Original Article

EFFECT OF RINSING WITH PHOSPHORYLATED CHITOSAN ON FOUR-DAY PLAQUE REGROWTH

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Abstract

This clinical investigation examined the effect of phosphorylated chitosan rinsing on plaque development and on the buffering capacity of plaque suspension. Three male adult subjects participated in the trial that was designed as a single blind study. Participants refrained from mechanical oral hygiene procedures during a four-day study and rinsed three times a day with 20 ml of test solutions. A wash-out period of three days was instituted between the placebo and phosphorylated chitosan rinsing period. Clinical evaluation and plaque sampling were performed at the end of each test period. We disclosed plaque accumulations on the buccal upper front teeth with a two-tone disclosing agent to distinguish between newly formed plaque and old plaque. After taking color slides, we then used a computerized image analysis. Tooth areas covered by plaque on the color slides were digitized and expressed as percentages of the tooth area. The buffering capacity of the collected plaque fluid was determined by using a β -titrator. A mouth rinse containing 0.5% phosphorylated chitosan significantly reduced both newly formed plaque areas (red disclosed; $p < 0.001$) and old plaque areas (blue disclosed; $p < 0.01$) compared to a placebo rinsing. However there was no significant difference in the plaque buffering capacity ($p > 0.05$) between the mouth rinse containing 0.5% phosphorylated chitosan and placebo. These findings might suggest that mouth rinse containing phosphorylated chitosan would be effective in reducing plaque formation and have a slight ability to enhance plaque buffering capacity.

Key words: Phosphorylated chitosan—Mouth rinse—Plaque formation—
Buffering capacity

INTRODUCTION

Plaque accumulation is initiated by rapid and selective adherence of oral bacteria on the tooth surface. The process of the adher-

ence is believed to involve electrostatic¹²⁾, hydrophobic⁹⁾, and lectin-like²⁾ interactions. Many researchers have attempted to reveal the mechanism of bacterial adherence on the tooth surface and to develop plaque-reducing

Fig. 1 Experimental design of this clinical study
C, Tooth cleaning; P, Plaque area measurement; B, Measurement of buffering capacity of plaque; PPS, Phosphorylated chitosan

agents based on disrupting the interactions from the electrostatic and the hydrophobic viewpoint. Agents such as poly-aspartic acid, poly-glutamic acid⁶⁾, phytate³⁾, and alkyl phosphates¹¹⁾ inhibit the adsorption of oral bacteria on saliva-coated hydroxyapatite beads by changing the ionic nature of bacterial cell walls and hydroxyapatite surfaces. It has also been reported that hydrophobic bond-disrupting agents such as Li^+ cation, sulfolane²¹⁾, and nonionic propoxylated surfactants⁴⁾ reduce the adherence of oral streptococci to saliva-coated hydroxyapatite beads. However, the clinical effects of these agents have not been examined thoroughly because of lack of safety, unpleasant taste, and incompatibility with conventional ingredients in oral hygiene products.

Chitosan derivatives have been receiving much attention as advanced functional materials in the pharmaceutical, cosmetic and food fields. Their unique properties are partially due to the polycationic nature of their free amino residues. We previously reported that water-soluble chitin derivatives have an inhibitory effect on adsorption of oral streptococci onto hydroxyapatite beads¹³⁾. Additionally low molecular chitosan is reported to have strong buffering capacity (pKa ; 6.5)¹⁵⁾ and cariostatic effects in rats¹⁶⁾.

In this study, we assessed the effect of rinsing with 0.5% phosphorylated chitosan on four-day plaque development and determined whether the rinse would enhance the buffering capacity of plaque fluid by the phosphorylated chitosan retained in the plaque

accumulation.

MATERIALS AND METHODS

1. Phosphorylated chitosan

Phosphorylated chitosan was prepared from commercially available chitosan (Kyowa Yushi Kogyo Co., Tokyo) by the reaction of chitin with phosphorous pentoxide¹⁰⁾. The molecular weight was determined by the GPC method with *N*-acetyl-*D*-glucosamine oligomer and pululan standards¹⁶⁾. Measurement of the degree of deacetylation was carried out by the colloid titration method⁵⁾. The degree of phosphorylation was estimated to be 0.7 by the quantitative analyses for phosphorus in the product. The molecular weight and degree of deacetylation of phosphorylated chitosan were 20,000 and 70%, respectively.

