Table 3 Reasons for visiting the dental office, reasons for selecting the dental office and information for selecting the dental office

(A)

Reason for visiting clinic	Regular visitors		Infrequent visitors		No answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
Tooth or gum disease	892	34.3	1677	64.4	35	1.3	2604	100 (50.7)
Denture problem	178	28.1	440	69.4	16	2.5	634	100 (12.4)
Health care	1146	78.9	295	20.3	11	0.8	1452	100 (28.3)
Trauma	13	41.9	18	58.1	0	0	31	100 (0.6)
Others	135	37.4	218	60.4	8	2.2	361	100 (7.0)
No answer	18	36.0	12	24.0	20	40.0	50	100 (1.0)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(B)

Reason for selecting dental office	Regular visits		Infrequent visits		No answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
Location	346	34.6	636	63.5	19	1.9	1001	100 (19.5)
Personal dental office	1150	58.0	806	40.6	27	1.4	1983	100 (38.6)
Technical competence of dentist	296	46.1	335	52.2	11	1.7	642	100 (12.5)
No waiting time	16	40.0	23	57.5	1	2.5	40	100 (0.8)
Explanation of treatment	59	49.6	60	50.4	0	0	119	100 (2.3)
Office open late or on holidays	0	0	4	100.0	0	0	4	100 (0.1)
Recommendation from associate	429	37.3	705	61.4	15	1.3	1149	100 (22.4)
Recommendation from doctor*	53	55.2	43	44.8	0	0	96	100 (1.9)
No answer	33	33.7	48	49.0	17	17.3	98	100 (1.9)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(C)

Information for selecting dental office	Regular visitors		Infrequent visitors		No answer		Total	
	n	%	n	%	n	%	n	% (% of Total)
Specialist	728	48.9	741	49.8	20	1.3	1489	100 (29.0)
Technological assessment	526	46.1	600	52.5	16	1.4	1142	100 (22.3)
Cost of treatment	112	38.2	177	60.4	4	1.4	293	100 (5.7)
Good reputation	805	45.9	932	53.1	17	1.0	1754	100 (34.2)
Staff	69	46.3	77	51.7	3	2.0	149	100 (2.9)
Background of the dentist	25	46.3	29	53.7	0	0	54	100 (1.1)
Others	52	55.3	40	42.6	2	2.1	94	100 (1.8)
No answer	65	41.4	64	40.8	28	17.8	157	100 (3.1)
Total	2382	46.4	2660	51.8	90	1.8	5132	100

(A) A high proportion of regular visitors visited dental offices for health care.
(B) 63.5%of infrequent visitors selected the dental office by location. There was little difference observed in the influence of the factors of technique, waiting time and explanation. * Doctor refers to a medical doctor or another dentist.
(C) There was little difference between regular and infrequent visitors with regard to the information used for dental office selection.

Questionnaires

The questionnaires consisted of items relating to demographics, regular check-up experience, desired cost of regular check-ups, reason(s) for visiting the dental clinics, the main reasons for dental clinic selection, information used for dental clinic selection, and monthly household incomes. All questionnaires were presented in a forced-choice format. (Table 1). In the case of patients aged 12 years and below the questionnaires were completed by family members.

Statistical analysis

To investigate the differences between those who visited clinics regularly (Regular check-up experience, Yes) and those who did not (Regular check-up experience, No), chi-square tests for the nominal scales and two-way ANOVA for ordinal scales were used. In addition, multiple logistic regression analysis was used to calculate the odds ratios for regular visitors to control for confounders. These analyses were performed using SPSS ver. 11.0 (SPSS, Tokyo, Japan).

Results

The number of patients who reported visiting clinics regularly (Regular check-up experience, Yes) was 2,382 (46.4%), and their demographic characteristics are shown in Table 2. There were more female regular visitors (49.9%) than male visitors (41.0%) (Table 2A). When comparing by age group, many of the regular visitors were found to be aged under 14 years. In contrast, a high proportion of the 15- to 29-year-old group did not visit regularly. From age 15 to 49, the proportion of infrequent visitors (Regular check-up experience, No) decreased, then from age 50 and above, it increased. (Table 2B) When comparing by occupational group, the proportion of infrequent visitors was especially high in office workers (Table 2C).

The three major reasons for attending dental clinics were tooth or gum disease, routine health care, and denture

Table 4 Willingness of regular and infrequent visitors to pay for regular check-ups

(A)

Desired cost for regular check-ups (yen)	Regular visitors		Infrequent visitors		No answer		Total	
	n	%	n	%	n	%	n	%
1000	673	29.4	930	36.4	18	37.5	1621	31.6
2000	923	40.3	1022	40.0	17	35.4	1962	38.2
3000	501	21.9	439	17.2	9	18.8	949	18.5
4000	27	1.2	14	0.5	0	0.0	41	0.8
5000	133	5.8	106	4.1	2	4.2	241	4.7
7000	7	0.3	6	0.2	0	0.0	13	0.3
10000	17	0.7	25	1.0	1	2.1	43	0.8
20000	7	0.3	13	0.5	1	2.1	21	0.4
No answer	95	4.2	104	4.1	42	37.5	241	4.7
Total	2288	100	2555	100	48	100	5132	100

(B)

Desired cost of treatment	less than 1,000		less than 2,000		more than 2,000		No answer		Total	
Household income	n	%	n	%	n	%	n	%	n	%
less than 200,000	181	11.2	139	7.1	55	4.2	116	48.1	491	9.6
200,000 - 300,000	353	41.5	339	17.3	148	17.4	11	1.3	851	16.6
300,000 - 400,000	355	21.9	459	23.4	277	21.2	26	10.8	1117	21.8
400,000 - 500,000	206	12.7	287	14.6	184	14.1	31	12.9	708	13.8
more than 500,000	280	17.3	459	23.4	444	33.9	37	15.4	1220	23.8
No answer	246	15.2	279	14.2	200	15.3	120	49.8	845	16.5
Total	1621	100	1962	100	1308	100	241	100.0	5132	100

(A) Willingness of regular and infrequent visitors to pay for regular check-ups. More than 60% of patients were willing to pay for regular check-ups if the cost were less than 2,000 yen. There was little difference between regular and infrequent visitors.
(B) Willingness to pay and household income per month. There were no clear differences in the willingness to pay based on household income.

Table 5 Ratios for regular check-up visits

	Odds Ratio	95% CI	*P*-value
Gender			
Male	0.65	0.77 - 0.55	< 0.01
Age group			
-9	5.22	1.91 - 14.27	< 0.01
10-19	7.39	3.11 - 17.53	< 0.01
20-29	1.22	0.48 - 3.10	0.68
30-39	1.01	0.70 - 1.46	0.95
40-49	1.31	0.97 - 1.77	0.08
50-59	1.59	1.18 - 2.13	0.00
60-69	1.31	0.99 - 1.74	0.06
70-	1.38	1.05 - 1.80	0.02
Occupation			
Office worker	0.97	0.67 - 1.41	0.89
Civil servant	1.74	1.12 - 2.70	0.01
Self-employed	1.24	0.83 - 1.85	0.29
Housewife	1.30	0.90 - 1.88	0.17
Student	0.66	0.29 - 1.51	0.33
Part-time job	1.02	0.68 - 1.54	0.91
Unemployed	1.02	0.68 - 1.53	0.94
Household income			
less than 200,000	0.91	0.68 - 1.23	0.55
200,000 - 300,000	1.14	0.69 - 1.91	0.61
300,000 - 400,000	1.15	0.66 - 1.99	0.62
400,000 - 500,000	1.14	0.65 - 1.99	0.64
more than 500,000	1.36	0.79 - 2.36	0.27

We included all the demographic factors for logistic regression analysis of regular check-up visits. The results indicated that men were inclined not to be regular visitors. Persons aged under 20, persons aged 50 to 59, and civil servants were inclined to be regular visitors. However, no statistically significant differences were observed with regard to household income.

problems in this study. The proportion of infrequent visitors was high for tooth or gum disease, and was higher then regular visitors for denture problems (Table 3A).

The major reasons for dental clinic selection were personal dental office (1,983; 38.6%), recommendation from an associate (1,149; 22.4%), and location (1,001; 19.5%). Infrequent visitors were more likely than regular visitors to include recommendations from an associate and location as reasons for dental clinic selection (Table 3B).

Information used for selecting a dental clinic included good reputation, specialist, and technological assessment. There was considerable variation for both regular and infrequent visitors with respect to the information used for dental clinic selection (Table 3C).

As shown in Table 3A, most visitors were willing to pay less than 2,000 yen (about $ 20). The proportion of regular visitors was not large in any of the groups. The difference was statistically significant based on two-way ANOVA. Table 4B compares the willingness of using household income to pay. As household income increased, the number of persons willing to pay less than 1,000 yen (about $ 10) decreased. In contrast, the number of persons willing to pay less than 2,000 yen (about $ 20) and the number of persons willing to pay more than 2,000 yen increased.

