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ORIGINAL ARTICLE

Two percent chitosan mouthwash: A microbiological and clinical comparative study

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KEYWORDS
Candida albicans;
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dental plaque;
drug delivery system;
Streptococcus mutans

Abstract *Background/purpose:* The aim of this study was to evaluate the microbiological and clinical effects of a chitosan (CH) mouth rinse on plaque inhibition.
Materials and methods: Thirty-six healthy participants were recruited. The following clinical data were recorded: a plaque index (PI), gingival index (GI), Quickley–Hein plaque index (QPI), and probing depth (PD). Volunteers were given oral hygiene (OH) instruction and trained on scaling and professional tooth cleaning (PTC). After the final PTC, volunteers were randomly allocated into three groups. Group A rinsed with 2% CH, group B rinsed with 0.2% chlorhexidine digluconate (CHX), and group C rinsed with 2% CH + 0.2% CHX. Plaque samples were collected and assayed for *Streptococcus mutans*, *Candida albicans*, and enterococci.
Results: After a non-brushing period, the full-mouth PI and QPI values between the CH and CHX + CH groups differed significantly. A higher PI score at sampling sites was seen in the CH group, but no significant differences were observed between groups. The *S. mutans* and *C. albicans* levels were statistically significant in each group on Days 0 and 4. Differences of *C. albicans* levels between groups were found to be significant; however, no statistical differences were obtained for *S. mutans* or enterococci levels among the groups at the various time intervals.
Conclusion: We conclude that further investigations are needed to evaluate the potential value of CH as an effective antiplaque mouth rinse.

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Introduction

Dental plaque is the primary etiologic factor of periodontal diseases. The removal of bacterial biofilms from tooth surfaces and plaque control are the main goals of the prevention and treatment of periodontal diseases.

In addition to mechanical plaque control, the use of different antiseptic compounds in mouth rinse formulations can complement or replace mechanical removal. Clinical studies showed that many of these antimicrobial agents have inhibitory effects on plaque and gingivitis compared to negative controls or a placebo, in the absence of tooth brushing.[1,2]

In most cases, during phase-I therapy, clinicians recommend that patients use an antimicrobial agent to reduce plaque accumulation. A variety of antimicrobial agents have been studied for their ability to prevent dental plaque formation and inhibit the development of gingivitis.[3–7] These microbial agents include metal salts (tin fluoride, zinc, and copper),[8] essential oils,[9] phenols,[10] fluorides (sodium fluoride and stannous fluoride),[11] bisbiguanides (chlorhexidine),[12] sanguinarine, oxygenating agents etc.

One of the most frequently studied antiplaque agents, chlorhexidine digluconate (CHX), has been used for more than three decades for both prevention and therapy of periodontal diseases owing to its bactericidal and bacteriostatic activities.[3,13,14] CHX at a 0.2% concentration can be considered the gold standard for its clinical efficacy in chemical plaque control.[2,15,16] CHX has broad antibacterial activities, with very low toxicity and a strong affinity for epithelial tissues and mucous membranes. Despite great benefits, the application of CHX is limited to short-term use because of some undesirable side-effects such as brown staining of the teeth and tongue, an unpleasant taste, enhanced supragingival calculus formation, and, rarely, painful desquamations of the oral mucosa, all of which have led to the search for new formulations.[17–19]

Chitosan (CH), a natural polysaccharide obtained by the deacetylation of N-acetyl glucosamine, has received much more attention as a chemical agent for mouthwashes that provide clinical benefits for plaque control.[20–23] In addition to its favorable properties, such as non-toxicity, biocompatibility, and biodegradability, CH has an extended retention time on the oral mucosa. Moreover, CH itself has an antimicrobial activity.[20,24–26] Recent studies have shown that chitosan has an *in vitro* antibacterial effect on *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*.[25,27,28] It has also been reported that low-molecular-weight chitosan prevents the adsorption of *S. mutans* onto hydroxyapatite.[25,27,28] As a bioadhesive polymer, CH provides an extended retention time on the oral mucosa. Previous studies have shown that a chlorhexidine (CHX)/CH combination has a synergistic antiplaque effect, based on the bioadhesive property of CH.[20–24,29,30] Despite these potent antibacterial and antiplaque properties, the application of CH as a chemical agent for mouthwashes or ices is limited because of its insolubility in water and incompatibility with mouthwash and dentifrice formulations. However, few studies have shown the antibacterial effects of different formulations of water-soluble CH in the dental field.[26,31]

