<table>
<thead>
<tr>
<th>Title</th>
<th>Effect of chitosan rinsing on reduction of dental plaque formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Sano, H; Shibasaki, K; Matsukubo, T; Takaesu, Y</td>
</tr>
<tr>
<td>Journal</td>
<td>Bulletin of Tokyo Dental College, 44(1): 9-16</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10130/339">http://hdl.handle.net/10130/339</a></td>
</tr>
</tbody>
</table>
EFFECT OF CHITOSAN RINSING ON REDUCTION OF DENTAL PLAQUE FORMATION

HIROSHI SANO, KEN-ICHIRO SHIBASAKI, TAKASHI MATSUZUKO* and YOSHINORI TAKAESU*

Oral-Care Research Laboratories, Lion Corporation, 3-7 Honjo, 1-Chome, Sumida-ku, Tokyo 130-8644, Japan
* Department of Epidemiology and Public Health, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Received 22 November, 2002/Accepted for Publication 5 February, 2003

Abstract

The purpose of the present study was to investigate whether the use of a chitosan mouthrinse could be efficacious in reducing plaque and saliva mutans streptococci level. A randomized crossover clinical trial was performed to evaluate the effect of a rinse with 0.5% chitosan for 14 days on plaque formation and mutans streptococci counts in saliva. Twenty-four subjects were randomly assigned either the chitosan rinse or a placebo rinse in addition to their usual oral hygiene procedures. Following the baseline examination, each subject was given a prophylaxis. They were instructed to rinse with 20 ml of the mouthrinse twice daily for 30 seconds. Plaque scores were measured after a 14-day rinsing period, and mutans streptococci counts in saliva were also determined at the start and the end of the each rinsing period. The procedures were repeated with the alternate rinse after a 14-day washout period. Rinsing with 0.5% chitosan was significantly more effective in plaque reduction using the Quigley & Hein Index (chitosan: 1.44, placebo: 1.62, p<0.001) and Plaque Severity Index (chitosan: 0.138, placebo: 0.186, p = 0.003). The mutans streptococci count in saliva was less after the chitosan rinsing ($\chi^2 = 13.51$, p = 0.035) than placebo rinsing. In conclusion, the chitosan rinsing was effective in reducing plaque formation and counts of salivary mutans streptococci after a 14-day rinsing period. These results would appear to warrant further investigation into the potential value of chitosan as an effective anti-plaque agent for use in oral hygiene products.

Key words: Chitosan—Randomized crossover test—Mouthrinse—Plaque formation—Mutans streptococci counts

INTRODUCTION

Dental plaque is believed to be a major etiologic factor in the development of dental caries and gingivitis. Specific types of microorganisms are associated with plaque as it...
develops and matures. Reduction in the accumulation of dental plaque has therefore been the objective of many in vitro and clinical investigations.\textsuperscript{2,3,14,24,35}

The ability of plaque organisms to attach to tooth surfaces or gingival tissues is commonly acknowledged to be a first step in the progression of oral diseases. The nature of this attachment appears to be complex and is ascribed, at least in part, to electrostatic and hydrophobic interactions.\textsuperscript{9,18,23,33} The inhibition of the adsorption of the bacteria to the oral tissues could be a promising approach to preventing their colonization and the progression of disease. This goal may be accomplished, to some extent, by either ionic or nonionic compounds such as alkyl phosphates,\textsuperscript{19} poly-L glutamic acid,\textsuperscript{10} phytate,\textsuperscript{7} sulfolane,\textsuperscript{36} and nonionic propoxylated surfactants,\textsuperscript{8} which modify the hydroxyapatite surface, thus reducing oral bacterial adsorption.