2. Subjects

Three male adults (aged 22–40) participated in this clinical study. All the subjects had a good oral hygiene, (i) showed no signs of periodontitis, (ii) had no extensive dental restorations, and (iii) had not received any systematic antibiotic treatment before or during the experimental period.

3. Study design

The study was designed as a single blind clinical trial (Fig. 1). In the first sequence, all subjects used a placebo rinse. At the start of each sequence of four-day experimental peri-

ods, the participants had their teeth professionally cleaned. They were instructed to perform no oral hygiene procedures during the clinical trials but to rinse with 20 ml of the allocated mouth rinse for one minute after the three meals of the day. The subjects were asked not to change their dietary habits during the course of the study. In the final day of the each sequence, all subjects rinsed with the test solution after lunch and then refrained from eating or drinking anything except water 2 h before the plaque assessment.

4. Test formulations

The following formulations were used. A): Phosphorylated chitosan mouth rinse: a solution containing 10% glycerin, 10% ethanol, 0.04% sodium saccharin, 0.48% nonionic surfactant (polyoxyethylene hydrogenated castor oil), 0.16% peppermint flavor, and 0.5% phosphorylated chitosan. B) Placebo mouth rinse: The solution A without 0.5% phosphorylated chitosan. Each test solution was adjusted to 100 w/w% by adding deionized water. All solutions had the same color and were kept in the same kind of bottle.

5. Plaque assessment

Plaque was disclosed with a two-tone disclosing agent (Dis-plaqueTM)¹⁾ at the end of each rinsing period. Plaque areas were measured on the upper labial front teeth (six surfaces per subject). We took standardized color slides for planimetric analyses¹⁷⁾. The slides were enlarged and projected onto white paper. We determined the areas of the stained plaque on the teeth surfaces by using a graphical tablet and a digitizer attached to a micro-computer. The proportions of red stained plaque areas and blue stained plaque areas on the tooth surface were calculated separately. The tooth area covered by plaque was expressed as percentage of the tooth area.

6. Buffering capacity of plaque

Supragingival plaque was collected from all teeth surfaces at the end of each rinsing period. Collected plaque samples were pooled in two ml of saline solution at 4°C. The buffering

capacity (β value) of 1.5 ml of each sample was determined using β -titrator assembly²⁰⁾ (BETA-1, ST-30A, ADT-1, XYR-2B, TOA Electronics Ltd., Japan). A β value is defined as equal to dCb/dpH , where dCb is the infinitesimal increase in the concentration of added strong alkali (mol/dm^3), and dpH is the resultant pH increase. The plot of β as a function of pH is called the buffer capacity curve (β -pH curve) of a sample solution. The titration assembly consisted of a divider-multiplier solid-state module (BETA-1), a conventional pH titrator (ST-30A), and a magnetically stirred titration vessel equipped with glass electrodes (ADT-1) that can produce β -pH curves of unknown samples on an X-Y recorder (XYR-2B) simultaneously with titration. For a monoprotic acid, this curve is bell-shaped with its center at a pH equal to pK_a . Its maximum height is equal to $0.576C_a$; C_a is the analytical concentration of the acid (mol/dm^3).

A 2 ml of collected plaque sample was mixed with 0.1 N sodium hydroxide and hydrolyzed at 70°C for 30 minutes. The sample protein content was then determined according to the method of Lowry *et al.*⁸⁾ with bovine serum albumin as a standard. Plaque buffering capacity was calculated by dividing the β -value at pH 6.5 in the plaque suspension by the protein content.

7. Statistical analyses

Statistical differences were calculated by the paired *t*-test. Statistical significance was accepted at the 5% probability level.

RESULTS

The three participants satisfactorily completed the two regimens. No side effects occurred during the rinsing period. Typical clinical appearances after plaque disclosure following use of phosphorylated chitosan or placebo are shown in Fig. 2. The mouth rinse containing 0.5% phosphorylated chitosan reduced the mean % of newly formed plaque by 26% after a four-day rinsing period compared to a placebo rinse ($p < 0.001$, Table 1).

Fig. 2

(A) Clinical appearance after plaque disclosure in a subject rinsing with placebo for 4 days.

(B) Clinical appearance of the same subject after rinsing with 0.5% phosphorylated chitosan for 4 days.