To control for confounders, we used all the demographic factors to calculate the odds ratio of the persons who visited regularly for check-ups. As shown in Table 5, men tended not to visit clinics regularly. Relatively more persons under 20 years old, 50- to 59-year-olds and civil servants visited clinics regularly. No statistically significant difference was observed in relation to household income. When comparing by occupation, the relatively high proportion of students who visit clinics regularly was found to be not statistically significant.

Discussion

The present survey demonstrated two important issues that should be improved in order to promote the willingness of patients to pay for regular dental check-ups in Japan. First, the environmental considerations for adult working males should be improved. According to our survey, the majority of those not receiving regular check-ups were male (Table 2A). The age group of 50 - 59 years comprised of more persons who did not visit clinics regularly than those who did. Logistic regression analysis revealed statistically significant differences for males. In the age groups for 20 to 49, there were no regular visitors and occupation was not always associated with regular attendance. However, as the prevalence of periodontal disease is high in this population (1), regular check-ups should be encouraged so as to minimize the prevalence of periodontal disease. One of the most important reasons deterring adult males from regular check-ups was the high opportunity cost. The survey of factors influencing regular check-ups revealed that the main criteria for selecting clinics included, location, appointment times for visits to the clinics, and whether the clinic held late hours. To meet these demands, clinics should be open for late hours and on weekends.

A method of payment for regular check-ups is also necessary. In this survey, 73.3% of the patients answered that they were willing to pay less than 2,000 yen (about $ 20) for regular check-ups. The national average hourly fee for a dental hygienist in Japan is 1,938 yen (about $ 19)(13). At this hourly rate, the treatment in professional preventive programs should be completed over 20 or 30 minutes. These facts indicate that professional preventive programs costing less 2,000 yen might have not profits in dental clinics, because the current insurance system in Japan does not provide for dental health maintenance. The system improved slightly in 2002, to cover regular check-ups, but only for patients with periodontal disease after treatment. This change may well promote regular check-ups.

In conclusion, the present survey indicates that the likelihood of attending regular dental check-ups is related to gender and age, but not to household income.

Acknowledgments

This study was supported by the Japan Health Care Dental Association. The authors wish to thank the thirty-nine private dental offices that participated in this study and the members of the study groups of the Japan Health Care Dental Association.

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Journal of Oral Science, Vol. 46, No. 3, 179-183, 2004

Original

Feasibility of eradication of mutans streptococci from oral cavities

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(Received 15 April and accepted 29 July 2004)

Abstract: Objectives: Dental caries prevention programs using chlorhexidine (CHX) have been proposed, but CHX's effect in reducing levels of mutans streptococci (*S. mutans* and *S. sobrinus*) appears to last for only a few months. The aim of this study was to attempt to eradicate mutans streptococci from the oral cavity using intensive professional mechanical tooth cleaning (PMTC) and topical application of CHX in custom-made trays. Methods: Seven adult dentate subjects participated in this study (mean age 53.7 +/- 5.6, age range 46 to 62, mean DMFT, 9.1 +/- 4.2). For each subject, PMTC was carried out eight times within ten days. After each PMTC, 1% CHX was applied twice to the tooth surface using custom-made trays. In addition, as home treatment, subjects were required to carry out tooth brushing three times a day, and apply 0.2% CHX in custom trays after brushing in the morning and evening. In addition, subjects rinsed with 0.2% CHX solution after lunch. Salivary levels of mutans streptococci were evaluated using Dentocult-SM at baseline and on days 9, 20, 70, 120. Results: Mutans streptococci were eradicated by day 120 from 4 of the 7 seven subjects participating in this study. Those 3 subjects still harboring mutans streptococci exhibited deep periodontal pocketing. Conclusions: Eradication of mutans streptococci from the oral cavity is feasible using a combination of CHX application in custom-made trays and intensive PMTC. (J. Oral Sci. 46, 179-183, 2004)

Keywords: mutans streptococci; chlorhexidine; eradication.

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Introduction

It is generally accepted that harboring mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) is a risk factor for dental caries. A number of in-vitro studies have demonstrated mechanisms by which these bacteria play a role in dental caries formation (1). In this regard, the production of water-insoluble glucans on the tooth surface seems a particularly important virulence factor (2-4).

Dental plaque is a biofilm, and the surface of it is covered with the matrix structure of dextran (5-8), which is resistant to penetration by most anti-microbial agents.

Chlorhexidine (CHX) is a topical anti-microbial agent that is effective in the elimination of mutans streptococci from the oral cavity (9). Application of CHX is performed using a rinsing solution, dentifrice, varnish on the tooth surface or gel application in individual trays. Of these methods, varnish application to the tooth surface and gel application using individual trays have been shown to be most effective (10,11) since they permit a high concentration of CHX to be maintained on the tooth surface. However, even using these methods, this agent is still unable to penetrate the biofilm (11). Eradication of mutans streptococci using CHX has been attempted in the clinical trial setting, but re-growth of the bacteria is generally observed within two to three months (12). There are no reports of permanent eradication of mutans streptococci from the oral cavity. Moreover, attempting to eradiate these bacteria using high concentrations of CHX over an extended time frame may result in local adverse effects on the oral mucosa (13,14).

We propose use of a combination of professional mechanical tooth cleaning (PMTC) and a dental drug delivery system (3DS) with CHX for elimination of mutans streptococci from the oral cavity (15,16). 3DS consists of drug retainers that contact the tooth surface and permit contact of the anti-microbial drugs directly with the tooth surface. Since mutans streptococci do not have receptors to permit adherence to the oral mucosa (17), it is not necessary to eradicate them from areas where teeth are absent. It was hypothesized PMTC would destroy the biofilm structure and permit the delivered CHX to reach the mutans streptococci remaining in micro-colonies.

Materials and Methods

Subjects and clinical examination

This study was approved by the ethical committee of the National Institute of Infectious Diseases of Japan. Members of the institute and co-researchers of this study were recruited, and all subjects gave written informed consent for participation in the study. In total, seven adult male subjects participated, with mean age 53.7 ± 5.6 years and mean DMFT 9.1 ± 4.2. Probing depths were measured a six sites per tooth using a WHO probe. Among the seven subjects, 2 subjects had deep periodontal pockets (> 4 mm). All subjects exhibited pockets of 3 mm.

Clinical and sampling procedures

Levels of salivary mutans streptococci were determined at baseline using Dentocult-SM strip methods (Orion Diagnostica, Finland). Alginate impressions were then taken and maxillary and mandible casts prepared. A polypropylene sheet (3.0 mm disk for mouth guard soft, Keystone, New Jersey, U.S.A.) was vacuum-adapted to each cast with a vacuum-forming machine (VACCUM ADAPTER I, Keystone). Vacuum-adapted drug retainers were individually fabricated to cover the complete arch of the dentition. The drug retainer was trimmed to be approximately 1.0 mm apical to the gingival margin.

Before CHX application, PMTC was carried out eight times within 10 days on each subject to remove the tooth biofilm. By the PMTC, dental plaque was disclosed prior to its removal using rubber cups with polishing paste (Prophy Paste;(RDA170); CCS Cleanchemical, Vasby, Sweden). The remaining inter-dental plaque was removed using dental floss and polishing using Eva chips with polishing paste. Complete plaque removal was confirmed by further disclosing. The tooth surface was varnished with a 0.2% NaF solution (FULORIDENT GEL, Stone Pharmaceuticals, Philadelphia, USA).

After each PMTC, 1% CHX gel (CORSODYL Zahn-gel, Smithkline Beecham, Thorishaus, Switzerland) was injected into those periodontal pockets deeper than 4 mm. This gel was also applied using a dental drug retainer for 5 minutes. During the 10 days after PMTC, subjects also applied 0.2% CHX gel (Plakout, Howe-Neos Dental, Bioggio, Switzerland) twice a day after tooth brushing (morning and evening) using a custom-made tray. In addition, after lunch, tooth brushing and mouth rinsing using 0.2% CHX mouth rinse was performed. Salivary mutans streptococci levels were determined using the Dentocult-SM system at days 9, 20, 70, 120. On days 70 and 120, bacteria were also cultured to determine salivary mutans streptococci levels.

Microbial procedures and saliva sampling

Salivary mutans streptococci and Lactobacillus were counted using a commercially available mutans streptococci evaluation kit, Dentocult-SM (Orion Diagnostica, Epsom, Finland). The levels were classified according to the manufacturer's instructions, that is: level 0 - 1: < 100,000 colony forming units (CFU) mutans streptococci /ml saliva; level 2: 100,000 < CFU/ml < 1,000,000; and level 3: > 1,000,000 CFU/ml.