This clinical study aimed to investigate the clinical efficacy of a water-soluble form of CH in a mouth rinse to act against plaque regrowth. Other objectives of this investigation were to determine its microbiological effects during a 4-day period of *de novo* plaque formation and to compare those with CHX.

Materials and methods

Study population

In total, 36 periodontally healthy participants (16 females and 20 males; with a mean age of 21.82 $\pm$ 0.57 years) were included in the study. The study was conducted by the Department of Periodontology at the Dental Faculty of Gazi University, Ankara, Turkey. This study design was approved by the Ethical Committee of the Faculty of Dentistry, Gazi University. After screening for suitability, all participants who fit the criteria volunteered, received verbal and written descriptions of the study design, and signed informed consent forms.

Study design

This randomized clinical study was conducted as a parallel-group design and was performed in a double-blinded manner.

Each volunteer had to fulfill the following inclusion criteria: (i) be in good general health; (ii) have undergone no antibiotic treatment during the last 6 months; (iii) be taking no regular medication containing anti-inflammatory compounds; (iv) not be using tobacco products; (v) have no regular use of oral antiseptics; (vi) have a minimum of 24 teeth and five teeth in each quadrant; (vii) have no fixed or removable prostheses, or orthodontic appliances; and (viii) have no signs of periodontal disease. Those reported to be allergic to CHX or CH derivates were not allowed to participate. Participants were comprised of non-smokers.

The study protocol is summarized in Fig. 1. Before the experimental phase, each participant received oral professional prophylaxis to remove all plaque, calculus, and stains from the teeth. This was performed using hand instruments and rotating brushes with polishing paste, and was repeated twice a week for a 2-week period. Participants were provided with a kit containing dental floss, a standard toothbrush, and conventional toothpaste for oral hygiene, and were instructed to use it after every meal until the next visit. Then, participants were asked to abstain from all mechanical plaque-control measures, but rinsed with one of three mouth rinses for the following 4 days.

Mouth rinsing was performed twice a day (after breakfast and in the evening), for 60 seconds with 10 mL of the assigned product. Rinsing with water for 30 minutes after this procedure was not allowed. Written instructions explaining how to use the mouth rinses were provided. The following mouth rinse preparations were tested: Group 1 used a CHX solution (0.2% chlorhexidine digluconate); Group 2 used a 2% CH solution; Group 3 used a 0.2% CHX/2% CH combination.

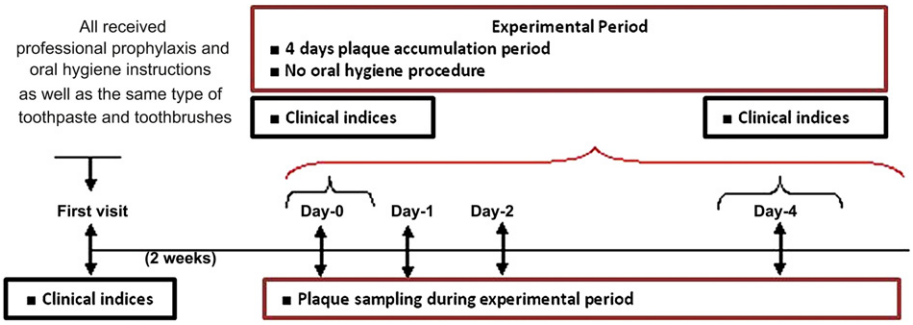

Figure 1 Study protocol.