Table 1 Mean % of newly formed plaque area after a 4-day rinsing period

group	n	Mean	S.E.	% reduction PPS vs. Placebo	p value
PPS	17	22.7	1.3	26	p<0.001
Placebo	17	30.5	1.6	—	

Table 2 Mean % of old plaque area after a 4-day rinsing period

group	n	Mean	S.E.	% reduction PPS vs. Placebo	p value
PPS	17	2.8	0.9	61	p<0.01
Placebo	17	7.2	1.8	—	

Table 3 Buffering capacity of the plaque suspension after a 4-day rinsing period

group	n	Mean*	S.E.	% increment PPS vs. Placebo	p value
PPS	3	3.0	1.1	36	p>0.05
Placebo	3	2.2	1.1	—	

(*; $\beta/\mu\text{g}$ protein)

The phosphorylated chitosan mouth rinse also decreased the mean % of the old plaque significantly by 61% compared to the placebo rinse ($p<0.01$, Table 2).

The mouth rinse containing phosphorylated chitosan enhanced the buffering capacity of the plaque suspensions by 36%, but the difference between the phosphorylated chito-

san and placebo was not significant ($p>0.05$, Table 3).

DISCUSSION

Short-term plaque regrowth studies are most commonly used to estimate oral hygiene

products. The study design usually uses the measurement of plaque regrowth under the influence of the test agent from a zero plaque baseline. The method avoids the confounding influences of toothbrushing, which is highly variable between individuals.

Indices used today to measure the accumulation of dental plaque are usually based on visual estimations of the plaque-covered area of the tooth surface. However, there is a need for more sensitive and objective indices to be used in clinical studies. Planimetric techniques giving measurements in a ratio scale have therefore been used recently to measure the plaque area.

In this study, we used a two-tone disclosing agent to distinguish thin plaque from thick, accumulated plaque. The thin plaque area corresponds to the newer plaque deposits and was relatively spread on the teeth surface. The thick, accumulated plaque was old plaque and had a high degree of architectural organization; it tended to strongly deposit at marginal sites of the tooth. It is believed that removal of marginal plaque deposits can be of critical importance in preventing dental caries and gingivitis^{7,14)}.

The results obtained in this study indicated that 0.5% phosphorylated chitosan mouth rinse suppressed the both new and old plaque formations after a four-day rinsing period compared to the placebo rinsing. Our previous study showed that phosphorylated chitosan strongly binds hydroxyapatite beads and inhibits adsorption of oral streptococci to saliva-coated hydroxyapatite beads¹³⁾. These results indicate that the inhibitory effect of phosphorylated chitosan on the plaque regrowth is partially due to a reduction of the initial attachment of oral bacteria. It was also suggested by Olsson *et al.*¹¹⁾ that an inorganic phosphate binds with high affinity to hydroxyapatite and might be an effective cleansing agent, displacing proteins and other organic substances from the enamel surfaces. Phosphorylated chitosan contains both phosphate groups and amino residues in the same glucosamine units. These amphoteric units might interfere with the binding of a variety of

plaque bacteria, which may be adsorbed to the enamel surface by electrostatic interactions. Further study will be needed to estimate the inhibitory effect by phosphorylated chitosan on the aggregation of oral bacteria and on the production of sucrose-dependant extracellular polysaccharide.

The buffering capacity of saliva is important for maintaining the pH and mineral dynamics of the enamel surface, counteracting dietary induced pH changes and neutralizing acids produced by oral bacteria. Phosphorylated chitosan has a buffering capacity in the neutral range of pH (pKa; 6.5) (unpublished data), similar to that of human saliva. Shibasaki *et al.*¹⁵⁾ reported that low molecular chitosan, which had a buffering capacity (pKa; 6.5), similar to that of phosphorylated chitosan, effectively inhibits a pH-fall in human dental plaque after application of glucose solution *in situ*. We assessed whether phosphorylated chitosan would enhance the buffering capacity of a plaque fluid after a four-day rinsing period by a direct buffering action of the phosphorylated chitosan. Our results showed that 0.5% phosphorylated chitosan mouth rinse tended to increase the buffering capacity of the plaque fluid compared to placebo rinsing but not significantly ($p > 0.05$). It has been reported that the diffusion rates of carbohydrates through dental plaque are inversely related to their molecular weight¹⁸⁾. The electric nature of the carbohydrates could also be a considerable factor in their permeability into a dental plaque¹⁹⁾. Phosphorylated chitosan has a relatively higher molecular weight (20,000) and contains bulky phosphate groups. These characters do not seem to facilitate the permeation of phosphorylated chitosan rapidly and efficiently into the plaque matrix during the four-day rinsing period. The influence of the molecular weight and the phosphate group on the diffusion in dental plaque is under investigation.

In conclusion, these findings might suggest that rinsing with phosphorylated chitosan would be useful for reducing dental plaque formation and subsequent dental caries development.