On days 70 and 120, mutans streptococci were also cultured. Paraffin-stimulated whole saliva samples were collected for 5 minutes. Saliva samples of 50 µl were then sonicated by ultrasonic dispersion (60 power output) for 10 seconds and spread onto Mitis-Salivarius agar (MS, Gibco, Tokyo, Japan) plates for growth of streptococci, and onto improved Mitis-Salivarius agar plates containing 0.02 M bacitracin (Wako Pure Chemicals, Osaka, Japan) (MSB) for selective growth of mutans streptococci (18), using an EDDY JET spiral system (Gunze Sangyo, Tokyo,

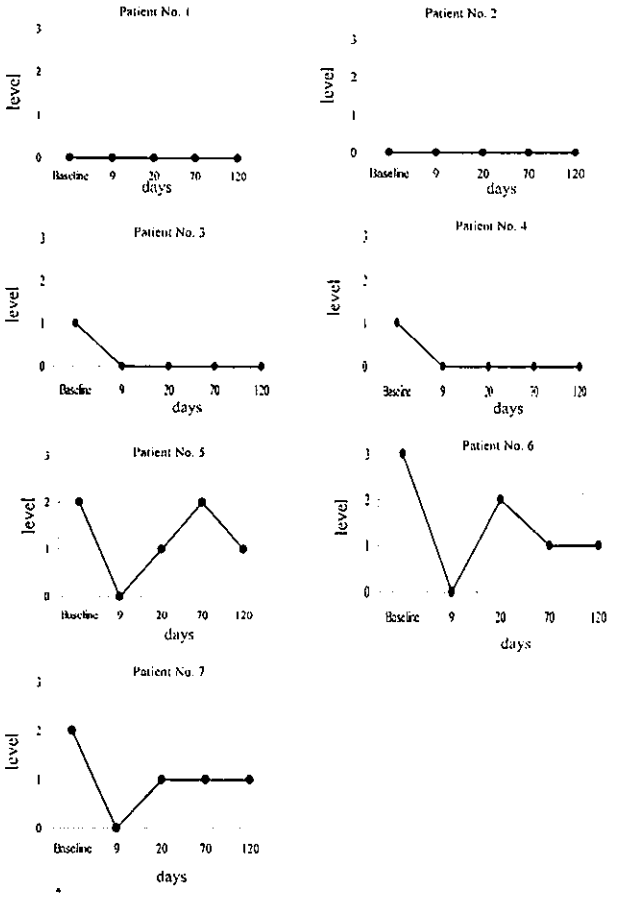

Fig. 1 Evaluation of the salivary levels of mutans streptococci by Dentocult SM.

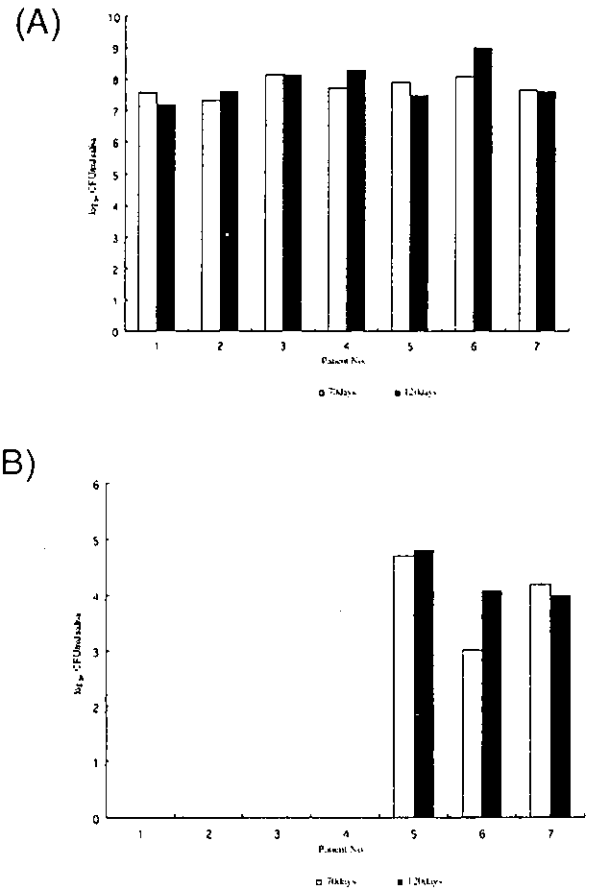

Fig. 2 Confirmation of the eradication of mutans streptococci from the oral cavity by culture.

Japan). After 48 hours anaerobic incubation, colonies were counted and the number of bacteria per ml of whole saliva calculated.

Statistical analysis

Mann-Whitney's *U*-tests were used for the evaluation of baseline differences between subjects in whom mutans streptococci was eradiated and those still harboring mutans streptococci.

Results

Figure 1 shows the mutans streptococci levels evaluated using the Dentocult SM. Two subjects with level 0 (no mutans streptococci detected)at baseline also had level 0 throughout the study. In addition, two subjects with level 1 at baseline decreased to the 0 level after 120 days. Three subjects above level 2 at baseline decreased to level 0 by day 9, and recovered to level 2 by day 120.

Figure 2 shows the mutans streptococci levels evaluated by the improved MSB culture system at 70 (A) and 120 days (B). Eradication of mutans streptococci was observed in three subjects.

Table 1 Baseline clinical characteristics (mean ± SD) of subjects according to success of mutans streptococci eradication methods

	Eradicated (n = 4)	Not eradicated (n = 3)	*P*-value
DMFT	10.00 ± 4.97	8.00 ± 5.29	0.31
Probing depth (% of pockets)			
> 3 mm	49.85 ± 19.92	54.76 ± 45.07	0.78
> 4 mm	6.40 ± 2.40	18.85 ± 18.46	0.70
> 5 mm	1.64 ± 0.89	6.94 ± 9.57	0.71
> 6 mm	0.89 ± 1.03	2.18 ± 3.78	0.58

The baseline characteristics of subjects in whom mutans streptococci were eradicated and those exhibiting mutans streptococci re-growth are shown in Table 1. There was a

tendency for those subjects with deep periodontal pockets to exhibit streptococci regrowth, although no statistically significant difference was observed.

Discussion

The results of the present study demonstrate that 3DS used in combination with intensive PMTC is effective in reducing salivary levels of mutans streptococci and in eradicating mutans streptococci. A number of dental caries preventive programs have been described (for review see Lewis et al.;19), including tooth cleaning, and fluoride or CHX application. Tooth cleaning and tooth brushing instruction have some benefit in caries prevention, although this benefit appears minor (20). A combination of oral hygiene instruction and fluoride application appears more effective (21), and if CHX is used in addition, an even greater favorable effect is evident (11). However, these preventive measures do not appear to result in complete inhibition of new dental caries.

Complete removal of dental plaque can be challenging, even in the case of chemical plaque removal (22). Tooth brushing in combination with tooth brushing instruction is also of questionable value, especially on tooth surfaces prone to caries (23). PMTC has been suggested as being one of the most effective methods for plaque removal, and PMTC performed by dental hygienists has been demonstrated to suppress dental caries (24,25) and reduce salivary levels of the mutans streptococci without the use of anti-microbial agents (26).

Elimination of mutans streptococci using CHX has been attempted in a number of studies, but the mean levels of these bacteria returned to baseline levels within two weeks when a rinsing solution was used (27), within 4 weeks using gel application or within 12 weeks using varnish (28). The results of these studies suggest that eradication of mutans streptococci from the oral cavity is not feasible. However, these results might be a consequence of inadequate plaque removal prior to CHX application. The dental plaque biofilm in which mutans streptococci are found (8) is resistant to anti-microbial agent penetration (29). Using the system described in the current study, pre-treatment of the tooth surface using PMTC to remove the biofilm appears to permit CHX to function more effectively in mutans streptococci removal.

The use of either PMTC or anti-microbial agents alone has some benefit in preventing dental caries, but is not effective in eradication of mutans streptococci. Using the system described in this study, that is complete removal of dental plaque by intensive PMTC and frequent applications of CHX within a short period of time, in some cases results in the eradication of mutans streptococci from the oral cavity. However, even using this system, mutans streptococci were eradicated from only 4 of 7 subjects.

Baseline differences in the periodontal condition between subjects in whom mutans streptococci were eradicated and those in whom they were not eradicated were not seen. This may be the result of the small sample size, and thus insufficient power to detect differences. However, in general, those subjects where mutans streptococci were not eradicated had deeper periodontal pockets. These results suggest periodontal treatment might be advisable prior to use of anti-microbial agents.

In conclusion, using appropriate methods, eradication of mutans streptococci from the oral cavity is feasible, but is not consistently achieved.

