

Table 1 mAb (KH5, P126, P136) used in the follow-up clinical test was mixed at a ratio of KH5 : P136 : P126 = 1 : 1 : 1 (V/V) and dissolved in PBS buffer to yield a final concentration of 1.55 mg/mL

KH5 (anti PAC) in PBS	5.67 mg/mL
P136 (anti GTF) in PBS	5.03 mg/mL
P126 (anti GTF) in PBS	3.25 mg/mL

tray termed as a drug retainer was used to allow 1,400 μ L of mAb solution to react over the tooth surfaces for 5 minutes. Eating and drinking was prohibited for two hours after the treatment. This treatment was given once every three days, for 6 times in total. A total of 12.6 mg of protein was used in the drug retainer for each person. The results show²³⁾, the amount of MS in the saliva of the subjects reduced through the first and second trial. An unexpected finding was observed in the first trial. After the first trial, in 4 out of 7 subjects, the amount of MS dropped below the detection limit sooner than we expected^{12,23)}.

The result of this follow-up clinical test demonstrates that MS are eliminated with physicochemical treatment alone. Therefore, an antibody is not a prerequisite in eliminating MS but rather plays a secondary role.

A New Clinical Inspection System of MS

In a follow-up clinical test, we initially used an MSB agar plate²⁶⁾, which is a commonly used selective medium for the examination of MS. However, specificity is poor when used with to clinical saliva samples as the MSB agar allows growth of other bacteria and necessitates colony counting based on the difference seen in colony configuration by eye. For a systematic bacterial test, it is necessary to make the distinction using an automatic colony counter. This problem was solved by adding gramicidin into the MSB agar (BML Co. Ltd., Patent No. JP2002027975 ; modified MSB agar ; Tables 2, 3). The modified MSB agar is more reliable than the MSB agar (see Table 3 (C, D)). The inspection system of MS calculates the

ratio between MS and total streptococci by using a stimulated saliva sample. Based on these data, a new clinical inspection system of MS for general practice has been developed by BML Co. Ltd in Japan.

Dental Drug Delivery System (3DS)

An MS elimination system has not yet been established despite many studies because previous tests have not incorporated a protocol that includes the intensive administration of a simultaneous physicochemical method. As a physical elimination method, Axelsson's group²⁷⁾ in Sweden attempted to physically control MS with an approach centering on PMTC. They divided 187 subjects (13-year-old) with a high level of the MS in their saliva into three groups to under go a program focusing on a physical elimination method. However, the difference among the test groups was temporary, and it had disappeared six months later²⁸⁾. With the treatment focusing on a physical treatment, the reduction in the number of the MS was transient. On the other hand, Sandham's group²⁸⁾ performed a chemical elimination method with 33 adult subjects with chlorhexidine varnish. This resulted in the reduction of the MS to below the detection limit in 21 subjects (64%). The average period of time that the MS remained below the detection limit was 34.6 months. Chlorhexidine varnish application was administered an average of 3.14 times, and the elimination of the MS was unsuccessful in 12 subjects (36%). In this study, the application of very concentrated chlorhexidine (40%) is necessary due to its dilution by saliva. This application may result in mucous membrane sensitivity and may destroy any commensal bacterial flora²⁹⁾. We attempted to create a protocol by summarizing Axelsson's group²⁷⁾, Sandham's group²⁸⁾ and our follow-up clinical test findings without using antibodies. The careful destruction of the biofilm with PMTC, followed by a disinfectant regionally applied with custom trays to the tooth surfaces, eliminated MS from the oral cavity without decreasing the population of oral commensal bacteria (see Table 2). These physicochemical methods described above have been designated as the Dental Drug Delivery System (3DS)¹⁴⁾.

Table 2 Development of modified MSB—Bacterial changes over the whole period of the first trial (2 of 7 subjects (A—G) ; raw data G, F)

Results of the first trial of subject G (stimulated saliva samples)											
	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1 (6/12)	7.36E+08	8.87	2.66E+08	8.42	36.1	4.01E+05	5.60	0.15	4.28E+05	5.63	0.16
D1-2 (6/14)	4.37E+08	8.64	2.65E+08	8.42	69.6	1.14E+06	6.06	0.43	1.12E+06	6.05	0.42
D1-3 (6/15)	2.09E+08	8.32	1.57E+08	8.20	75.1	7.06E+05	5.85	0.45	5.38E+05	5.80	0.41
D1-4 (6/19)	1.04E+09	9.02	2.95E+08	8.47	28.4	1.24E+06	6.09	0.42	1.18E+06	6.07	0.40
D1-5 (6/20)	5.54E+08	8.74	2.14E+08	8.33	38.6	8.66E+05	5.94	0.41	1.05E+06	6.02	0.50
D2 (6/29)	5.61E+08	8.75	2.19E+08	8.34	39.0	2.00E+02	2.30	0.00009	1.00E+02	2.00	0.00005
D3 (7/21)	4.11E+08	8.61	2.76E+08	8.44	60.3	1.30E+03	3.11	0.0005	1.00E+03	3.00	0.0004
D4 (7/31)	7.23E+08	8.86	3.36E+08	8.53	46.5	1.80E+03	3.26	0.0005	2.50E+03	3.40	0.0007
D5 (8/14)	3.64E+08	8.56	1.59E+08	8.20	43.7	5.10E+02	2.71	0.0003	5.60E+02	2.75	0.0004
D6 (9/6)	2.99E+08	8.48	1.23E+08	8.09	41.1	1.02E+03	3.01	0.0008	1.32E+03	3.12	0.0011
D7 (10/18)	1.59E+09	9.20	1.00E+09	9.00	62.9	1.27E+04	4.10	0.0013	9.84E+03	3.99	0.0010

Results of the first trial of subject F (stimulated saliva samples)											
	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1 (6/12)	7.70E+08	8.89	2.96E+08	8.47	38.4	7.50E+04	4.88	0.028	7.50E+04	4.88	0.028
D1-2 (6/14)	5.62E+08	8.75	1.39E+08	8.14	24.7	8.40E+04	4.92	0.032	1.50E+04	4.18	0.006
D1-3 (6/15)	6.87E+08	8.84	2.25E+08	8.35	32.8	3.20E+04	4.51	0.020	4.50E+04	4.65	0.035
D1-4 (6/19)	8.50E+08	8.93	1.51E+08	8.18	17.7	6.20E+04	4.79	0.021	6.00E+04	4.78	0.020
D1-5 (6/20)	3.27E+08	8.51	1.47E+08	8.17	45.0	8.50E+04	4.93	0.040	1.16E+05	5.06	0.054
D2 (6/29)	2.73E+08	8.44	1.29E+08	8.11	47.2	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D3 (7/21)	3.06E+08	8.49	1.79E+08	8.25	58.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D4 (7/31)	1.02E+09	9.01	1.28E+08	8.11	12.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D5 (8/14)	4.65E+08	8.67	8.21E+07	7.91	17.7	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D6 (9/6)	1.97E+08	8.29	5.02E+07	7.70	25.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D7 (10/18)	4.28E+08	8.63	2.02E+08	8.31	57.3	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00

Blood agar ; total bacteria, MS agar ; total streptococci, MSB and modified MSB agar ; mutans streptococci (MS).