Participants were divided randomly into the three groups described above. All participants were instructed to refrain from using any other form of oral hygiene and not to change their dietary habits during the course of the study. Following the 4-day period of no oral hygiene, participants resumed optimal mechanical plaque control.

Preparation of the rinse formulations

Water-soluble CH (Protasan UP CL213, Novamatrix, Norway) with molecular weight of 272 kDa and a deacetylation degree of 84% was used to prepare the solutions at a 2% concentration (weight/volume) in distilled water. CHX at 0.2% was incorporated into the CH solutions.[24,31] All study products were blinded. All three mouth rinses were delivered in brown bottles. The bottles were labeled A, B, and C by an investigator: bottle A contained 0.2% CHX, bottle B contained 2% CH, and bottle C contained the 0.2% CHX/2% CH combination.

Clinical assessments

Whole-mouth recordings in each participant served as a basis for the clinical periodontal diagnosis. The following clinical parameters (in sequential order) were recorded at the baseline, on Day 0, and after 4 days of no oral hygiene: a plaque index (PI),[32] gingival index (GI),[33] Quigley—Hein plaque index (QPI), and probing depth (PD). All clinical parameters were measured with a Goldman—Fox Williams probe calibrated in millimeters at six sites per tooth (mesio-, mid-, and distobuccal, and mesio-, mid-, and distopalatal) according to the criteria given below.

The PI system score criteria are as follows: 0 indicates no plaque; 1 indicates a film of plaque adhering to the free gingival margin and adjacent area of the tooth; 2 indicates moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin could be seen with the naked eye; and 3 indicates an abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

The GI system score criteria are as follows: 0 indicates normal gingiva; 1 indicates mild inflammation, a slight change in color, and slight edema, with no bleeding on probing; 2 indicates moderate inflammation redness, edema, and glazing, with bleeding on probing; and 3 indicates severe inflammation, marked redness, edema with ulceration, and a tendency for spontaneous bleeding.

Plaque scoring by the QHI is as follows: 0 indicates no plaque; 2 indicates separate flecks of plaque at the cervical margin of the tooth; 3 indicates a thin continuous band of plaque (up to 1 mm) at the cervical margin of the tooth; 4 indicates a band of plaque wider than 1 mm but covering less than one-third of the crown of the tooth; 5 indicates plaque covering at least one-third but less than two-thirds of the crown of the tooth; and 6 indicates plaque covering two-thirds or more of the crown of the tooth.

All examinations were performed by a single experienced dental examiner (Duygu Boynueğri) who was blinded with respect to the mouth rinses. First premolars were used to evaluate clinical parameters and microbiological sampling because they were more accessible, and such sites are less prone to saliva contamination. Third molars were excluded from the analysis.

Microbiological sampling and analysis

After the sampling sites had been dried and isolated with cotton rolls, supragingival plaque was sampled from each patient with a sterile curette. Samples were obtained from the following surfaces of teeth 14, 24, 34, and 44: mesiobuccal on Day 0; distobuccal on Day 1; mesiopalatinal on Day 2; and distopalatinal surfaces on Day 4 to avoid disturbing plaque regrowth. Plaque samples were collected from different surfaces of each tooth at various time intervals. The different test sites on each tooth during the experiment period were used to clarify total bacterial counts to assess the antibacterial activity of the chitosan mouth rinse. This selection was made owing to standardization of clinical and microbiological evaluation of plaque compositions.