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J. Appl. Glycosci., **51**, 129–134 (2004)

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Abstract: Phosphate ester groups are known to link to some glucosyl residues in starch molecules. We have prepared phosphoryl oligosaccharides (POs) from potato starch hydrolysates. The POs were composed of two fractions, PO-1 and PO-2. Fraction PO-1 was the main fraction, and it was composed of maltotriose, maltotetraose, and maltopentaose to which one phosphoryl group was attached. Fraction PO-2 was predominantly composed of maltopentaose and maltohexaose to which at least two phosphoryl groups were attached. POs had the ability to form a soluble complex with calcium and had an inhibitory effect on the formation of a calcium-phosphate precipitate. Based on the function of the POs, described above, we applied the POs of calcium (POs-Ca) as a food ingredient. POs-Ca was an advantageous food ingredient as a soluble calcium source. In relation to prevention of dental caries, POs cannot be fermented by cariogenic microorganisms or mutans streptococci, and they reduce the fall in plaque pH *in vitro*. Moreover, POs-Ca effectively enhanced the remineralization of enamel lesions. The aim of this study was to develop the application of POs-Ca for dental health to the enamel remineralization through the chewing of a sugar-free gum.

Key words: phosphoryl-oligosaccharides, saliva, remineralization, enamel, chewing-gum

The wide distribution of the ester phosphorus is observed in starches from various sources. Potato starch is known to contain an esterified phosphoryl group amoung its components.[1,2] Takeda and Hizukuri have reported that the phosphate groups were located mostly in the B-chain of amylopectin, whereas the phosphorylation of amylose was very little.[3] Potato amylopectin contains 100–1000 ppm of the ester phosphorus.[2] Furthermore, approximately 60% to 70% of the phosphate groups were linked to C-6 of the glucosyl residues, almost all the rest being linked to C-3 and a very small part possibly being linked to C-2 of the glucosyl residues.[2] Our attention was focused on the utilization of the esterified phosphoryl group in potato starch, and we succeeded in preparing new phosphoryl oligosaccharides (POs) from the starch hydrolysate.[4] In this article, we introduce our recent achievements in a new function of the oligosaccharides focusing on the application for soluble calcium and the effect of remineralization on enamel lesion.

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Abbreviations: POs, phosphoryl oligosaccharides; POs-Ca, phosphoryl oligosaccharides of calcium; Ca, calcium; P, phosphate; BSA, bacterial saccharifying α-amylase; GA, glucoamylase; BLA, bacterial liquefying α-amylase; PPA, porcine pancreatic α-amylase; HSA, human saliva α-amylase; TAA, Taka-amylase A; Glc-6-P, D-glucose-6-phosphate; 6^2-phosphoryl maltose, *O*-6-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranose; 6^3-phosphoryl maltotriose, *O*-6-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose; 6^2-phosphoryl maltotriose, *O*-α-D-glucopyranosyl-(1→4)-*O*-6-phosphoryl-α-D-glucopyranosyl-(1→4)-D-glucopyranose; 6^3-phosphoryl maltotetraose, *O*-α-D-glucopyranosyl-(1→4)-*O*-6-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose; 6^4-phosphoryl maltopentaose, *O*-α-D-glucopyranosyl-(1→4)-*O*-6-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose; 3^3-phosphoryl maltotetraose, *O*-α-D-glucopyranosyl-(1→4)-*O*-3-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose; 3^4-phosphoryl maltopentaose, *O*-α-D-glucopyranosyl-(1→4)-*O*-3-phosphoryl-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose.

Structure and characterization of POs.

We developed a method of producing POs from potato starch, using bacterial liquefying α-amylase (BLA) [EC 3.2.1.1], glucoamylase (GA) [EC 3.2.1.3] and pullulanase [EC 3.2.1.41]. The actions of the amylolytic enzymes were hindered by the phosphoryl groups linked to the glucosyl residues, and POs were obtained as indigestible components by the enzymes. The components of the POs were analyzed by high-performance anion-exchange chromatography and pulsed amperometric detector system.[4] The substance-linked phosphoryl group is detected by the system at different retention times according to the number and the positions of phosphate groups linked to each molecule. The POs were fractionated into two fractions; PO-1 and PO-2.[4,5] Fraction PO-1 was the major component of POs and was composed of maltotriose, maltotetraose and maltopentaose to which one phosphoryl

group is attached.[5] Fraction PO-2 was predominantly composed of maltopentaose and maltohexaose to which at least two phosphoryl groups were attached.[6] The average degree of polymerization of dephosphorylated PO-1 and PO-2 was evaluated to be 4.02 and 5.82, respectively.[4] The detailed structure of the components of the PO-1 fraction was analyzed by using the different hydrolytic properties of bacterial saccharifying α-amylase (BSA) and GA on the phosphoryl oligosaccharides. The limiting cleavage points of BSA on PO-1 components were the same sites as those of porcine pancreatic α-amylase (PPA) and human saliva α-amylase (HSA), as reported by Takeda *et al.*[7] The phosphate group linked at C-6 of the glucosyl residue was detected as the content of the Glc-6-P after acid hydrolysis. The spectrometric analysis by ^{13}C-NMR also distinguished the phosphate groups linked at C-3 and C-6 of the glucosyl residues, respectively. In conclusion, the PO-1 fraction was made up of oligosaccharides phosphorylated at C-3 (3^3-phosphoryl maltotetraose and 3^4-phosphoryl maltopentaose) and oligosaccharides phosphorylated at C-6 (6^3-phosphoryl maltotriose, 6^2-phosphoryl maltotriose, 6^3-phosphoryl maltotetraose, and 6^4-phosphoryl maltopentaose) (Fig. 1).[5] In the case of the PO-2 fraction, a little of these enzymes' treatment-resistant PO-2 remained. It clearly indicated that two of the phosphoryl groups attached to C-6 and C-3 existed in PO-2 components.[6] From these results, the possible structures of PO-2 components were as shown in Fig. 1.

The POs can form solubilized complexes with Ca and iron.[4,8] The inhibitory effect of POs on the formation of Ca-P precipitate was dependent upon the covalently bound phosphoryl groups in the molecule.[4,8-10] POs-bound calcium was thought an advantageous food ingredient as a soluble calcium source.[11] In addition, POs can not be metabolized by cariogenic bacteria as mutans streptococci, as is true for xylitol, and the preventive effect was shown on reducing the fall in plaque pH despite the buffering power sucrose-dependent fermentation.[12] Sugar alcohols such as xylitol are widely used as sweeteners in chewing-gum to prevent dental caries and to promote remineralization.[13,14] The POs can also reduce the amount of artificial plaque and demineralization on enamel, even in the presence of sucrose.[15] Furthermore, the effects of POs on remineralization of caries-like lesions in enamel were examined *in vitro*.[16] The results showed the possibilities that POs may have a synergistic effect with fluoride on the rate of remineralization.[16] Based on the previously revealed features of POs, we examined the effect of remineralization on enamel by a chewing gum containing a calcium salt of POs (POs-Ca).

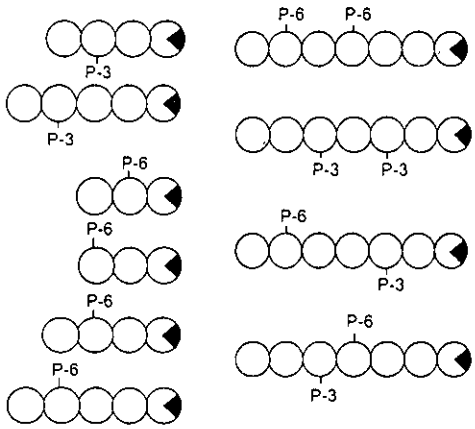

Fig. 1. Structure of phosphoryl oligosaccharides.

Symbols: P-3 and P-6, phosphoryl groups linked at C-3 and C-6 of glucosyl residues; ○, glucosyl residue; ○, glucosyl residue which possibly exists; ◑, reducing end.

Application of POs-Ca for oral health.

The effects of daily application of a sugar-free chewing gum containing 2.5 wt% POs-Ca on remineralization of enamel were examined. POs-Ca was prepared as 5 wt% calcium in the molecule. The gum was concluded to be a non-cariogenic product since it was proven by intraoral plaque pH-telemetry tests in four human volunteers not to depress the pH of interdental plaque below 5.7 by bacterial fermentation, either during consumption or during a period of 30 min following consumption by the general method of the Association for Toothfriendly Sweets.[17] First, the effect of the gum containing POs-Ca on remineralization of caries-like lesions in enamel was examined by using a human saliva immersing (HSI) test.[18] The HSI-test would be a useful system for detection and evaluation of the remineralization effect using human saliva, since it is easy to control condition for the test and light demands are made on volunteers, making it preferable to an intraoral study. The results suggested that the HSI-test and intraoral study have relevance to the effect on enamel remineralization of POs-Ca.[18-20] It is thought that human saliva plays some important role in oral health.[21] In particular, stimulated saliva for chewing would have some influence on remineralization.