B/A ; total streptococci/total bacteria. C/B ; MS/total streptococci. D/B ; MS/total streptococci. Bacterial examination before physicochemical treatment ; D1-1 to D1-5. Bacterial examination after physicochemical treatment ; D2 to D7. Actual days are indicated in the parenthesis.

This consists of two phases : (1) physical destruction of the biofilm, and (2) chemical elimination of the remaining bacteria using custom trays (a drug retainer). Since the detection of MS is very rare in the predentate group, MS are considered to proliferate over the tooth surfaces rather than mucous membranes and saliva³⁰. Therefore, MS have an ecological localization in the oral cavity. If a non specific disinfectant is delivered regionally to the tooth surfaces, it is

possible to selectively reduce the MS population without damaging the commensal bacteria on the mucous membrane and saliva. Based on the follow-up clinical test, we concluded that it was possible to eliminate MS using commercial disinfectants. Our finding has become the foundation for the development of a more practical system (3DS unit). 3DS differs from FMD which does not utilize a custom tray. FMD for periodontal diseases should not be used in the prevention

Table 3 Development of modified MSB—Bacterial changes over the whole period of the second trial (2 of 7 subjects (A—G) ; raw data of A, D)

Results of the second trial of subject A (stimulated saliva samples)												
	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)			
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)	
D1-1 (7/2)	8.20E+07	7.91	3.20E+07	7.51	39.02	5.10E+02	2.71	0.00	1.50E+03	3.18	0.00	
D1-2 (7/5)	6.10E+07	7.79	2.20E+07	7.34	36.07	3.10E+03	3.49	0.01	3.40E+03	3.53	0.02	
D1-3 (7/6)	3.40E+07	7.53	1.80E+07	7.26	52.94	4.50E+03	3.65	0.03	5.00E+03	3.70	0.03	
D1-4 (7/7)	1.80E+07	7.26	7.80E+06	6.89	43.33	1.40E+03	3.15	0.02	4.60E+03	3.66	0.06	
D1-5 (7/8)	4.00E+07	7.60	9.40E+06	6.97	23.50	2.70E+03	3.43	0.03	4.70E+03	3.67	0.05	
D2 (6/27)	2.50E+07	7.40	1.40E+07	7.15	56.00	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D3 (7/18)	4.57E+07	7.66	1.69E+07	7.23	36.98	0.00E+00	0.00	0.00	5.10E+01	1.71	0.00	
D4 (7/28)	3.50E+07	7.54	1.10E+07	7.04	31.43	0.00E+00	0.00	0.00	1.52E+02	2.18	0.00	
D5 (8/15)	3.82E+07	7.58	8.33E+06	6.92	21.81	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D6 (9/4)	6.00E+07	7.78	1.46E+07	7.16	24.33	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D7 (10/16)	4.52E+07	7.66	9.82E+06	6.99	21.71	2.54E+02	2.40	0.00	1.52E+02	2.18	0.00	

Results of the second trial of subject D (stimulated saliva samples)												
	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)			
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)	
D1-1 (7/2)	5.60E+07	7.75	1.80E+07	7.26	32.14	1.80E+03	3.26	0.01	2.80E+03	3.45	0.02	
D1-2 (7/5)	8.70E+07	7.94	3.20E+07	7.51	36.78	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D1-3 (7/6)	6.50E+07	7.81	1.70E+07	7.23	26.15	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D1-4 (7/7)	7.90E+07	7.90	2.90E+07	7.46	36.71	0.00E+00	0.00	0.00	5.00E+01	1.70	0.00	
D1-5 (7/8)	7.20E+07	7.86	2.00E+07	7.30	27.78	0.00E+00	0.00	0.00	5.00E+01	1.70	0.00	
D2 (6/27)	9.10E+06	6.96	6.80E+06	6.83	74.73	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D3 (7/18)	1.22E+08	8.09	6.61E+07	7.82	54.18	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D4 (7/28)	5.60E+07	7.75	1.40E+07	7.15	25.00	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D5 (8/15)	4.27E+07	7.63	1.55E+07	7.19	36.30	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D6 (9/4)	9.86E+07	7.99	2.80E+07	7.45	28.40	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	
D7 (10/16)	1.25E+80	8.10	2.17E+07	7.34	17.37	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00	

See Table 2 for explanation

of a dental caries epidemic.

Clinical Use of 3DS (3DS Unit)

Outpatients at risk of having a high ratio of MS received a clinically modified 3DS treatment, called a 3DS unit. The 3DS unit is indicated in the diagram (Fig. 2). 41 subjects who had consented to receive a 3DS unit treatment were involved. The MS population and their ratio were evaluated before and after 1 cycle of the 3DS unit. The conditions for the initia-

tion of the 3DS unit treatment were an MS number greater than 1×10^5 CFU/mL and/or an MS ratio (number of MS/total streptococci $\times 100$) of over 2%. The conditions for terminating the treatment were an MS number less than 1×10^4 CFU/mL and/or an MS ratio of less than 0.2% (data in preparation). PMTC was performed prior to the application of 0.2% chlorhexidine gel (Fig. 3). Subjects were instructed to refrain from sucrose ingestion immediately after the administration of 3DS. After 1 cycle of 3DS unit treatment, MS number and MS ratio were suppressed to