Immediately after removal, plaque samples were pooled and transferred to an Eppendorf tube containing 1 mL of reduced transport fluid (RTF). RTF was prepared to transport plaque samples to a microbiology laboratory. The composition of the RTF/L was as follows: 75 mL of stock saline solution A (containing 0.6% K_2HPO_4); 75 mL of stock saline solution B (containing 1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, and 0.25% $MgSO_4$); 10 mL 0.1 M EDTA; 5 mL 8% Na_2CO_3; 20 mL 1% dithiothreitol; and 814 mL distilled water. This medium was sterilized using a membrane filter. To collect samples, all transport media were dispensed in 1 mL samples in Eppendorf tubes. Trypticase yeast-extract cysteine sucrose bacitracin (TYCSB) selective medium for *S. mutans*, Saboraud dextrose agar (SDA) for *C. albicans*,

and 5% sheep blood agar (SBA) with 6.5% NaCl for *Enterococcus* spp. were used to recover microorganisms from the dental plaque samples. The composition of the TYCSB/L medium was as follows: 15 g casein; 0.2 g L-cysteine; 5 g yeast extract; 0.1 g Na_2CO_3; 1 g NaCl; 2 g Na_2HPO_4; 2 g $NaHCO_3$; 20 g Na acetate; 200 g sucrose; and 15 g agar. The medium was autoclaved at 115°C for 15 minutes, and after cooling to 55°C, 200IE bacitracin was incorporated. Within an hour, samples were transferred to a microbiology laboratory, and glass beads were added to the Eppendorf tubes containing dental plaque and vortexed for 1 minute at full speed to homogenize the samples. Twenty microliters of the homogenized suspension was plated on TYCSB, SDA, and SBA plates. The SDA and SBA plates were incubated aerobically at 37°C for 48 hours, and the TYCSB plates were incubated in an atmosphere of 7% CO_2 at 37°C for 5 days. After incubation, colonies on the plates were counted, and results are presented as colony-forming units (CFU)/mL.

Statistical analysis

Statistical analysis of the clinical and microbiological data collected was performed using the statistical package, SPSS version 16.0 (SPSS for Windows, Chicago, IL, USA). All values were reported as the mean and standard deviation (S.D.). Repeated-measures analysis of variance (ANOVA) was used to evaluate the study data. Pearson rank correlations were used to analyze correlations of clinical periodontal variables and microbiologic parameters. Each point on the graphs shows the ID number of the person whose variable value is an outlier of the distribution. In all statistical evaluations, 0.05 was taken as the cutoff for the level of significance.

Results

All participants completed the study with no complications.

Results of full-mouth clinical indices from all groups are presented in Table 1. There were no significant differences in clinical parameters between groups at the baseline. PI values ascertained on Days 0 and 4 were found to significantly differ ($P < 0.05$) in each group. The CHX + CH combination group showed the lowest PI scores on Day 4. No significant differences in QPI scores were observed in any groups during the experimental period. However, the 2% CH group showed higher QPI values on Days 0–4 than the others groups. Variations in PI and QPI values on days 0–4 were found to significantly different between the 2% CH and CHX + CH groups ($P < 0.05$). In each group, full-mouth GI scores showed significant differences between measurements ($P < 0.05$). However, no significant differences were observed in GI values between groups at any measurement time ($P > 0.05$). When comparing groups, GI scores were higher in the CHX + CH group than the others, but this was not statistically significant.

For sample site clinical observations, changes in PI scores of sampling sites are shown in Fig. 2. At sampling sites, PI scores were observed to significantly differ in each group at various time intervals ($P < 0.001$). On Day 4, higher PI scores were obtained in both the 2% CH and CHX + CH-combination groups. However, these differences between groups were not statistically significant ($P > 0.05$). GI scores at sample sites at the measurement times are shown in Fig. 3. Differences in GI scores were found to be significant in each group ($P < 0.001$). However, the 2% CH group had a higher GI score than the CHX and CHX + CH combination groups on Day 4. These differences were not statistically significant ($P > 0.05$). Values of the QPI from sampling sites are presented in Fig. 4. Significant differences were observed in QPI values between Days 0 and 4 in each group ($P < 0.05$). QPI scores were not found to significantly differ

Table 1 Full-mouth PI, QPI, GI and PD values for all groups at different time intervals.