Chitosan is a natural polysaccharide derived from chitin by N-deacetylation. The glucosamine backbone produced by the N-deacetylation process gives chitosan a polycationic character. Because of these positive charges, such derivatives have been receiving much attention as potential candidates for stimulants for regenerating oral soft tissues\textsuperscript{17} and some blood coagulant factors.\textsuperscript{6}

Our previous study demonstrated that five chitin derivatives reduced the adsorption of oral streptococci onto saliva-treated hydroxyapatite and detached these bacteria from hydroxyapatite beads.\textsuperscript{25} Tarsi \textit{et al.}\textsuperscript{31} also demonstrated that low molecular weight chitosan had a selective action against \textit{Streptococcus mutans} adhesive properties and reduced the adsorption to saliva-treated hydroxyapatite beads. They suggested that the mechanism of anti-adherence activity of chitosan involved (1) bacterial surface modifications, (2) alterations in the expression levels of bacterial surface ligands, and (3) chitosan adsorption to host surfaces to change in hydroxyapatite ionic properties.

We also showed that chitosan inhibited the initial adherence of oral streptococci onto the human tooth surfaces \textit{in situ}.\textsuperscript{26} Moreover, it was found that chitosan with molecular mass of 5–6 kDa and a degree of deacetylation of 50–60\% produced a maximal inhibitory effect on both the adsorption and desorption of \textit{S. sobrinus} 6715 in saliva-treated hydroxyapatite binding assay.\textsuperscript{27}

Therefore, the aim of this study was to therefore investigate the inhibitory effect of chitosan with a molecular mass of 6 kDa and a degree of deacetylation of 60\%, on plaque formation and salivary mutans streptococci level.

\section*{MATERIALS AND METHODS}

\subsection*{1. Preparation of chitosan}

The chitosan used in the clinical evaluation was prepared by depolymerization of commercially available chitosan (Fronac NA-500; Kyowa Yushi Kogyo Co., Japan) with sodium nitrite.\textsuperscript{5} A solution of chitosan (200 g) in 2\% acetic acid (10 liter) was treated with a solution of 8.5\% sodium nitrite (100 ml) at 20\°C. The sodium nitrite solution was added in small increments over 30 min with stirring and the mixture was then neutralized. After dialysis against deionized water with tubes having a cut-off value of 1.5 kDa, the watersoluble chitosan was freeze-dried. The molecular mass of this chitosan preparation was determined by the gel permeation chromatography method with N-acetyl-d-glucosamine oligomer and pullulan standards.\textsuperscript{30} We measured the degree of deacetylation of chitosan using the MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride) method.\textsuperscript{32} The molecular mass and degree of deacetylation of chitosan were 6 kDa and 60\%, respectively.

\subsection*{2. Subjects}

The subjects were twenty-four male adults (age range 20–40 years). At the screening visit, individuals were given verbal and written information concerning the study and signed consent forms. All the subjects had good oral hygiene (average plaque score\textsuperscript{22}: 1.42; average PMA index\textsuperscript{28}: 7.2). They had at least 20 teeth without artificial crowns or bridge prosthetics, resin or composite fillings or caries.
Their mean number of decayed, missing, and filled (DMF) teeth was 3.6, and it ranged between 0 and 13. They had not been received any medication in the past one month which might influence plaque accumulation.

3. Rinse formulation

The chitosan mouth rinse formulation in this study had the following composition (expressed as a w/w%); 0.5% chitosan, 15% ethanol, 10% glycerin, 0.008% sodium saccharine, 1% polyoxyethylene hydrogenated castor oil, and 0.5% flavor in deionized water. The placebo rinse was the same as the chitosan mouthrinse without 0.5% chitosan. Because it was a double blind study, the rinse solutions were supplied in identical 350 ml plastic containers marked only with subject codes, and the examiner was unaware of subject assignments.