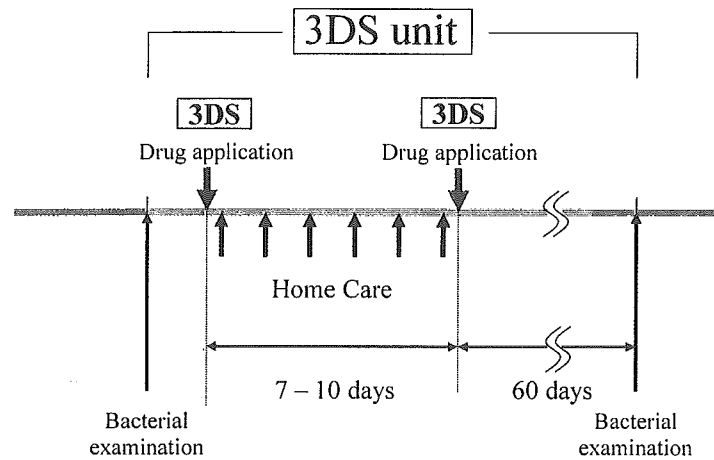


Fig. 2 Clinical protocol of 3DS

At first, bacterial examination must be carried out to decide on appropriate application of 3DS. Second, PMTC is carried out for the physical elimination of biofilm. Third, as a chemical treatment, 0.2% chlorhexidine gel is applied via a customized tray called a drug retainer that fits onto the dentition for 5 minutes. A 3DS unit involves 2 chemical treatments over approximately 1 week. The subjects use the drug retainer once a day for 5 minutes. After 60 days, bacterial examination was reperformed.

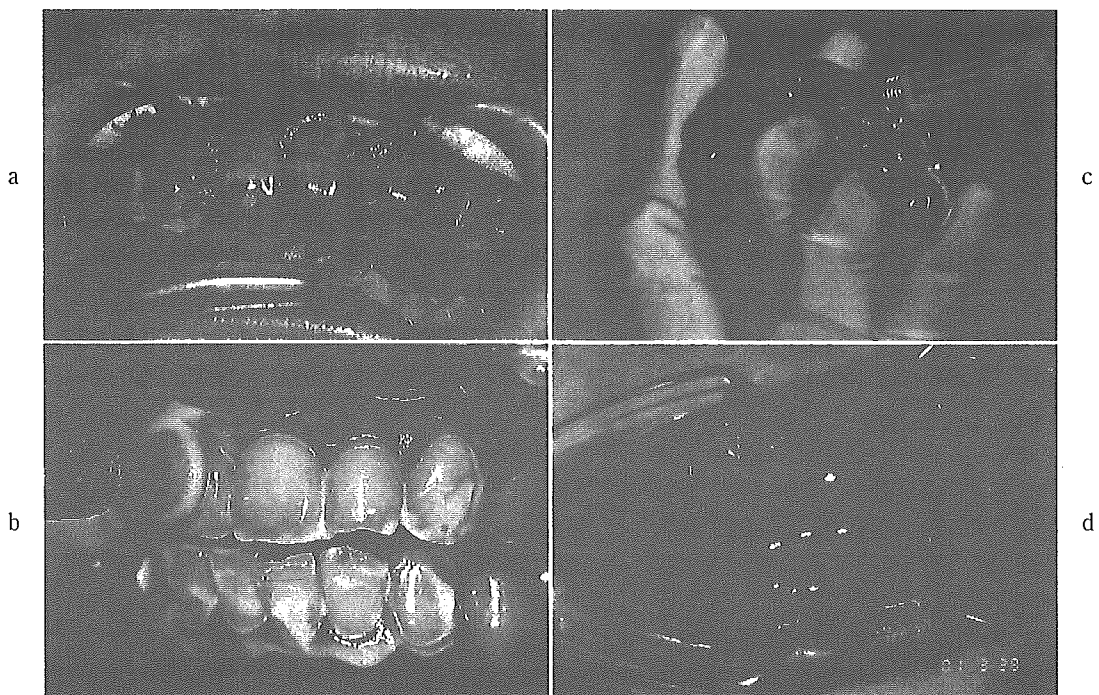


Fig. 3 Partial view of 3DS

a : Disclosing tooth surfaces. b : PMTC. The device destroys dominant biofilms and makes bacteria susceptible to contact with drugs. c : A custom tray (drug retainer) with 0.2% chlorhexidine gel. d : Fitting the tray onto tooth surfaces for 5 minutes. The drug retainer delivers the disinfectant directly on to tooth surfaces.

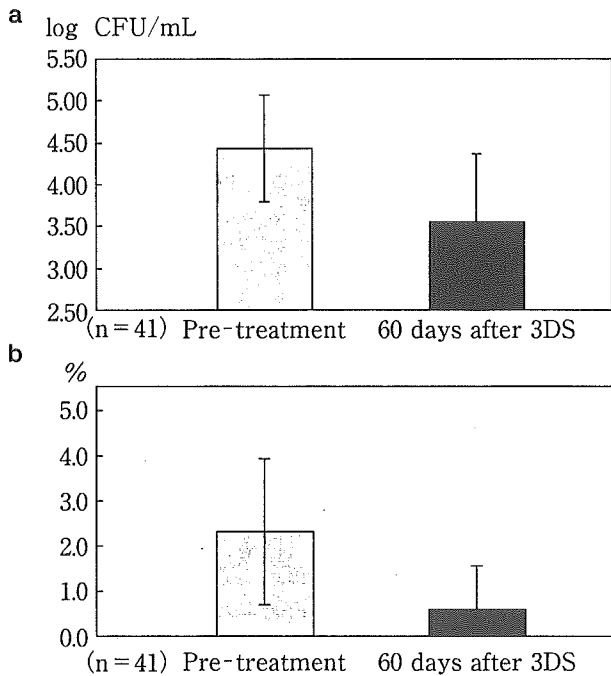


Fig. 4 Bacterial changes before and after the 3DS unit

Numbers (a) and ratios (b) of MS at the pre-treatment stage and 60 days after the final treatment of 3DS.

below the risk values ($p < 0.05$) as compared to the values pre-treatment (Figs. 4, 5). However, the number of total streptococci before and after a cycle of 3DS unit treatment commonly remained at the same level (Fig. 5). Destruction of commensal bacteria and unpleasant oral symptoms were not observed in any of the subjects.