Group	Visit	PI[a]	QPI[a]	GI[a]
0.2% CHX	Day 0	0.03 (0.02)	0.41 (0.10)	0.02 (0.02)
$n = 12$	Day 4	0.49 (0.18)	0.66 (0.38)	0.42 (0.28)
2% CH	Day 0	0.03 (0.01)	0.83 (0.19)	0.02 (0.01)
$n = 12$	Day 4	0.66 (0.17)[b]	0.87 (0.22)[c]	0.54 (0.27)
CHX + CH	Day 0	0.06 (0.03)	0.41 (0.14)	0.04 (0.02)
$n = 12$	Day 4	0.37 (0.13)[b]	0.50 (0.36)[c]	0.59 (0.31)

CH = chitosan; CHX = chlorhexidine; GI = gingival index; PI = plaque index; QPI = Quigley–Hein plaque index.

[a] Normally distributed data are expressed as means (standard deviations). Repeated ANOVA test for comparisons.

[b] PI: difference between CH and CHX + CH groups was statistically significant, $P = 0.019$ ($P < 0.05$).

[c] QPI: difference between CH and CHX + CH groups was statistically significant, $P = 0.026$ ($P < 0.05$).

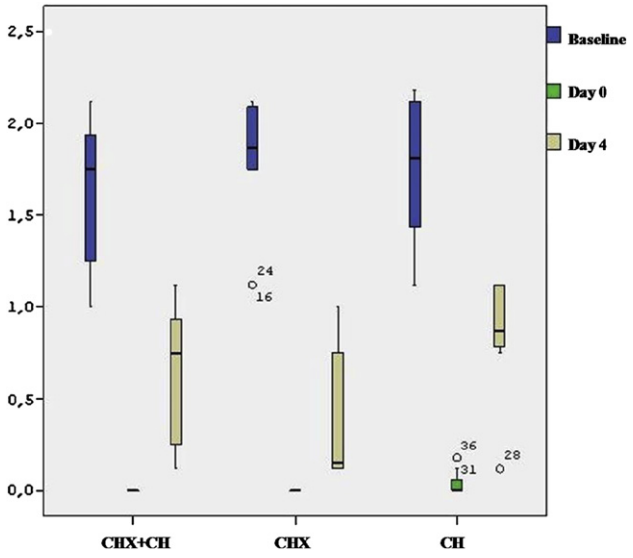

Figure 2 The values of PI in sample sites for each group. The data are expressed as means and standard deviations. B = baseline; CH = chitosan; CHX = chlorhexidine; CHX + CH = chlorhexidine + chitosan; PI = plaque index. Difference between B and Day 0 in each group was statistically significant ($P < 0.05$). Difference between B and Day 4 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 4 in each group was statistically significant ($P < 0.05$). Difference between groups at time intervals was not statistically significant ($P > 0.05$).

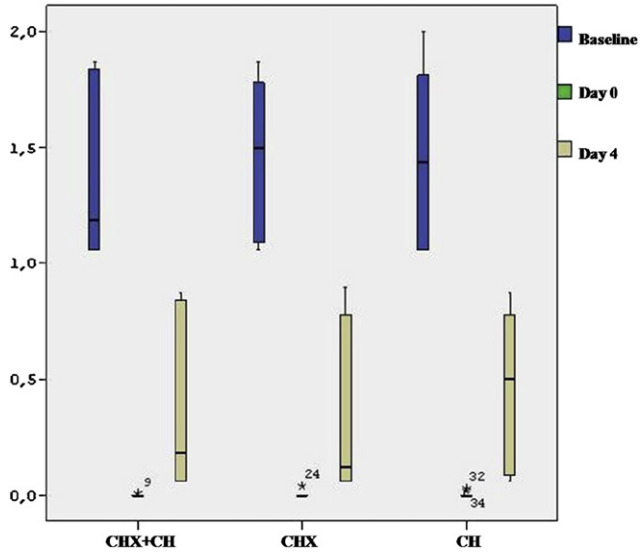

Figure 3 The values of GI in sample sites for each group. The data are expressed as means and standard deviations. B = baseline; CH = chitosan; CHX = chlorhexidine; CHX + CH = chlorhexidine + chitosan; GI = gingival index. Difference between B and Day 0 in each group was statistically significant ($P < 0.05$). Difference between B and Day 4 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 4 in each group was statistically significant ($P < 0.05$). Difference between groups at time intervals was not statistically significant ($P > 0.05$). *Represents the samples which are outliners in the dataset.