4. Protocol for clinical evaluation

This study was a randomized, double blind, placebo-controlled crossover design with two rinsing periods of 14 days duration (Fig. 1). The washout time between the rinsing periods was 14 days of self-performed mechanical oral hygiene. After screening, the subjects were randomly assigned to use either the chitosan rinse or the placebo rinse according to a randomized code. Allocation of the respective mouthrinse was through the laboratory personnel, where a sealed code-breaker was kept. A clinician other than the recorder instructed the subjects in the use of the mouthrinses. Prior to commencement of both the first and second treatment period, all subjects underwent a scaling to remove all plaque. Unsupervised rinsing with 20 ml of the mouthrinse was performed for 30 sec in the morning and in the evening after toothbrushing. Each subject was instructed to continue normal hygiene procedures using an assigned toothbrush and a commercially available dentifrice without fluoride. After 14 days of each of the rinse periods, subjects reported to the clinical facility without having rinsed or brushed on that morning. Subject diaries and the return of test bottles, which were weighed to determine the amount of remaining test solution, finally monitored compliance. The entire procedure was repeated after a washout period but subjects were assigned to use the opposite test solution.

5. Plaque assessment

Supragingival plaque was recorded using a disclosing agent at the end of each rinse period according to the criteria of the Quigley & Hein Index (QHI) with scores of 0 to 5. The scoring was made at the buccal and lingual surfaces of all teeth except the third molars. The mean QHI score for each subject was determined by adding the indi-
individual plaque scores (two per tooth) and dividing this sum by the total number of measurements. Mean Plaque Severity Index (PSI) scores were also calculated for each subject by dividing the total number of tooth measurement surfaces (two per tooth) that received numerical QHI scores of either three, four, or five by the total number of measured tooth surfaces in the entire mouth.

6. Measurement of mutans streptococci counts in saliva

Samples of unstimulated whole saliva were collected at the beginning and the end of each rinsing period before plaque assessment. A 0.1 ml aliquot of each saliva sample was placed in a sterile vial containing MSB broth, and the vials were incubated aerobically at 37°C for 24 hours. The number of mutans streptococci was determined macroscopically comparing the degree of cell adherence to the inner surface of the vial with an evaluation chart (range of 1 to 5). The scores in the evaluation chart were set according to the following criteria: score 1 = <10^4 CFU/ml; 2 = 10^4 – 5×10^4 CFU/ml; 3 = 5×10^4 – 10^5 CFU/ml; 4 = 10^5 – 10^6 CFU/ml and 5 = >10^6 CFU/ml15).

7. Statistical analysis

Total and subgroup (buccal and lingual) analyses were performed. For each site, analysis of variance (ANOVA) was used to determine the effects of treatment, period, and subject on both QHI and PSI scores. All data were available, maintaining the orthogonal design, and means did not require adjustment for possible confounding effects of treatments or subjects. Statistical significance was accepted at the 5% probability level.

The difference in the distribution of the mutans streptococci categories between the two treatments was analyzed with the chi-square method. The minimum acceptable level for statistical significance was the 5%

| Table 1 Mean QHI scores (S.E.) and reductions (%) compared to placebo |
|-----------------------------|-------------|------------|-----------------------------|
| % reduction | vs. placebo | p-value |
| chitosan total | 24 | 1.44 | 0.08 | 11.1 | p<0.001 |
| buccal | 24 | 1.09 | 0.08 | 16.7 | p<0.001 |
| lingual | 24 | 1.81 | 0.11 | 7.2 | p = 0.022 |
| placebo total | 24 | 1.62 | 0.08 |  |
| buccal | 24 | 1.31 | 0.09 |  |
| lingual | 24 | 1.95 | 0.12 |  |

Analysis of variance for the total QHI scores showed significant differences between treatments (p<0.001), and subjects (p<0.001) but not between periods (p = 0.461).

| Table 2 Mean PSI scores (S.E.) and reductions (%) compared to placebo |
|-----------------------------|-------------|------------|-----------------------------|
| % reduction | vs. placebo | p-value |
| chitosan total | 24 | 0.138 | 0.02 | 25.8 | p=0.003 |
| buccal | 24 | 0.110 | 0.02 | 32.5 | p<0.001 |
| lingual | 24 | 0.213 | 0.03 | 14.5 | p = 0.185 |
| placebo total | 24 | 0.186 | 0.02 |  |
| buccal | 24 | 0.163 | 0.03 |  |
| lingual | 24 | 0.249 | 0.04 |  |

Analysis of variance for the total PSI scores showed significant differences between treatments (p=0.003), and subjects (p<0.001) but not between periods (p = 0.309).
probability level.