1. Salivary Assessment.

We produced two types of sugar-free chewing gum (tablet type) for the experiments. One contained 2.5% POs-Ca (POs-Ca (+) gum) and 46% xylitol, and the other contained 48.5% xylitol without POs (POs-Ca (−) gum). The average weight of each chewing gum tablet was about 1.5 g. All saliva stimulated while chewing 2 tablets of POs-Ca (+) gum or POs-Ca (−) gum was collected from 12 healthy adult volunteers (6 males and 6 females; mean age=29.9 y old). Each volunteer chewed 2 pieces of gum for 20 min and the whole saliva was collected for the first 10 min (Fs) and last 10 min (Ls) separately. Demineralized bovine enamel slabs were immersed in the Fs for 10 min and subsequently in the Ls for 10 min at 37°C. Immediately after the salivary treatments, the enamel slabs were rinsed with deionized water. This procedure was repeated 4 times a day for 4 days. During the study period, no fluoride agent was used and great care was taken not to dry the enamel disk samples. The human saliva was used in the HSI-test within 1 h after sampling. Salivary volume and mineral contents were compiled in Table 1. The volume and pH of saliva from each volunteer were measured immediately after sampling. Subsequently, an aliquot of the saliva was centrifuged (10,000 $\times g$) for 5 min and the supernatant was prepared 0.1 N HCl solution with addition of 1 N HCl solution. After re-centrifugation, the supernatant was filtrated with an ultra filter (0.45 μm). The filtered saliva was assayed for the

Table 1. Analysis of volumes and mineral contents in saliva.

	POs-Ca	Fs		Ls		P[a]
		Means±SD	P[b]	Means±SD	P[b]	
Salivary volume (mL)	+	20.34±4.13	ns	9.35±3.24	ns	**
	−	20.74±4.43		9.65±3.35		**
Ca (mM)	+	6.29±2.44	**	1.72±0.27	*	**
	−	1.69±0.41		1.39±0.37		ns
P (mM)	+	5.62±1.41	ns	6.22±1.31	ns	ns
	−	6.15±1.35		6.49±1.15		ns
Ca/P	+	1.12±0.31	**	0.27±0.05	*	**
	−	0.28±0.08		0.22±0.05		ns

Fs, collected whole saliva for the first 10 min; Ls, collected whole saliva for the last 10 min. [a]p for POs-Ca (+) *vs.* POs-Ca (−), [b]p for FS *vs.* LS. ns, not significant; $^{**}p<0.0001$, $^{*}p<0.05$.

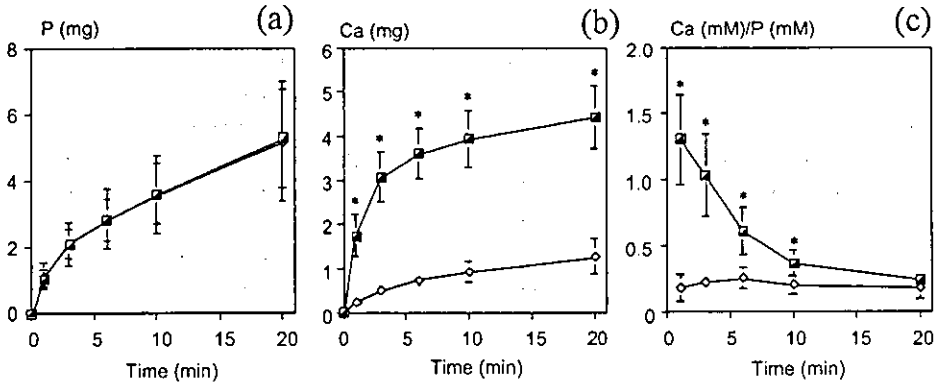

Fig. 2. Time course of salivary soluble phosphate (a), calcium (b), and Ca/P ratio during chewing gum with (◪) or without (◇) POs-Ca.

Human whole saliva was collected from 17 healthy adult volunteers (9 males and 8 females; mean age=29.0 y old) by chewing 2 tablets of POs-Ca (+) gum or POs-Ca (−) gum. Each volunteer chewed the tablets for 20 min and the whole saliva was collected. Vertical bars, SD.

concentrations of inorganic phosphate (P) and calcium (Ca). The concentrations of P and Ca were measured by the methods using molybdenum reagent[22] and by *o*-cresolphthalein complexion (OCPC) method using the calcium-C-test (Wako Pure Chemical, Japan), respectively. The salivary volume of Fs and Ls secreted by chewing of POs-Ca (+) gum and that by POs-Ca (−) gum were nearly equal values, and no significant difference was observed. The concentration of Ca in Fs in POs-Ca (+) gum was much higher than that of POs-Ca (−) gum ($p<0.0001$). However, the Ca concentration of Ls in POs-Ca (+) gum was similar to that of Ls and Fs from POs-Ca (−) gum. The difference in chewing gum did not largely influence the concentration of P in saliva samples. Neither gum included any ingredients containing P. Human saliva includes abundant P, compared with Ca. The time course of soluble Ca and P during 20 min was measured in chewing each gum (Fig. 2a, b). In the experiment, human whole stimulated saliva was collected from 17 healthy adult volunteers (9 males and 8 females; mean age=29.0 y old) including the former 12 volunteers during chewing 2 tablets of POs-Ca (+) gum or POs-Ca (−) gum. The results indicated that most Ca in POs-Ca (+) gum was extracted into saliva within the first 10 min. However, the concentration of P is almost fixed in saliva and increases in proportion to the amount of saliva. The Ca/P ratio values in Fs from POs-Ca (+) gum (1.12±0.31) were significantly higher than the values in the other ($p<0.0001$). Especially at the beginning, the Ca/P ratio ranged to 1.67, which is the value of hydroxyapatite in enamel (Fig. 2c). The pH of saliva was measured at 1, 3, 6, 10 and 20 min during saliva sampling from 17 volunteers. The pH was about 7.0 at the beginning, rose to about 7.5 in the first 6 min, and thereafter remained around 7.5 during the chewing period in both cases. After human salivary treatments, planoparallel sections of about 500 μm thickness were cut from the enamel samples using a water-cooled diamond coated saw (Isomet, Buhler, USA). These sections were ground planoparallely on a wet 800-grit abrasive paper to a thickness of about 200 μm. The sections were fitted on a high resolution positive film (Fuji, Japan) together with an aluminum step wedge and microradiographed (PW-1830, Philips, The Netherlands) by Cu-Kα x-ray generated at 25 kV and 25 mA for 24 s. The films were developed, fixed, and rinsed under standardized conditions. The degree of remineralization was evaluated on digitized microradiographic images by combined means of computer-assisted videodensitometry (CAV) and a mineral distribution analysis program (MDA) developed by Inaba *et al.*[23,24] Finally, the mineral distribution parameters, namely

the lesion depth (ld, μm) and mineral loss (ΔZ, vol%·μm), were measured. The data values were analyzed statistically by the repeated measure ANOVA followed by the Tukey-Kramer test for multiple comparison. The ld and ΔZ values are shown in Fig. 3 by individuals in types of gum. In all the individuals participating, significantly lower ld and ΔZ values were observed in the case of POs-Ca (+) gum indicating enhanced enamel remineralization. No remarkable mineral recovery was observed in the POs-Ca (−) gum group. The ld (means±SD=81 ±15 μm) and ΔZ (2,825±593 vol%·μm) in the POs-Ca (+) gum group were significantly lower ($p<0.001$) compared with those after initial demineralization and in POs-Ca (−) gum group. The saliva secreted by chewing POs-Ca (+) gum had higher remineralization-enhancement activity than that from POs-Ca (−) gum. No difference was observed in volume, time-course change in pH level or contents of soluble P of saliva between chewing gum types or among volunteers. There were adequate amounts of P compared with Ca in saliva collected by chewing of the POs-Ca (−) gum. In the case of POs-Ca (+) gum, the Ca content was higher than that from chewing the POs-Ca (−) gum. The initial Ca/P ratio value in POs-Ca (+) gum-induced saliva was higher than that in POs-Ca (−) gum-induced saliva. The Ca/P ratio was 0.3 or less for POs-Ca (−) gum. These results suggested that remineralization enhanced by chewing the POs-Ca (+) gum was due to the increased soluble calcium in saliva that resulted in a higher Ca/P ratio value corresponding with the value (1.67) of hydroxyapatite.