REFERENCES

- 1) Block, P.L., Lobene, R.R. and Derdivanis, J.P. (1972). A two-tone dye test for dental plaque. *J Periodontol* **43**, 423–426.
- 2) Gibbons, R.J. (1984). Adherent interactions which may affect microbial ecology in the mouth. *J Dent Res* **63**, 378–385.
- 3) Hoppenbrouwers, P.M.S., Borggreven, J.M.P.M. and van der Hoeven, J.S. (1984). Adherence of oral bacteria to chemically modified hydroxyapatite. *Caries Res* **18**, 1–6.
- 4) Humphries, M., Jaworzyn, J.F., Cantwell, J.B. and Eakin, A. (1987). The use of non-ionic ethoxylated and propoxylated surfactants to prevent the adhesion of bacteria to solid surfaces. *FEMS Microbiol Lett* **42**, 91–101.
- 5) Kina, K., Tamura, K. and Ishibashi, N. (1974). Use of 8-anilino-1-naphthalene sulfonate as a fluorescent indicator in colloid titration. *Japan Analyst* **23**, 1082–1083. (in Japanese).
- 6) Lamberts, B.L., Pederson, E.D. and Simonson, L.G. (1985). The effect of basic and acidic synthetic polypeptides on the adherence of the oral bacteria, *Streptococcus mutans* and *Streptococcus sanguis*, to hydroxyapatite. *Archs Oral Biol* **30**, 295–298.
- 7) Listgarten, M.A. (1988). The role of dental plaque in gingivitis and periodontitis. *J Clin Periodontol* **15**, 485–487.
- 8) Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951). Protein measurement with the folin phenol reagent. *J Biol Chem* **193**, 265–275.
- 9) Nesbitt, W.E., Doyle, R.J. and Tayler, K.G. (1982). Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxyapatite. *Infect Immun* **38**, 637–644.
- 10) Nishi, N., Nishimura, S., Ebina, A., Tsutsumi, A. and Tokura, S. (1984). Preparation and characterization of water-soluble chitin phosphate. *Int J Biol Macromol* **6**, 53–54.
- 11) Olsson, J., Carlen, A. and Holmberg, K. (1991). Inhibition of *Streptococcus mutans* adherence to hydroxyapatite with combinations of alkyl phosphates and nonionic surfactants. *Caries Res* **25**, 51–57.
- 12) Rølla, G., Robrish, S.A. and Bowen, W.H. (1977). Interaction of hydroxyapatite and protein-coated hydroxyapatite with *Streptococcus mutans* and *Streptococcus sanguis*. *Acta Path Microbiol Scand Sec B* **35**, 341–346.
- 13) Sano, H., Matsukubo, T., Shibasaki, K., Itoi, H. and Takaesu, Y. (1991). Inhibition of adsorption of oral streptococci to saliva treated hydroxyapatite by chitin derivatives. *Bull Tokyo dent Coll* **32**, 9–17.
- 14) Shaw, J.H. (1987). Causes and control of dental caries. *N Eng J Med* **316**, 996–1004.
- 15) Shibasaki, K., Sano, H., Matsukubo, T. and Takaesu, Y. (1994). Effects of low molecular chitosan on pH changes in human dental plaque. *Bull Tokyo dent Coll* **35**, 33–39.
- 16) Shibasaki, K., Matsukubo, T. and Takaesu, Y. (1995). Cariostatic effect of low molecular weight chitosan in rats. *Shikwa Gakuho* **95**, 1–17. (in Japanese)
- 17) Söder, P.-Ö., Jin, L.J. and Söder, B. (1993). Computerized planimetric method for clinical plaque measurement. *Scand J Den Res* **101**, 21–25.
- 18) Tatevossian, A. (1979). Diffusion of radiotracers in human dental plaque. *Caries Res* **13**, 154–162.
- 19) Tatevossian, A. and Newbrun, E. (1983). Diffusion of small ionic species in human saliva, plaque fluid and plaque residue *in vitro*. *Archs oral Biol* **28**, 109–115.
- 20) Tsuji, K. (1982). β -Titration, a device for direct recording of buffer capacity curves: I, Theory and apparatus. *Agric Biol Chem* **46**, 677–682.
- 21) Zhang, X.-H., Rosenberg, M. and Doyle, R.J. (1990). Inhibition of the cooperative adhesion of *Streptococcus sanguis* to hydroxyapatite. *FEMS Microbiol Lett* **71**, 315–318.

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