RESULTS

1. Plaque score

All volunteers satisfactorily completed the rinsing regimens without any noted side effects. The mean QHI scores and mean PSI scores for each of the three sites are shown in Tables 1 and 2.

Analysis of variance for total QHI scores showed significant differences between treatments (p=<0.001) and subjects (p=<0.001) but not between periods (p=0.461). Compared to the placebo rinse, the chitosan rinse significantly reduced the mean QHI scores by 11.1% on the total tooth surfaces. Subgroup analysis also indicated that the chitosan rinse significantly reduced the scores by 16.7% on the buccal tooth surfaces and by 7.2% on the lingual surfaces (Table 1).

Analysis of variance for the total PSI scores also showed significant differences between treatments (p=0.003) and subjects (p=<0.001) but not between periods (p=0.309). The chitosan rinse showed a significant reduction of the mean PSI scores by 25.8% on the total and 32.5% on the buccal tooth surfaces compared with the placebo rinse (Table 2). For lingual mean PSI scores, although there was a trend toward less plaque with the chitosan rinse (by 14.5%) compared to the placebo rinse, the difference was not statistically significant (p=0.185).

There were no significant differences between chitosan first rinsing group and placebo first rinsing group for QHI and PSI scores.

2. Salivary bacterial counts

Changes in mutans streptococci counts in saliva from baseline to day 14 are outlined in Table 3. The MSB method revealed a statistically significant difference between the chitosan rinse and the placebo rinse ($\chi^2_{cal} = 13.51$, p=0.035). In the chitosan rinse group, 16 subjects (changes in scores 1, 2 and 3) showed reductions of mutans streptococci counts, whereas, in the placebo group, only 5 subjects (change of score 1 and 2) showed reductions of the bacterial counts.

There was no significant difference between the chitosan first rinsing group and the placebo first rinsing group for changes in mutans streptococci counts in saliva.

DISCUSSION

The effect of a chitosan-containing mouth rinse on reducing plaque formation and mutans streptococci level in saliva was evaluated. We undertook a crossover design that provides considerable power for detecting significant differences with relatively small numbers compared to the usual parallel design of home use studies. The results of the crossover clinical test showed that a treatment with chitosan rinse was more effective in reducing plaque formation and mutans streptococci counts in saliva than the placebo rinse.

The effects of mouth rinse may be confounded by a number of variables including the Hawthorne effect, improved oral hygiene, the influence of a pre-study prophylaxis, and...
possible interactions between the mouth rinse and toothpaste ingredients. The design of the study was not to avoid such factors as much as possible, but rather to show that the product as a whole was significantly better than placebo rinse. Therefore, we attempted to simulate normal home use conditions as far as possible within the restrictions of a clinical trial.

Our previous study demonstrated that chitosan with a molecular mass of 5–6 kDa and a degree of deacetylation of 50–60% is effective in inhibiting of adsorption of \textit{S. sobrinus} 6715 onto saliva-treated hydroxyapatite beads. Therefore, we prepared for the chitosan with a molecular mass of 6 kDa and a degree of deacetylation of 60% to be used as a promising active ingredient in a test mouth rinse.

Findings of the clinical trial showed that the 0.5% chitosan rinse resulted in 11.1% lower mean OHI scores (1.44) than the placebo rinse (1.62). Although it is difficult to make direct comparisons with other experiments because of different protocols, these results are consistent with those in previous rinsing studies, which have shown 10–20% lower mean QHI scores than placebo rinsing when using 0.05–0.1% delmopinol\textsuperscript{4}. Previous evidence has suggested that cationic compounds like chlorhexidine, delmopinol, and chitosan sometimes have a smaller effect on plaque growth than expected when using as adjuncts to toothpastes. Sodium lauryl sulfate, the anionic surface-active agent introduced in toothpaste, is the most frequently used detergent for toothpaste. A possible explanation for relatively small reduction in the plaque score of our subjects brushing with the toothpastes could be the chemical interaction between the chitosan and an anionic forming detergent\textsuperscript{20}, resulting the lack of an additional plaque inhibitory effect.