2. *Intraoral evaluation.*

Based on the former results, we investigated the effects of the POs-Ca (+) gum on the remineralization of enamel *in situ*. Twelve healthy adult volunteers (6 males and 6 females; mean age, 21 years old) were randomly divided into 3 groups and participated in a double-blind intraoral study. In first, each volunteer wore a palatal appliance containing 3 demineralized enamel disks, and chewed one of the following experimental gums 4 times a day (after meals and before bed time) for up to 4 weeks.[18] The three groups were (i) the POs-Ca (−) gum group, (ii) the POs-Ca (+) gum group or (iii) sugar gum containing 62 wt% sucrose (the sucrose gum group). The chewing time was always 20 min and the palatal plate was preserved in the oral cavity for an additional 20 min. Except for the time of chewing the gum and the subsequent 20 min, the appliances were stored in a plastic container with 100% humidity. The results were that the remineralization rates (ld reduction percentage with respect to the mean ld value after initial demineralization) in the POs-Ca (+) group were about 67, 54 and 76% at the 1st, 2nd and 4th week, respectively. The remineralization rates in the POs-Ca (−) group ranged from 12 to 23%, being much lower than that in the POs-Ca (+) group. The sucrose group showed a negative value by the 4th week, indicating progression of demineralization. The remineralization rate of the POs-Ca (+) group was higher than that of the POs-Ca (−) group at the 1st, 2nd, and 4th week.[18] The present results were well consistent with the results of the *in vitro* evaluations with the HSI-test. Furthermore, the promoting of enamel remineralization of the POs-Ca (+) group was reconfirmed *in situ* by a two-week double-blind and cross-over design intraoral study.[25] The ld of the POs-Ca (+) group was significantly reduced by 51% compared with that in the sucrose group and by 44% compared with that in the POs-Ca (−) group (Fig. 4a). The ΔZ in the POs-Ca (+) group was also significantly lower by 30% compared with that in the sucrose group and by 25% compared with that in the POs-Ca (−) group (Fig. 4b). The effects of the gum on remineralization of dentin were also confirmed in a double-blind cross-over design intraoral study.[26]

Conclusion and perspective.

The promoted remineralization of enamel and dentin lesions by the POs-Ca (+) group can be explained as follows. The pH of the saliva during the chewing of the gums is estimated to increase from about 7 to 7.5. Since, in general, this relatively higher pH is not suitable for the solubilizing of Ca and phosphate, it is considered that POs-Ca in the saliva would aid to maintain the solubility of mineral ions even at pH 7–7.5 and, thereby, ionized Ca and P had potential to redeposit onto the residual hydroxyapatite crystals in enamel and dentin lesions. Thus, under the presence of POs, soluble Ca in saliva increases efficiently and, thereby, the salivary Ca/P ratio can increase nearly up to the rate of hydroxyapatite (1.67). In a former study, some ratios of Ca/P was investigated *in vitro*.[27] The Ca/P ratio of 1.67 showed significant reduction in ld and ΔZ of enamel lesion compared with other ratios. It was also suggested the elevation of the Ca/P ratio

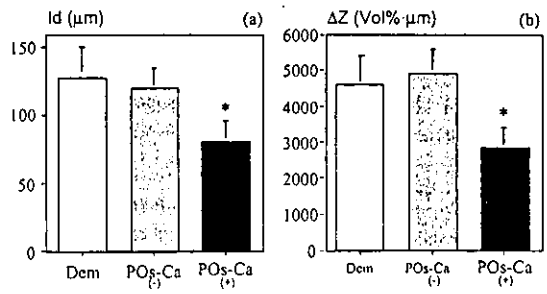

Fig. 3. Comparison of the lesion depth (a) and mineral-loss (b) values after salivary treatments.

Numbers of volunteers were 12 persons (6 males and 6 females; mean age=29.9 y old). Vertical bar, SD; $^{*}p<0.001$; Dem, initial demineralized enamel.

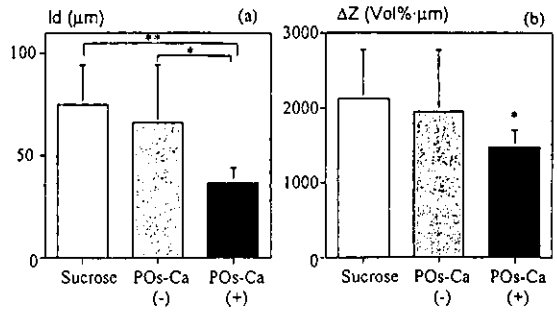

Fig. 4. Comparison of the lesion depth (a) and mineral loss (b) values after *in situ* experiments.

Numbers of volunteers were 12 persons (6 males and 6 females; mean age, 21 y old). Vertical bar, SD; $^{*}p<0.05$; $^{**}p<0.01$.

in saliva enhanced the remineralization. The gum containing POs-Ca would be able to compensate the remineralization effect of saliva itself. The results suggested that POs may be a novel and unique substance to enhance enamel remineralization, and could be utilized for caries prevention by a nutritional approach. It is generally known that a gastrointestinal disorder would occur when we take a food containing an excess amount of sugar alcohol at one time. We have already shown that the consumption of an excess amount of POs-Ca does not cause a gastrointestinal disorder.[28,29] We also confirmed that the orally administered POs-Ca was hydrolyzed and then absorbed completely in the small intestine in rats.[30] In conclusion, daily use of a sugar-free chewing gum containing POs-Ca can effectively enhance the remineralization both in enamel and dentin lesions. POs-Ca enhanced enamel remineralization by increasing the solubility of Ca in the oral environment and could be a beneficial material for oral health.

We thank Drs. Shigetaka Okada and Reiichiro Sakamoto for helpful suggestions through out the course of the study.

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馬鈴薯澱粉由来リン酸化オリゴ糖カルシウムのオーラルヘルスへの応用

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澱粉には，その構成糖にリン酸基がエステル結合している糖を含むことが知られている．著者らは馬鈴薯澱粉の加水分解物より，リン酸基がエステル結合している糖，つまり，リン酸化オリゴ糖カルシウム（POs-Ca）を調製してきた．このリン酸化オリゴ糖は二つの画分 PO-1 画分および PO-2 画分から構成されていた．PO-1 画分はリン酸化オリゴ糖の主な成分であって，マルトトライオース，マルトテトラオース，およびマルトペンタオースから構成されており，分子内に 1 個のリン酸基を有していた．PO-2 画分は主にマルトペンタオースおよびマルトヘキサオースから構成されており，少なくとも 2 個のリン酸基を分子内に有していた．リン酸化オリゴ糖はカルシウムと水溶性の複合体を形成し，カルシウム-リン酸の沈澱形成を阻害する効果を有していた．以上の結果をもとにリン酸化オリゴ糖のカルシウム塩（POs-Ca）を食品素材として開発してきた．POs-Ca は，水溶性カルシウム供給のための食品素材として優れていた．また，う蝕予防の観点から，リン酸化オリゴ糖はう蝕原因細菌であるミュータンス連鎖球菌の栄養源にならず，本菌の産生する酸によるプラーク内の pH の低下も抑制する作用を有していることを明らかにした．さらに，POs-Ca は初期う蝕を誘発したエナメル質の再石灰化を効果的に促進する作用も有していることがわかった．ここでは，POs-Ca を関与成分としたシュガーレスガムの初期う蝕の再石灰化効果を明らかにし，POs-Ca の口腔保健への応用開発について紹介する．

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〔質 問〕 信州大 北畑

1）再石灰化率から見ますと POs-Ca の濃度が低い方が良いということでしょうか？

2）ガムを噛んで 10 分後，20 分後で Ca/P 濃度比を比較すると，10 分で十分ではないでしょうか？

〔答〕

1）再石灰化には試験溶液中の Ca/P 濃度比が大きく影響します．*In vitro* の試験系において，POs-Ca を高濃度添加しますと Ca/P 濃度比が高値になり，再石灰化率が低下するものと考えております．よって，Ca/P 濃度比を考慮した POs-Ca の利用が重要です．つまり実際の唾液には唾液用の POs-Ca 濃度が有効ということです．

2）個人差はありますが，ガムの殆どの成分は約 10 分間で唾液中に溶出するものと考えられます．POs-Ca も 10 分間で殆ど溶出してきておりました．再石灰化効果に関しても 10 分間の咀嚼で効果が得られることも確かめております．このことから，ご質問のように 10 分間の咀嚼で十分効果が発揮されることも考えられます．一方，唾液量を考えた場合，10 分間の咀嚼で約 20 mL の唾液量になり，20 分間の咀嚼では約 30 mL の量に増加します．唾液には再石灰化のみではなく，口腔機能維持にとって様々な重要な役割を担っております．総合的に考え，20 分程度のガムの咀嚼を推奨しております．

〔質 問〕 食総研 山本

1）POs は口腔内のアミラーゼによってどのような影響を受けるか？

2）Ca を結合して可溶化する物質には POs 以外にも CPP(カゼインホスホペプチド)，クエン酸，ポリグルタミン酸など様々あるが，POs がそれらに比べて優れている点はどんなところか？

3）歯の再石灰化の際には，アモルファスリン酸カルシウム(ACP)から出発して結晶転移を経てハイドロキシアパタイト(HA_p)になるが，Ca/P の最適値は HA_p の 1.67 で良いか．ACP や第二リン酸カルシウム(DCPD)の Ca/P でなくても良いか？

〔答〕

1）POs は α-アミラーゼを馬鈴薯澱粉に作用させて得られてきた産物ですので，これ以上の加水分解作用は唾液中のアミラーゼでも受け難いと考えております．私たちのこれまでの研究でもヒト唾液由来 α-アミラーゼの POs への作用特性を詳細に報告しております．