A Paradigm Shift in the Role of Caries Vaccine

Although the ability of initial attachment of MS is lower than for oral commensal bacteria, it is possible for MS in saliva to resettle over tooth surfaces. Since it is beyond the scope of 3DS to prevent a resettlement of MS, the development of immunological methods has become necessary. Specific antibodies can effectively bind and block this kind of process. Dental caries is not a lethal illness, and any side effects of the vaccine will always pose a problem to the use of an active immunity method. In 1976, a cross reaction between human cardiac muscles and antibody derived

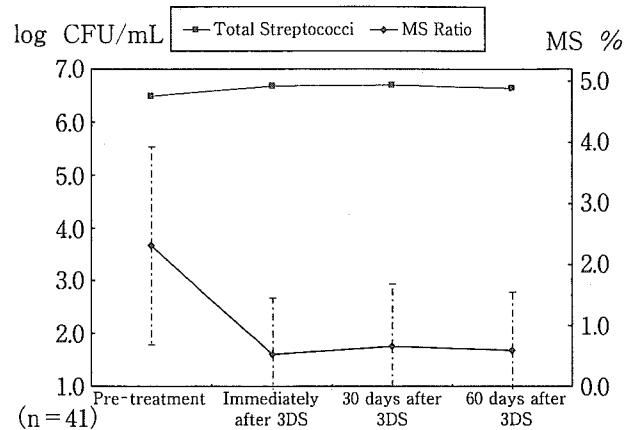


Fig. 5 Bacterial changes over the whole period of 3DS unit

This graph shows the clinical result of 3DS treatment for MS. The salivary bacterial samples were taken at each stage of pre-treatment, immediately after 3DS, 20 days after 3DS, and after 60 days. The y-axis on the left side shows the amount of total streptococci (CFU/mL), and on the right side shows the ratio of MS (%). The ratio of MS was apparently reduced after the 3DS treatment, and was still at a low level after 60 days. However, the total number of streptococci remained at the same level during the whole stage.

from the immunity against whole MS cells was reported³¹). In the active immunity approach, a peptide antigen, which is an antigenic epitope and the smallest unit of a adhesive protein molecule, is a safe consideration. After suppressing the formation of biofilm over the tooth surfaces by using 3DS, a steady secretion of inhibitory antibodies can suppress the process of resettlement of MS to the tooth surfaces and colonization. Antibody proteins remain effective for 72 hours by non-specifically coupling to the surface layer of the enamel via the pellicle, with intermolecular and van der Waals forces²⁶). During this process, MS are specifically bound and absorbed by mAb²²). However, the current viewpoint states that antibodies themselves may prevent the initial adhesion of planktonic bacteria. Therefore, antibodies should be present in the saliva at all times to adhere to and absorb planktonic MS to prevent their adhesion to tooth surfaces. Antibody characteristics were

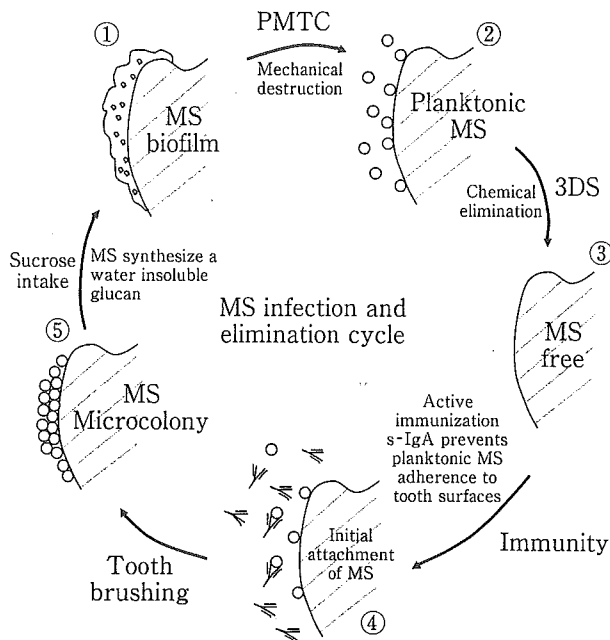


Fig. 6 A schematic diagram of the MS infection and elimination cycle

The MS biofilm covering tooth surfaces is destroyed by PMTC (1). Planktonic MS remains on the tooth surface (2). 3DS eliminates the remaining MS (3). Antibodies absorb against planktonic MS and protect the tooth surface from resettlement of the MS (4). The MS escape from the antibodies to form microcolonies (5). MS synthesize a water insoluble glucan catalyzed by glucosyltransferase (GTF) using sucrose as a substrate to form a cariogenic biofilm (1).

not utilized to their full potential because they were used inside a tray. On the other hand, a disinfectant should only be administered regionally to the tooth surface, and not to the full mouth. Since we have developed an approach to remove the biofilm formed over the tooth surfaces using 3DS, we have succeeded in creating an environment where antibodies can work effectively. The next strategy is to prevent the initial adhesion of MS. If 3DS becomes widely used, the actual need to develop a vaccine will become more pressing as antibodies work more effectively against planktonic bacteria (Fig. 6). A combination of passive immunity as well as active immunity will be most desirable.

Conclusion

We have shown that it is possible to eliminate the MS that have adhered to tooth surfaces using 3DS. However, to effectively eliminate MS altogether, it is important to prevent the resettlement of MS to tooth surfaces. In particular, the synergistic effect of combined physicochemical and immunological treatments is desirable for the effective inhibition of MS.

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Original

Effect of 3.8% Ag(NH₃)₂F Solution as an Anti-carries Agent on Dentin in Artificial Mouth Model System using *Actinomyces naeslundii*

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Abstract : The object of this study was to evaluate the effect of 3.8% and 38% Ag(NH₃)₂F solutions as a caries-preventive agent on dentin surfaces in the artificial mouth model system. A total of 36 bovine dentin slabs were divided into three groups : 3.8% Ag(NH₃)₂F and 38% Ag(NH₃)₂F application groups and a control group. *Actinomyces naeslundii*, selected as having an important role in dentin caries, was used in this experiment. Several parameters concerning caries development were evaluated by the following methods : the amount of artificial biofilm, the changes in pH, the changes in dentin microhardness measured by a hardness tester, and element quantitative analysis for silver and fluorine elements by an energy dispersive X-ray micro analyzer. The dentin specimens in the 3.8% and 38% Ag(NH₃)₂F application groups had significantly less hardness reduction than the specimens in the control group ($p < 0.05$). Little difference, however, was observed for the dentin hardness reduction between the two test solution groups. The amounts of silver and fluorine elements on the specimen surface between the two groups were not significantly different after a lapse of 60 hours. The outcomes in this study suggest that 3.8% Ag(NH₃)₂F solution might have similar effectiveness as 38% Ag(NH₃)₂F solution as a preventive agent for dentin caries.

Key words : Ag(NH₃)₂F, *Actinomyces naeslundii*, Dentin microhardness, Element quantitative analysis, Artificial mouth system

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Introduction

With the increase of the dentate elderly, root caries has become an important clinical issue in dentistry. In spite of emerging as a common cause of tooth loss in the elderly, the initiation and progression of root caries remains unclear^{1,2)}. In contrast, the caries lesion on enamel has been studied thoroughly. Moreover, many studies have shown that various fluoride compound applications prevent caries and retard caries lesion progress³⁻⁶⁾. However, the prevention of root caries by fluorides has been supported by little evidence in past studies, due to compositional and anatomical complexities of dentin^{7,8)}.

