chlorhexidine in reducing gingival inflammation by virtue of its antimicrobial effect (3). The antimicrobial effect has also been proposed to be caries preventative by reducing the levels of *S. mutans* in the oral cavity (3, 8). Unfortunately, some investigators have had difficulties reproducibly reducing *S. mutans* levels with chlorhexidine (7, 11).

Other studies have demonstrated that a combined solution of chlorhexidine and peroxide resulted in a more effective decrease in short-term plaque growth than seen with each rinse individually (1, 5, 6). In spite of this evidence, the present study did not observe any significant reduction in salivary *S. mutans* levels when a combination rinse of chlorhexidine followed by hydrogen peroxide was used as a mouthrinse, as compared to chlorhexidine alone. Although a decreased *S. mutans* trend was observed after the rinse with chlorhexidine and chlorhexidine + hydrogen peroxide in the four successive rinse studies used in this cross-over design (Fig. 1), there was no indication that hydrogen peroxide improved the anti-*S. mutans* effect of chlorhexidine. The large variability in the microbial counts may have impaired the ability to demonstrate an antimicrobial effect on *S. mutans*.

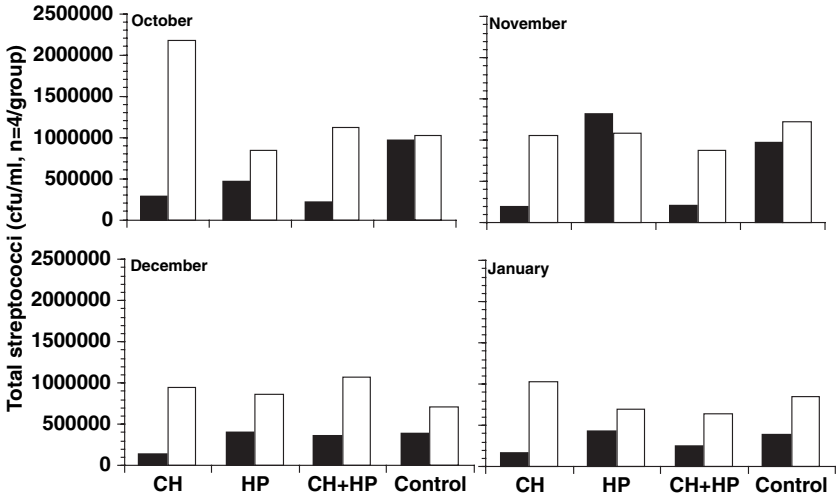

Fig. 4. Levels of total streptococci in saline rinse samples from individual groups per month. Sixteen subjects rinsed for 7 days (four subjects each rinsing with chlorhexidine, hydrogen peroxide, chlorhexidine + hydrogen peroxide, or water [control]) and samples collected on days 7 (■) and 21 (□) after rinsing began. Every participant was randomly assigned to a rinse group. Each rinse group (different rinsing solution) was initiated every 5 weeks.

Nonetheless, the observations with total streptococci indicate that the combination did not improve the antimicrobial effect on streptococci, specifically *S. mutans*. There have been some reports that *S. mutans* may be more susceptible to antimicrobial effects than other oral streptococci (9). This conclusion was not supported by the results of this study. Alternatively, the lower concentration of chlorhexidine used in the US (0.12%) may not be sufficiently strong to reduce *S. mutans* (even in combination with hydrogen peroxide) compared to other concentrations (i.e. 0.2%) and delivery formulations (i.e. gels, varnishes).

A 4-cell crossover design was used in this study because of its increased statistical power when using paired samples (each person was randomly assigned to all four groups). However, a carryover effect of the preceding mouthrinse protocols may have negatively affected the advantages of such a design. Emilson et al. (2) previously reported using a 1% chlorhexidine gel applied once via one of three different ways (in trays, by flossing and with a combination of polishing and flossing). They showed that even though the *S. mutans* levels were significantly reduced, they increased to levels at or above baseline by 2 weeks. Therefore, based on previous published and unpublished studies, we expected that *S. mutans* levels would recover to baseline by waiting 4 weeks between rinse schedules. Nevertheless, a significant time effect was observed. A possible additive or carryover effect of successive mouthrinses may have diminished *S. mutans* levels during the study. However, this was not supported by the analysis of *S. mutans* levels on a monthly basis (Fig. 3). For the month of October, the levels of *S. mutans* on day 7 were lower than on day 21 in the chlorhexidine and chlorhexidine + hydrogen peroxide groups. Conversely, in the untreated control group, the levels of *S. mutans* on day 21 were lower than on day 7. These results and the general variability in *S. mutans* levels in the various groups each month do not support a direct carryover effect. The results with total streptococci levels further indicate a lack of evidence for a carryover effect; the mouthrinse resulted in significant reductions in the chlorhexidine and chlorhexidine + hydrogen peroxide groups on day 7 (Fig. 2) that did not result in an overall decrease in total streptococci levels with time from saline samples (Fig. 4). It is possible that other unidentified factors such as seasonal events, changes in diet, or variations in oral hygiene practices altered the levels of *S. mutans* as the studies progressed.

In summary, this study demonstrates that the combination of chlorhexidine and hydrogen peroxide did not have a greater effect than chlorhexidine alone in decreasing oral *S. mutans* or streptococci levels. Furthermore, in none of the treatment groups were significant decreases in *S. mutans* levels found compared to the control. The findings of this study continue to demonstrate the difficulty of reproducibly reducing oral *S. mutans* levels by antimicrobial treatment.

Acknowledgments

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