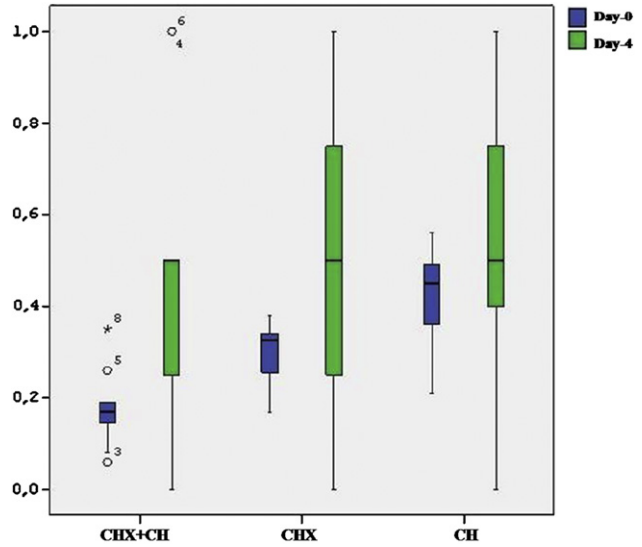

Figure 4 The values of QPI in sample sites for each group. The data are expressed as means and standard deviations. CH = chitosan; CHX = chlorhexidine; CHX + CH = chlorhexidine + chitosan; QPI = Quigley—Hein plaque index. Difference between Day 0 and Day 4 in each group was statistically significant ($P < 0.01$). Difference between groups at time intervals was not statistically significant ($P > 0.05$). *Represents the samples which are outliners in the dataset.

between groups, but the 2% CH group had a higher level than the CHX + CH-combination group on Day 4 ($P > 0.05$).

For microbiological observations, results from plaque samples collected are presented in Figs. 5 and 6 for changes in *S. mutans* and *C. albicans* levels, respectively. Changes in *S. mutans* and *C. albicans* levels were statistically significant in each group on Days 0 to 1 ($P < 0.05$), Days 0 to 2 ($P < 0.05$), and Days 0 to 4 ($P < 0.001$). When comparing the three groups, a higher *S. mutans* amount was obtained in the CHX group on Day 0, in the CHX + CH combination group on Days 0 and 1, and in 2% CH group on Day 0, whereas no statistical differences were observed in *S. mutans* amounts between groups at any time interval ($P > 0.05$). The lowest *C. albicans* amounts were obtained in the CHX group for all measurements, and the 2% CH group had a higher *C. albicans* amount on Day 0. On all experiment days, the amount of *C. albicans* was significantly reduced in all groups. Differences between groups were found to be significant on Day 0 ($P < 0.001$). No differences were observed among all groups on Day 4. *Enterococcus* spp. was not found in any clinical plaque samples during the experimental period. No correlation was found between clinical indices and *S. mutans*, *C. albicans*, or *Enterococcus* spp. in any group during the experimental period. A positive correlation was observed between PI and GI scores in the CHX ($r = 0.665$, $P < 0.05$), CHX + CH ($r = 0.833$, $P < 0.05$), and 2% CH ($r = 0.928$, $P < 0.05$) groups on Day 0 in experiment. No correlation was found between clinical indices on Days 0 and 4 in any group.