The 3.8% and 38% of silver diamine fluoride solutions,

Ag(NH₃)₂F, were developed in Japan⁹⁾. The 38% Ag(NH₃)₂F solution has been studied mostly in Japan and used in Asian countries as a caries arresting agent¹⁰⁾ and a dentin desensitizing agent¹¹⁾. The 3.8% Ag(NH₃)₂F solution, however, was approved only as a root canal treatment agent by the Ministry of Health, Labour and Welfare in Japan in 1979^{12,13)}.

Therefore, we decided to examine of the anti-cariologic effect of two different concentrated Ag(NH₃)₂F solutions on dentin caries *in vitro*. The purpose of this study was to evaluate and to compare the anti-carries effect of the 3.8% and 38% Ag(NH₃)₂F solutions in an artificial mouth model system.

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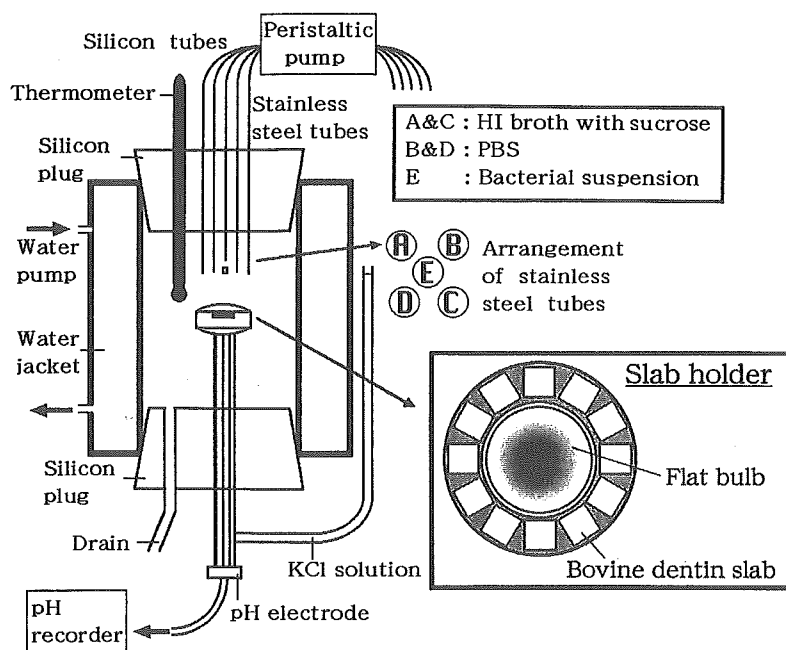


Fig. 1 A diagram of the artificial mouth model system

Materials and Methods

1. Reagent

The 3.8% and 38% of silver diamine fluoride solutions, $\text{Ag}(\text{NH}_3)_2\text{F}$ (Saforide RT and Saforide, Bee Brand Medico Dental Co. Ltd., Osaka, Japan), were used in this experiment. The 38% $\text{Ag}(\text{NH}_3)_2\text{F}$ solution contains 30 w/v % of AgF and approximately 8 w/v% of NH_3 ⁹.

2. Preparation of bacterial broths

Actinomyces naeslundii JCM 8349 was selected. Following pre-culture, *A. naeslundii* was anaerobically incubated in a brain heart infusion broth (BHI : Difco Laboratories, Detroit, MI) at 37°C for 24 hours. The cell suspension of bacteria in 0.1 M phosphate-buffered saline (PBS) solution was prepared to optical density at 500 nm (OD_{500}) = 2.0 (5.0×10^8 colony forming units/ml).

3. Preparation of dentin slabs

Bovine dentin slabs, 3.5 × 3.5 × 1.2 mm in size, with flat surfaces polished by a 1 micron grade polishing film, were prepared from bovine unerupted lower incisors cleaned of soft tissue debris. All slabs were sonicated at 11 W for 20 seconds for removal of debris from the dentinal tubules. Then, all slabs were measured for hardness at nine symmetrical points on the test surface of

each slab with a diamond Vickers indenter on a micro-hardness tester loaded with 50 g or 100 g (Hardness Tester MVK-E, AKASHI, Japan). The mean and standard deviation of dentin hardness of each slab were calculated as a baseline. Dentin slabs of 55 to 65 in average Vickers hardness number were used in this experiment. Finally, 36 dentin slabs were selected.

4. Treatment groups

A total of 36 dentin slabs were equally divided into two experimental groups and a control group. One application of 38% $\text{Ag}(\text{NH}_3)_2\text{F}$ for 10 seconds by a cotton-tipped swab was applied to 12 dentin slabs dried out by an air-syringe prior to placement in the artificial mouth. In the same preparation, three applications of 3.8% $\text{Ag}(\text{NH}_3)_2\text{F}$ at 12 hour intervals were applied to 12 dentin slabs prior to placement in the artificial mouth. The remaining 12 dentin slabs served as controls and did not receive application of 38% $\text{Ag}(\text{NH}_3)_2\text{F}$ solution.

5. Artificial mouth model system and sampling

Three identical artificial mouths were used. Figure 1 shows a diagram of an artificial mouth. Each artificial mouth consisted of an 18 mm diameter Teflon holder around a 9 mm flat bulb of a pH electrode. The pH electrode was set upside down in the center of the artificial

mouth constantly supplied with Heart Infusion broth medium (HI : Difco Laboratories, Detroit, MI) supplemented with 2.5% sucrose, PBS, and *A. naeslundii* suspension at 37°C for 60 hours, continuously dropped at 3 ml per hour per tube. There were five tubes in the artificial mouth system ; two for HI medium, two for PBS, and one for bacterial suspension. The prepared dentin slabs and control slabs were seated on the Teflon holder. Change in pH underneath artificial biofilm was automatically recorded. All procedures were carried out under aseptic conditions. Three slabs at each interval were sampled for post-examinations at 24, 36, 48, and 60 hours.

6. Determination of total amount of artificial biofilm

The specimens were sonicated in 1 ml of PBS at 11 W for 10 seconds for removal of artificial biofilm. Optical density of artificial biofilm at 540 nm was measured to assess the accumulated artificial biofilm on surface of dentin specimens (Double-beam Spectrophotometer, UV-190, SHIMADZU, Japan).