In this study, the mean difference of QHI scores of 0.18 between chitosan rinse and placebo seemed to be of relatively small magnitude with respect to the clinical significance. However, this might be explained by the relatively short duration of the study and the low plaque forming ability of the subjects, who had average plaque scores at the baseline of 1.42 compared to the previous clinical study\textsuperscript{4}.

We formulated a mouth rinse containing a nonionic surfactant (polyoxyethylene hydrogenated castor oil) as a solvent for a flavor. The cationic groups of chitosan might have been partially inactivated by being incorporated into the surfactant micelles in the test solution. The same phenomenon was reported by Mukasa \textit{et al.}\textsuperscript{10}. They revealed that micelles of polyoxyethylene hydrogenated castor oil reacted with cetylpyridinium chloride, a cationic anti-plaque agent, and extensively reduced the bactericidal effect. Waaler \textit{et al.}\textsuperscript{34} proposed that the nature of the micelles of the detergents that dissolve the active ingredients could be of significant importance. Therefore, formulation of products containing polycationic chitosan must be carefully considered before any judgment concerning their effect is clinically made.

In addition to calculating a QHI score for each subject, a Plaque Severity Index score (PSI) was also calculated. This PSI score permits comparisons of the tooth surfaces from each treatment group that received the most severe QHI scores, that is, scores of 3 and greater. Therefore, the PSI score is a measure of plaque removal efficacy of the “more difficult to brush surfaces” of the teeth\textsuperscript{12}. Table 2 shows a comparison of mean PSI scores for the two rinse groups after 14 days of rinsing. The placebo rinse had a mean PSI score of 0.186, while the chitosan rinse had a mean PSI score of 0.138. Thus, the chitosan rinse decreased plaque 25.8% more from the more difficult brush surfaces of the teeth than the placebo rinse.

The results from this study also showed that daily use of a chitosan rinse significantly reduced the number of mutans streptococci in saliva. The same finding was obtained in our previous study in rats\textsuperscript{29}. The possible mechanism for reduction of plaque formation and mutans streptococci in saliva by chitosan might be a modification of the electrostatic interaction between the bacterial cell surface in saliva and tooth pellicle surface, thereby permitting only a less favorable inter-
action. This electrostatic interaction is usually repulsive due to the fact that, in nature, both the bacteria and the pellicle surface are predominantly negatively charged\textsuperscript{19}. On the basis of the mutans streptococci surface charge and characteristics of chitosan, we can say that chitosan interaction with mutans streptococci cells is electrostatic: polymer chains attach themselves to the negatively charged bacterial cell surface by means of their positively charged groups. If these chains are of a sufficient length to bind more than one cell, bridges are formed between bacterial cells. As soon as the bridging becomes effective, flocs are formed\textsuperscript{1}, and the mutans streptococci cannot colonize the tooth surfaces. There have also been suggestions that bacterial aggregates are removed more easily from the oral cavity than individual bacterial cells\textsuperscript{11,13}. These comments are in line with the clinical effect of chitosan rinsing in this study.

Chitosan showed very little antibacterial activity against the metabolism of plaque bacteria\textsuperscript{30}. Based upon this evidence, the effect of chitosan rinse on the reduction of plaque and mutans streptococci level is considered to derive from inherent polycationic nature.

In conclusion, within the limits of this short-term study, we clearly demonstrated that chitosan rinsing is effective in reducing plaque formation and counts of salivary mutants streptococci. The results would appear to warrant further investigation into the potential value of chitosan as an effective anti-plaque agent for use in oral hygiene products.

ACKNOWLEDGMENTS

The author appreciates the support of Dr. Kenji Kaneko towards the design of this study and the writing of the paper.

REFERENCES


Reprint requests to:
Dr. Hiroshi Sano
Oral-Care Research Laboratories,
Lion Corporation,
1-3-7 Honjo, Sumida-ku,
Tokyo 130-8644, Japan