2）再石灰化とは，次の二つのプロセスで進むと考えられます．①歯の脱灰患部にカルシウムイオンとリン酸イオンが供給される．②供給されたカルシウムイオンとリン酸イオンが脱灰患部の結晶成長に使用される．その観点から，再石灰化促進物質とは，①カルシウム-リン酸の不溶化を抑制するが，②脱灰患部の結晶成長を助長するという機能が必要と考えられます．本観点からカルシウムイオンとのイオン結合能力が重要な問題になります．分子内に過剰の結合リン酸基が存在する場合には，キレート能力が高すぎて，結晶成長を阻害することも確認してきております．ただし，詳細な情報を得ることは今後の課題と考えております．

3）HA_p が形成されるまでには，DCPD，トリカルシウムホスフェイト(TCP)およびオクタカルシウム(OCP)などが関与していることが知られております．POs-Ca を咀嚼した際の唾液中の Ca/P 比 1.67 を目標にしておりますが，ガム食品の性質上，咀嚼開始直後から経時的に Ca/P 比は低下してゆきます．そして，10 分以降には通常の唾液組成の Ca/P 比 0.3 程度に戻ってしまいます．一般的に唾液に再石灰化の機能が備わっていることは周知の事実であることから，必ずしも Ca/P 比 1.6 でなくとも再石灰化は生じます．しかし，短期間での効果的な再石灰化を生じさせるためには，Ca/P 比を 1.67 にすることであると考えております．

Oral Microbiology Immunology 2004: 19: 386–389
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Oral Microbiology and Immunology

Molecular analysis of age-related changes of *Streptococcus anginosus* group and *Streptococcus mitis* in saliva

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Morita E, Narikiyo M, Nishimura E, Yano A, Tanabe C, Sasaki H, Hanada N. Molecular analysis of age-related changes of Streptococcus anginosus *group and* Streptococcus mitis *in saliva.*
Oral Microbiol Immunol 2004: 19: 386–389. © Blackwell Munksgaard, 2004.

The purpose of this study was to survey the prevalence of streptococcal species, especially *Streptococcus anginosus* (which has been reported to be associated with cancer in the upper digestive tract), *Streptococcus constellatus*, and *Streptococcus intermedius* in the saliva of different age groups. A sequence analysis of 16S rDNA was performed and DNA quantified using real-time polymerase chain reaction. The *S. anginosus* level increased with age, whereas the levels of *S. constellatus* and *S. intermedius* did not change. *Streptococcus mitis* was the predominant species in the saliva of all the age groups but, unlike the *S. anginosus*, the proportion of *S. mitis* in the salivary bacteria decreased with age. The increase in *S. anginosus* with age should be carefully monitored because of its association with diseases, including cancer.

Key words: real-time polymerase chain reaction; saliva; *Streptococcus anginosus*; *Streptococcus mitis*

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Accepted for publication July 1, 2004

Streptococcus anginosus is a currently recognized species of the '*Streptococcus milleri*' group, the name used for heterogeneous oral streptococcal strains associated with purulent infections (14). The *S. milleri* group comprises at least three different species: *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (14). Awareness of the clinical importance of *S. anginosus* has gradually increased because several studies have reported a close association between *S. anginosus* infection and cancer in the upper digestive tract (3, 7, 8, 12). In spite of this clinical significance, information on the prevalence of *S. anginosus* in the oral cavity is limited because most studies on this subject were performed before the current classification criteria of the *S. anginosus* group was established (11). Recent analyses using real-time polymerase chain reaction (PCR) found extremely low levels of *S. anginosus* in the saliva (2, 10). However, these studies did not consider the variety of *S. anginosus* strains. Nor was age considered, although microbial infection in the oral cavity appears to change with age (5). It was reported that the streptococcal salivary colony-forming units were higher in adults than in children, and that the isolation frequency and proportion of streptococcal species change with age (11). The incidence of cancer is highest in people in their sixties (1). The oral cavity may act as a reservoir of *S. anginosus*, a potential pathogen, and saliva is the most probable carrier. The emphasis of this study was the molecular analysis of subsets of the salivary microbiota in different age groups, focusing on *S. anginosus*.

Material and methods

Saliva samples

Saliva samples were obtained from systemically healthy volunteers aged 25–70 years. These samples were centrifuged, then frozen and stored at −80°C until use. Written informed consent was obtained from all the volunteers.

Bacterial strains

S. anginosus ATCC 33397, *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, *Streptococcus mutans* LM 7, *Streptococcus sobrinus* AHT, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii*

Table 1. S. anginosus, S. constellatus, and *S. intermedius* strains and accession numbers for the DDBJ/EMBL/GenBank nucleotide sequence databases from which DNA sequence data were used for designing specific primers

Species/strains	Accession numbers
S. anginosus strain ATCC33397	AF352808
S. anginosus genotype VA8466	AF306838
S. anginosus strain 920	AF145246
S. anginosus strain 1007	AF145245
S. anginosus strain 414	AF145243
S. anginosus strain 21	AF145242
S. anginosus strain 1204	AF145240
S. anginosus strain 367	AF145239
S. anginosus strain GTC822	AB006121
S. anginosus strain GTC821	AB006120
S. constellatus strain 1259	AY277942
S. constellatus strain 919	AY277941
S. constellatus strain 1192	AY277940
S. constellatus strain 15	AY277939
S. constellatus strain 857	AY277938
S. constellatus strain 1	AY277937
S. constellatus strain ATCC27823	AF104676
S. constellatus strain 206	AF104677
S. constellatus strain VAMC3868	AF169356
S. constellatus strain VAMC5464	AF169353
S. intermedius strain 488	AF104673
S. intermedius strain 125	AF104672
S. intermedius strain 535	AF104674
S. intermedius strain ATCC27335	AF104671
S. intermedius strain B33	AJ491836

ATCC 10558, *Streptococcus mitis* ATCC 6249, and *Streptococcus salivarius* ATCC 9759 were cultured.

DNA extraction

Genomic DNA was isolated from saliva and bacteria by a standard phenol–chloroform method. DNA content was determined spectrophotometrically.

PCR cloning and sequence analysis

Equal amounts of DNA extracted from the saliva of 10 people were mixed for each age group (25–49-year-old group, 50–69-year-old group and 70-year-old group) and used as templates. For amplification of a portion of the 16S rDNA gene of many oral bacteria from saliva, PCR was performed with primers Ust1X and Ust2E (Tables 1 and 2), and PCR cloning and sequence analysis of 16S rDNA were performed as described previously (4). A species was determined when its sequence had greater than 90% homology to bacteria.

Alignment and primers

16S rDNA sequences of 10 strains of *S. anginosus*, 10 strains of *S. constellatus*, 5 strains of *S. intermedius* (Table 1) and other streptococcal species were aligned by Clustal W (13) to design specific primers for amplification of 16S rDNA of *S. anginosus*, *S. constellatus*, and *S. intermedius*. Primers Ust1 and Ust2 modified from Ust1X and Ust2E were used for amplification of the 16S rDNA gene of many oral bacteria (Table 2).

Quantitative real-time PCR

Real-time PCR was performed on the ABI Prism Sequence detection System 7700 (Applied Biosystems, Foster City, CA) using SYBR green chemistry. The reaction mixture in a total volume of 25 μl contained SYBR Green Core Regent (Applied Biosystems), 3 mM $MgCl_2$, 200 nM of each primer, and 5 μl of DNA solution. The reaction was started with an incubation of 2 min at 50°C, followed by 10 min at 95°C, then 50 cycles of 15 s at 95°C and 1 min at 68°C.

Statistical analysis

Differences in the levels of *S. anginosus*, *S. constellatus*, and *S. intermedius* DNA in the three age groups were statistically analyzed using the Mann–Whitney *U*-test.

Results

Distribution of *Streptococcus* in saliva

The diversity of the bacterial flora in saliva was examined in three age groups. Table 3 describes species that had more than 90% similarity to partial sequences of 16S rDNA obtained for clones of salivary DNA. In all, 119 of 192 clones were identified as *Streptococcus*. *Streptococcus* accounted for 93% of identified strains in 25–49-year-olds, 45% in 50–69-year-olds, and 58% in 70-year-olds. *S. mitis* was the most frequently detected species in all age groups, and the proportion decreased with increasing in age. *S. mitis* accounted for 76% of *Streptococcus* in subjects aged 25–49 years, 46% in 50–69-year-olds, and 43% in 70-year-olds. Diversity of bacterial species increased as age increased. *S. anginosus* was not detected in any age group. *S. constellatus* was the only species detected among the *S. anginosus* group.