7. Measurement of microhardness

The change in microhardness for each dentin specimen after exposure to the bacterial environment for each interval was measured as already described. The change in dentin hardness (Δ DH) between baseline and post-experiment hardness values for the control and test specimens was calculated [Δ DH = (baseline-hardness) - (post-hardness)]. Next, the percentage of changed hardness (%CDH) was calculated [%CDH = Δ DH \times 100 / (baseline-hardness)].

8. Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis

After sonication with 11 W for 10 seconds, 24 specimens were prepared for scanning electron microscopy and element quantitative analysis. They were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffered saline solution (pH 7.4) for 1 hour at room temperature. Each fixed specimen was then dehydrated through a graded ethanol series until a value of 99.5% was reached, and dried in a critical point dryer (HCP-2, Hitachi, Japan). Next, they were osmium plasma coated by a Neoc Pure Osmium Coater (Meiwa Shoji, Japan) for element quantitative analysis using an energy dispersive X-ray micro analyzer (EMAX EX-

220, Horiba, Japan) and for scanning electron microscope observation (S-5200, Hitachi, Japan). Energy dispersive X-ray micro analyzer was performed at an acceleration voltage of 10 kV and magnification of \times 5000.

9. Statistical analysis

A One-Way Analysis of Variance and Tukey Post-hoc Test were performed to assess the differences found in the outcomes among control group and two treatment groups at each interval. Moreover, Student's t-test was done for the results of the element quantitative analysis (Statcel, 2nd ed., OMS, Saitama, Japan).

Results

1. The change in pH

Figure 2 expresses the change in pH on an electrode covered with accumulated artificial biofilm in each artificial mouth. Compared to the control whose terminal pH was 5.93, the terminal pHs of the 3.8% and 38% Ag (NH₃)₂F solution groups had inhibited decline, and were 6.09 and 6.44 respectively.

2. The change in total amount of artificial biofilm

Figure 3 shows the amount of artificial biofilm accumulation on specimens. There was statistically significantly less artificial biofilm accumulation in the experimental groups compared to the control group. On the other hand, there was no statistical difference between 3.8% and 38% Ag (NH₃)₂F solution groups.

3. The change in dentin microhardness

Figure 4 shows the change in microhardness of dentin at each interval in the artificial mouth system. Terminal changed dentin hardness of specimens in the control group became more than 75% ($75.87 \pm 2.52\%$), while the figures for the 3.8% and 38% Ag (NH₃)₂F solution groups were more than 50% ($51.94 \pm 3.77\%$) and 30% ($32.53 \pm 1.76\%$) respectively. For specimens treated with Ag (NH₃)₂F solutions, there was statistically significantly less reduction of dentin hardness than in the control group. However, there was no statistically significant difference at most of the intervals between the specimens treated with 3.8% Ag(NH₃)₂ and 38% Ag(NH₃)₂ solutions, except for the value of reduced hardness at 60 hours.

4. Silver and fluorine elements microanalysis

Figure 5 presents an example of the images of element quantitative analysis by the energy-dispersive X-

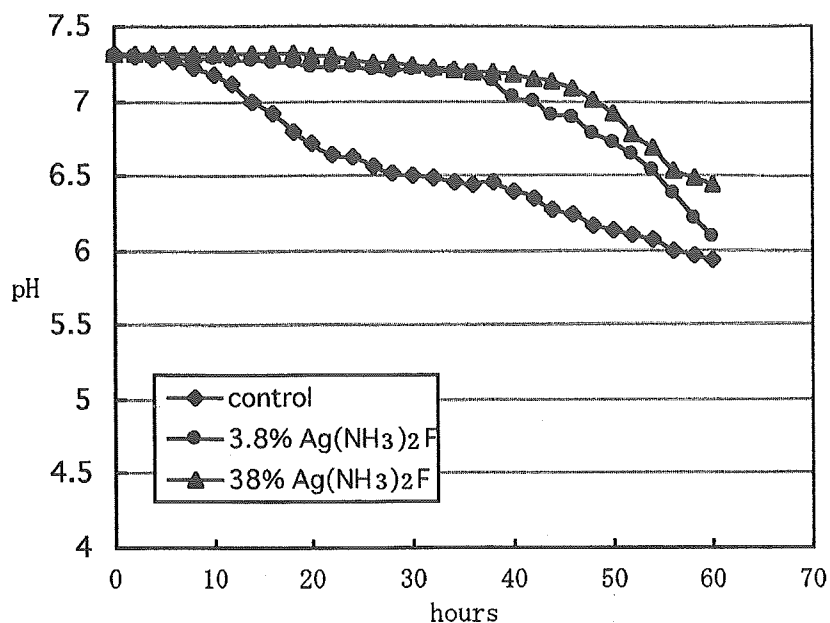


Fig. 2 The change in pH underneath artificial biofilm on an electrode in each artificial mouth.

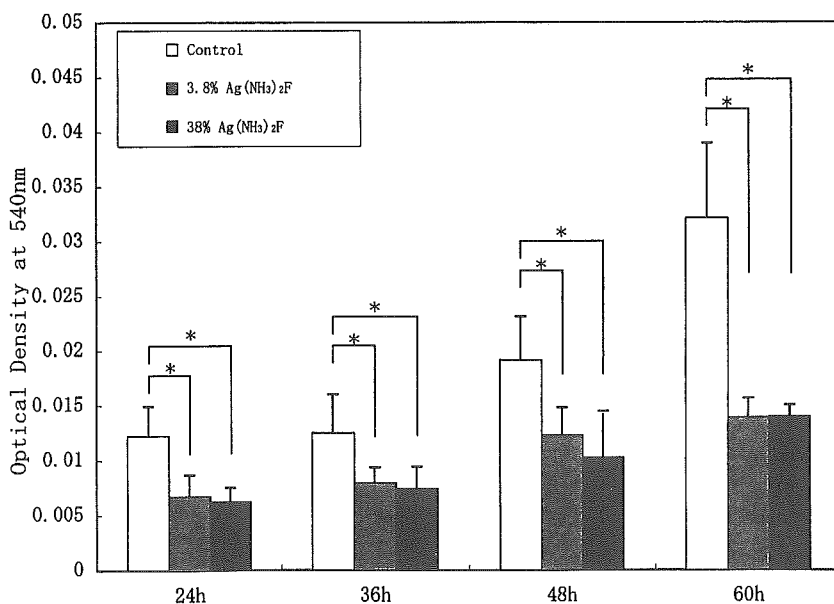


Fig. 3 Means and standard deviations of change in total amount of artificial biofilm at each interval, 24, 36, 48, and 60 hours

*Denotes a significant relationship at the 0.05 level

ray micro analyzer. Table 1 shows the percentage of weight of residual silver and fluorine elements on the specimen surfaces at 0, 24, 48, and 60 hours. The amounts of silver elements on the specimen surface of

the two groups were not significantly different after a lapse of 60 hours. Also there was mostly no statistically significant amount of residual fluorine from the 3.8% and 38% Ag(NH₃)₂F applications on the dentin surfaces.