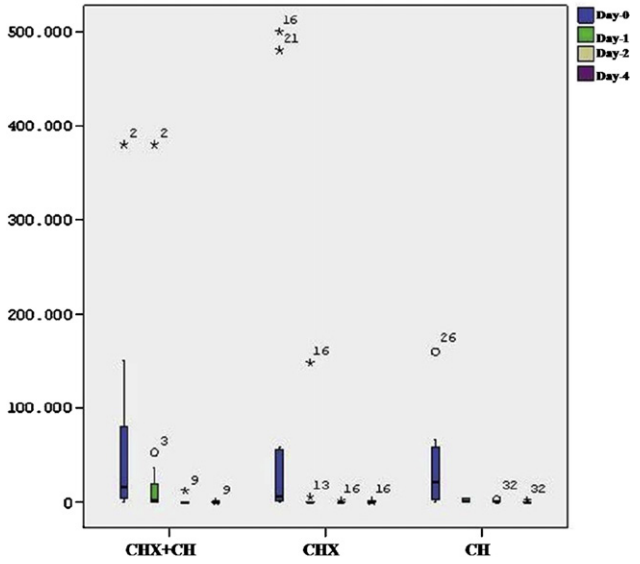

Figure 5 The values of SM in sample sites for each group. The data are expressed as means and standard deviations. CH = chitosan; CHX = chlorhexidine; CHX + CH = chlorhexidine + chitosan; SM = *S. mutans*. Difference between Day 0 and Day 1 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 2 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 4 in each group was statistically significant ($P < 0.001$). Difference between groups at time intervals was not statistically significant ($P > 0.05$). *Represents the samples which are outliners in the dataset.

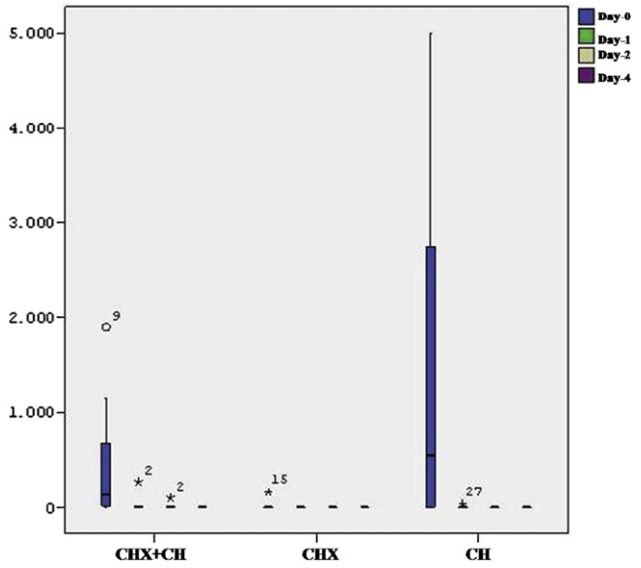

Figure 6 The values of CA in sample sites for each group. The data are expressed as means and standard deviations. CA = *C. albicans*; CH = chitosan; CHX = chlorhexidine; CHX + CH = chlorhexidine + chitosan. Difference between Day 0 and Day 1 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 2 in each group was statistically significant ($P < 0.05$). Difference between Day 0 and Day 4 in each group was statistically significant ($P < 0.05$). Difference between CHX and CH at time intervals was statistically significant ($P < 0.01$). *Represents the samples which are outliners in the dataset.

Discussion

The aim of this study was to evaluate and compare the antiplaque effects of a 60 second rinse with 0.2% CHX and the 2% CH/CHX combination during 4 days (96 hours) of plaque accumulation.

Results of this study indicated that the mouth rinses we evaluated demonstrated a range of inhibitory effects on the test bacteria. These conclusions were supported by the clinical parameters and microbiologic outcomes.

In the present study, when comparing gingival indices, the lowest scores were obtained by rinsing with CHX + CH after 96 h of the plaque accumulation period ($P < 0.001$), suggesting that the combination treatment offered a better antiplaque effect than CHX alone. With regard to plaque accumulation for 4 days, although the PI revealed that the combination treatment provided a better antiplaque activity, the difference was not significant. As expected, for probing pocket depths, no significant alterations were detected. This observation can be explained by the short experimental period and patient selection. However, when the GI scores were compared, the best antiplaque effect was obtained with the use of CHX + CH combination treatment ($P < 0.001$).