Table 2. Primers used in this study

Primer	Purpose	Bacterial specificity	Sequence	Position[a]
F13	Real-time PCR	*S. anginosus*	CTAATACATGCAAGTAGG	48
F6	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTT	58
F8	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTC	58
R3	Real-time PCR	*S. anginosus*	CAAGCATCTAACATGTGTTAC	186
ConF2	Real-time PCR	*S. constellatus* (*S. intermedius*)	CACCGTAGTTTACTACACCGTATT	78
ConR4	Real-time PCR	*S. constellatus* (*S. intermedius*)	CTACCATGCAGTAAATGTTC	181
Ust1	Real-time PCR	Oral bacteria	GAACGGGTGAGTAACGCGTAGGT	106
Ust2	Real-time PCR	Oral bacteria	CACTCACGCGGCGTTGCTCGGTC	387
Ust1X	PCR cloning	Oral bacteria	GCTCTAGAGAACGGGTGAGTAACGCGTAGGT	106
Ust2E	PCR cloning	Oral bacteria	GGAATTCCACTCACGCGGCGTTGCTCGGTC	387

[a]The 5′ position in *Escherichia coli* 16S rDNA numbering convention.

Table 3. Bacterial species inferred from PCR cloning analysis of the saliva of healthy people in the three age groups

Species	No. of clones			
	25–49 years	50–69 years	70 years	Total
S. mitis	32	13	21	66
S. salivarius	2	0	7	9
S. infantis	3	3	6	12
S. sanguinis	1	4	2	7
S. parasanguinis	0	4	4	8
S. australis	0	2	5	7
S. cristatus	1	1	2	4
S. constellatus	0	1	0	1
S. anginosus	0	0	0	0
Unidentified *Streptococcus*	3	0	2	5
Other bacterium	3	34	36	73

Primer design

An alignment of *S. anginosus*, *S. intermedius*, *S. constellatus*, and other *Streptococcus* revealed a variety of *S. anginosus* strains in 16S rDNA (Fig. 1). In previous studies, a variable region was used as primer to detect *S. anginosus* (2, 3, 10). Three different forward primers were designed to determine if this variable region is suitable as an *S. anginosus*-specific primer (Fig. 1, Table 2). F6 and F8 primers are highly specific but include the variable region used for detecting *S. anginosus* in former studies (2, 3, 10). F13 primer is less specific but does not include this variable region. In order to maintain specificity to *S. anginosus*, these three forward primers were used with a specific reverse primer, R3 (Fig. 1, Table 2). The alignment also revealed a strong similarity between *S. intermedius* and *S. constellatus*, making it difficult to design specific primers to distinguish *S. constellatus* from *S. intermedius*. Therefore, a primer set (ConF2, ConR4) that detected both *S. constellatus* and *S. intermedius* was designed (Table 2).

Quantification system of *S. anginosus*, *S. constellatus*, and *S. intermedius*

Real-time PCR was used to analyze DNA extracted from *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sanguinis*, *and S. sobrinus* in order to examine quantification systems with new primer sets of *S. anginosus* and *S. constellatus* (*S. intermedius*). When 10^1–10^6 fg DNA from *S. anginosus* was added in serial dilutions to the reaction mixture for real-time PCR systems of *S. anginosus*, detection and quantification were linear over the range of the DNA concentration examined. The real-time PCR system of *S. constellatus* (*S. intermedius*) was examined in the same way, and a similar result was obtained. When 10^4 fg of DNA from other species were assayed, the calculated values derived from a standard curve were lower than the detectable level, 10^1 fg. It was therefore concluded that each system was specific for each species. Three *S. anginosus* forward primers were then examined to determine whether they were suitable for the *S. anginosus* quantification system. *S. anginosus* DNA in saliva samples was quantified, but the quantified values derived from each system were different. Figure 2 depicts quantified values of *S. anginosus* in the saliva from the 50–69-year-old group. The system using F6 and F8 primers often exhibited completely different values from one another (6 of 18 samples showed more than 10 times difference), and they were not proportional. The system using the F13 primer usually had the highest values, which were comparatively close to the second highest values. As these results suggested that F6 and F8 primers are too specific to quantify various types of *S. anginosus* present in the oral cavity, F13 was chosen as the primer to quantify *S. anginosus* in the present study.

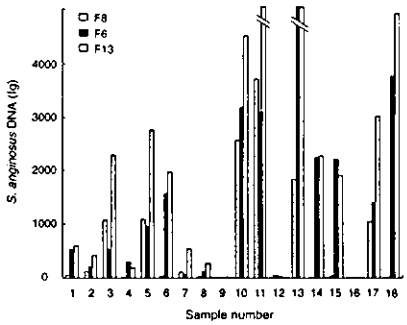

Fig. 2. Quantified values of *S. anginosus* DNA in 50–69-year-olds obtained using three different systems of real-time PCR. Ten ng of salivary DNA was used.

Quantification of *S. anginosus* and *S. constellatus* in saliva

The quantities of *S. anginosus*, *S. constellatus* (*S. intermedius*), and oral bacteria in saliva samples from 65 healthy people aged 25–70 years were determined, and the proportions of *S. anginosus* and *S. constellatus* (*S. intermedius*) in oral bacteria were calculated (Table 4). Results demonstrated that the average proportion of *S. anginosus* in oral bacteria increased with age: 0.38% at 25–49 years of age, 1.12% at 50–69 years, and 2.02% at 70 years. Statistical analysis indicated that the level was significantly higher at age 70 than at ages 25–49. Additionally, the oral bacteria in the saliva of 25% of 50–69-year-olds contained more than 2% *S. anginosus*, whereas the highest

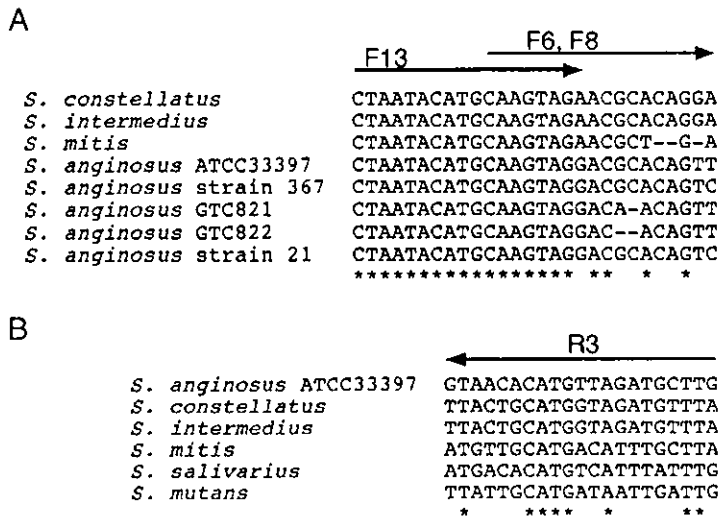

Fig. 1. Alignments of 16SrDNA of streptococcal species and *S. anginosus* strains. Arrows indicate primers. Stars represent identical nucleotides.

Table 4. Average proportion of *S. anginosus* or *S. constellatus* (*S. intermedius*) to oral bacteria in the three age groups

	Average age	Sample no.	*S. anginosus* (%)	*S. constellatus* (*S. intermedius*) (%)
25–49 years	33.2	17	0.38 ± 0.32	0.06 ± 0.04
50–69 years	58.3	18	1.12 ± 1.74	0.11 ± 0.26
70 years	70	30	2.02 ± 3.49	0.07 ± 0.09

level found in the 25–49-year-old group was only 1.7% (data not shown). In contrast, the average ratio of *S. constellatus* (*S. intermedius*) to oral bacteria was about 10–40 times lower than that of *S. anginosus*, with no significant difference between age groups.

Discussion

Previous studies demonstrated very low levels of *S. anginosus* in saliva (2). In the present study, the distribution of streptococcal species in the saliva of healthy people was determined by clonal analysis of 16S rDNA sequence. It was demonstrated that *S. anginosus* as well as other *S. milleri* group species, *S. constellatus* and *S. intermedius* were minor species in all three age groups. However, quantification of *S. anginosus* DNA demonstrated that most people possess *S. anginosus* to some extent in their saliva. These results were obtained with the use of our new primer.

The average level of *S. anginosus* in the oral bacteria increased with age, whereas the average level of *S. constellatus* (*S. intermedius*) did not change and the proportion of *S. mitis* in the oral bacteria decreased. *S. mitis* was the predominant oral streptococcal species in infants, both in its prevalence and in its proportion of the oral streptococci. It may thus be the major component of the initially colonizing streptococcal microbiota of infants (9). The trend toward a decreasing proportion of *S. mitis* might start from an early age. Viridans group streptococci, including *S. mitis*, are known to induce inflammation of renal tissue (15). However, at least in the oral cavity, a prevalence of *S. mitis* may indicate young healthy microflora. In contrast, *S. anginosus*, which increased with increasing age in inverse proportion to *S. mitis*, should be carefully monitored due to its association with various kinds of infectious diseases such as endocarditis and cancer in the upper digestive tract.

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