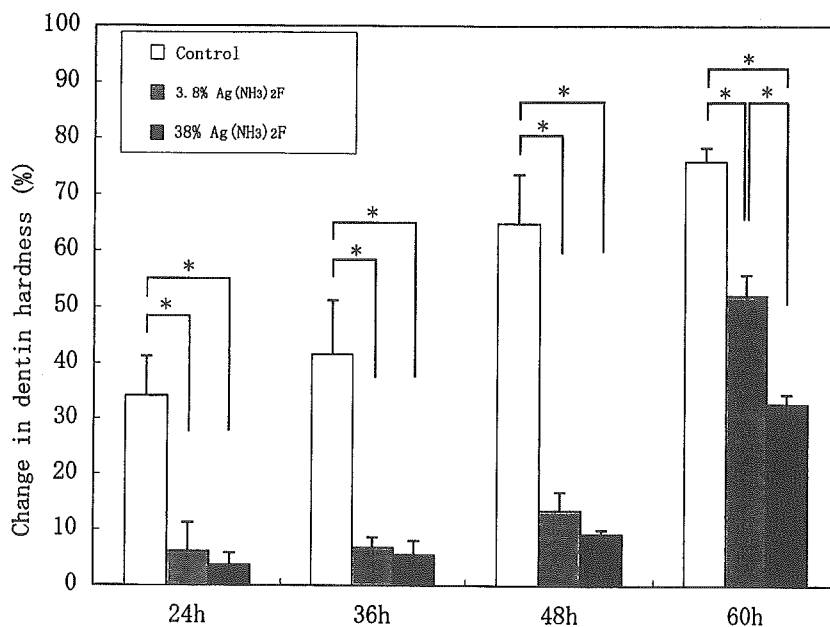
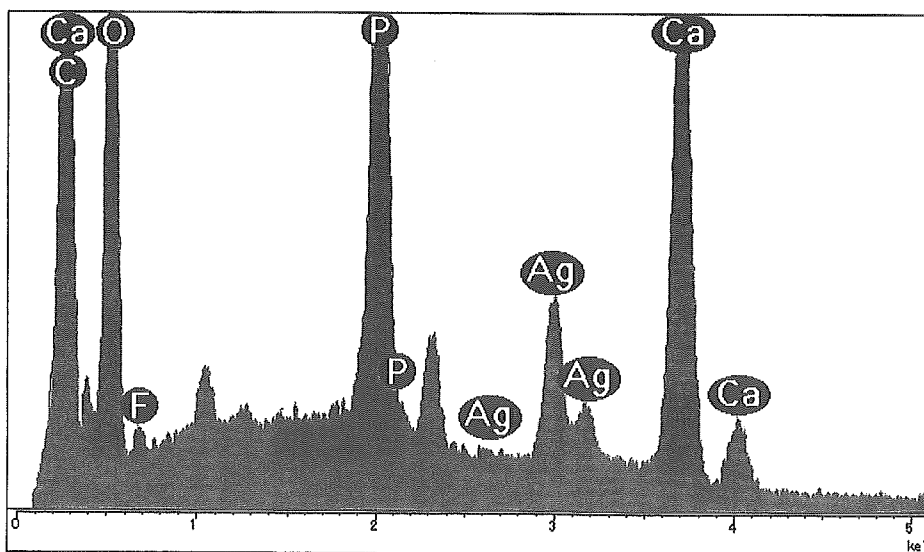


Fig. 4 The percentage of means and standard deviations of changed dentin microhardness for 3.8% Ag(NH₃)₂F, 38% Ag(NH₃)₂F, and control at each interval, 24, 36, 48, and 60 hours
*Denotes a significant relationship at the 0.05 level



Elements	C	O	Ca	P	F	Ag	total
Weight %	19.95	27.61	24.63	13.13	1.52	13.17	100

Fig. 5 An example of the images of element quantitative analysis for silver and fluorine elements by an energy dispersive X-ray micro analyzer
Specimen with 38% Ag(NH₃)₂F (48 hours) : full scale 792, count cursor : 5.012 keV

Table 1 Means and standard deviations of residual silver (A) and fluorine (B) (w%) on specimens

A : Silver				
	0 hour	24 hours	48 hours	60 hours
3.8%Ag (NH ₃) ₂ F	8.77 ± 2.52	1.78 ± 0.23	2.00 ± 1.32	1.70 ± 1.68
38%Ag (NH ₃) ₂ F	16.2 ± 3.22	13.30 ± 5.12	13.32 ± 1.09	2.68 ± 1.49
B : Fluorine				
	0 hour	24 hours	48 hours	60 hours
3.8%Ag (NH ₃) ₂ F	14.33 ± 4.00	10.36 ± 1.18	3.45 ± 0.47	2.56 ± 0.43
38%Ag (NH ₃) ₂ F	14.43 ± 4.91	11.17 ± 3.32	2.24 ± 0.33	2.02 ± 1.00

* Denotes a significant relationship at the 0.05 level

Discussion

1. Artificial mouth system and bacteria used

Artificial mouth laboratory models were developed in the late 19th century. Since then, artificial mouth model systems have been investigated and improved by overcoming several problems, such as contamination of the system and difficulty to mimic complicated human oral cavity¹⁴⁾. Moreover, as ethical problems related to studying dental caries in humans have recently been raised, the artificial mouth model systems have been useful not only to investigate the etiology of dental caries, but also to seek preventive methods. Thus, the use of artificial mouth systems for dental caries experiments has been extremely useful¹⁵⁾.

The artificial mouth model system in our laboratory was developed in 1984¹⁶⁾. The characteristic of it is that the changes in pH in the artificial biofilm are constantly measured with a glass electrode. Many experiments to evaluate anti-cariogenic food and medicine with this system have been done in our laboratory. The results from the repeated experiments have had good reproducibility of the pH curves¹⁷⁾. Thus, it is clearly suggested that this system has reliability as an *in vitro* system.