Several *in vitro* and *in vivo* studies have proved the long-term efficacy of CHX mouth rinses,[3,34,35] and 0.2% CHX has been accepted as the gold standard as it shows bacteriostatic action for 12 hours.[36] In order to reduce local side effects of CHX, the use of CHX mouth rinses of lower concentrations was considered, and decreased side effects were reported.[17,18] As for plaque inhibition, no differences were observed between 0.1%, 0.12%, and 0.2% CHX mouth rinses.[13,37] However, it has been suggested in one study that evaluated 0.1% CHX mouth rinse, that the degree of CHX inactivation was caused by its formulation.[38] Concentration of 0.12% CHX appeared to be as effective as 0.2%, if the volume of the rinse was increased to 15 mL.[4] The optimal dose of CHX is generally considered to be a regime of 20 mg twice a day,[18,39] which balances efficacy against local side effects and user acceptability. In order to reach an optimal dose, we used 10 mL of 0.2% CHX twice a day in our study design. In a recent study. Charles et al[7] evaluated the antiplaque effectiveness of a CHX and essential oil mouth rinse. All participants used one of the two mouth rinses as an adjunct to their usual mechanical oral hygiene procedures for 6 months. After 6 months, both of the mouth rinses showed comparable antiplaque activities. Similarly in this study, we compared 0.2% CHX to 2% CH, and after 4 days of a plaque accumulation period, no significant differences were observed with regard to PIs or GIs, suggesting that CH may be an alternative chemical agent for managing patients who show side effects associated with CHX. These results might be explained by the favorable bioadhesive properties of CH and its good retention on oral surfaces.

CHX was used in different concentrations and in different formulations. Van Strydonck et al[40] evaluated two commercial CHX mouth rinses (0.12% CHX in a non-alcohol base with 0.05% cetyl pyridinium chloride (Cpc) vs. 0.2% CHX in an alcohol base). After 3 days of a plaque accumulation period, there was no significant difference in plaque accumulation between the two groups. In our study comparing the antiplaque effects, the combination of CHX + CH was slightly more effective than either CHX or CH alone, but the difference was not significant. However with regard to GI scores, the combination of CHX + CH showed the best antiplaque effect, and the difference was statistically significant.

So far, there have only been a few studies reporting the effects CH and CHX combinations on oral microorganisms. Giunchedi et al[20] evaluated CHX buccal tablets prepared using drug-loaded CH microspheres. Combining CH microspheres as controlled drug delivery systems with CHX not only prolonged the release of the drug in the oral cavity but also improved the antimicrobial activity of CHX. In a recent study, Decker et al[21] evaluated CHX on plaque combination to improve antiplaque strategies. In that study, CHX (0.1%) was used as the positive control, saline was the negative control, and two CH derivates together with their CHX combination were attached to *Streptococci sanguis* for 2 minutes. In their results, the CHX + CH combination was stronger than CHX alone, because it united the bioadhesive properties of CH with the antibacterial activity of CHX which resulted synergistically in a superior antiplaque effect to CHX alone. In our study, we evaluated *S. mutans* levels during 96 h of a non-brushing period. The results from plaque samples collected revealed that the reduction in *S. mutans* was more pronounced when using CHX + CH, but this reduction did not significantly differ when the groups were compared. On Day 1, *C. albicans* was significantly lower in the CHX group compared to the 2% CH

group. During the experimental period, the CHX + CH combination showed higher *C. albicans* levels; this difference did not reach statistical significance.

This 4-day randomized clinical trial demonstrated that all of the treatment modalities had comparable antiplaque activities. The promising outcomes of this study could allow CH to be considered as a mouth rinse either alone or in combination with CHX. The precise antibacterial mechanism of CH is still unknown. Further studies will help optimize CH formulations either alone or combined with other antiplaque agents.

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