Actinomyces spp. has been implicated as a root caries pathogen although their initiation and progression have not been fully understood¹⁸⁾. From those results, however, the control group finally reached pH 5.93, which was below a critical point of dentin decalcification, and had more than 75% (75.87 ± 2.52) of dentin hardness reduction after a lapse of 60 hours. Moreover, when pH of

the control group reached below pH 6.2 after a lapse of 48 hours, more dentin hardness reduction occurred. Similarly, drastic hardness reduction started at around pH 6.3 for both experimental groups. Although those pH values might not demonstrate accurate pHs located on the specimens in our experimental model, this finding verifies previous studies that *A. naestlundii* itself is capable of producing acidic substances to decalcify dentin structure.

2. Silver diamine fluoride : Ag(NH₃)₂F

Silver has been used as a disinfectant with a long history. The efficacy of silver includes anti-bacterial, antiviral and anti-fungal effects. Silver is thought to be non-toxic in humans at standard rates of consumption. Moreover, the efficacy of the silver compound for periodontal pathogens has been reported recently^{19,20)}.

Ag(NH₃)₂F was developed by Yamaga et al. in 1966. The safety and toxicity of 38% Ag(NH₃)₂F solution has been well-evaluated and it has been proven to be a safe anti-caries agent^{21,22)}.

The primary purpose of development of 38% Ag(NH₃)₂F solution was to reduce deciduous tooth caries lesions clinically²³⁾. Chu et al. discussed the effectiveness of 38% Ag(NH₃)₂F solution comparing to NaF varnish for deciduous teeth of Chinese pre-school children. They indicated that the examiner could not distinguish in post-experiment evaluation either application of NaF varnish or Ag(NH₃)₂F solution, because all arrested caries lesions on the subjects were similar; that is, the appearance of arrested dentin caries seemed to be blackening and hardening. Moreover, they concluded that an-

nual application of 38% Ag (NH₃)₂F solution for pre-school children was more effective in hardening or arresting dentin caries lesions in upper anterior deciduous teeth than the three-month applications of a 5% NaF varnish²⁰.

The clinical use of 3.8% Ag (NH₃)₂F solution is a medication for root canal treatment. The studies have revealed that 3.8% Ag (NH₃)₂F solution has capability to occlude dentinal tubules and to resist oral bacterial growth^{25,26}. We recognized the advantage of this characteristic to treat flat dentin surfaces.

From this experiment, outcomes show that the percentage of the reduction of post-hardness of 3.8% Ag (NH₃)₂F and 38% Ag(NH₃)₂F groups were not significantly different although the hardness of 3.8% and 38% Ag (NH₃)₂F-coated specimens were remarkably maintained compared to the hardness of controls. Moreover, artificial biofilm formation with test solutions was significantly inhibited due to the anti-bacterial effect of silver. Furthermore, residual elements of silver and fluorine on the dentin surfaces with both test solutions were not significant after a lapse of time. Thus, it indicates that 3.8% Ag (NH₃)₂F solution might have a similar effectiveness to 38% Ag (NH₃)₂F solution as an anti-caries agent.

The overall impression from this study is that the usage of 3.8% Ag (NH₃)₂F solution may be one of the solutions for the prevention of dentin caries, especially for the dentate elderly and persons with difficulty in proper home care. It might be expected that 3.8% Ag (NH₃)₂F solution would be less harmful to oral soft tissue and less staining on teeth. Future investigations are expected to determine whether 3.8% Ag (NH₃)₂F solution is clinically suitable for the prevention of root caries and to define the frequency of application of 3.8% Ag (NH₃)₂F solution to get maximum efficacy. Moreover, its capability and limitation for the root-caries-preventive effect need to be investigated. This study, however, may help develop clinical strategies to prevent the initiation and progression of root caries.

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Actinomyces naeslundii を用いた人工口腔装置における 3.8% フッ化ジアミン銀の象牙質う蝕抑制効果

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概要 : 有歯顎高齢者が増加している今日にあって、加齢や歯周病の進行などの原因により根面露出した歯は、潜在的に根面カリエス罹患のリスクが高くなるといわれている。Actinomyces naeslundii は、根面カリエスの重要な原因菌のひとつと考えられている。本研究は、A. naeslundii をう蝕誘発菌として使用し、人工口腔装置内における3.8および38% フッ化ジアミン銀水溶液の象牙質う蝕抑制効果を比較検討することを目的とした。

36個のウシ象牙質歯片を3.8% フッ化ジアミン銀水溶液3回塗布群および38% フッ化ジアミン銀水溶液1回塗布群とコントロール群の3つのグループに分け、人工口腔装置中で60時間観察した。う蝕抑制効果の評価として、pH測定、象牙質硬度測定、人工バイオフィーム蓄積量の測定、象牙質表面における残留銀量およびフッ素量の微小X線元素分析を経時的に行った。

その結果、3.8および38% フッ化ジアミン銀水溶液塗布群はコントロール群と比べ、著しく人工バイオフィームの蓄積と象牙質の硬度減少を抑制することがわかった。一方、3.8と38% フッ化ジアミン銀水溶液塗布群間には統計学的な有意差はみられなかった。また、残留銀およびフッ素の微小X線元素分析の結果により、60時間経過後の銀とフッ素の残留量は、3.8と38%のフッ化ジアミン銀水溶液塗布群間に有意差がないことが明らかとなった。

以上のことから、3.8% フッ化ジアミン銀水溶液に38% フッ化ジアミン銀水溶液と同等の象牙質う蝕抑制効果が期待されることが示唆された。

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索引用語 : フッ化ジアミン銀, Actinomyces naeslundii, 象牙質硬度測定, 元素分析, 人工口腔装置

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Relationships between Breath Odors and Odor Emission Sources in the Elderly Investigated using the Electronic Nose

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Abstract : The aim of this study is to compare breath odors and odor emission sources (saliva, tongue coat, and dentures in current use) of the elderly and to define the relationships between the odors. Fifteen patients visiting the outpatient clinic, Tokyo Medical and Dental University Geriatric Dentistry, were recruited. The breath, saliva, denture, and tongue coat of each individual were collected as samples. Then, the gas evaporating from each sample was saved in a sampling bag. The sampling gas was measured by an electronic nose (Odor Discrimination Analyzer FF-1, Shimadzu, Japan). In principle component analysis, principle component 1 showed significant differences between three pairs of items : breath odor (BR) and the odor of the saliva (SL), breath odor and the odor of the denture (DE), and breath odor and the odor of tongue coat (TC) ($p < 0.001$). There were no significant differences between SL and TC, SL and DE, and TC and DE. The mean differences between BR and the each odor emission source showed that DE was the nearest to BR, then SL, and TC lastly. In this study, TC, previously named as one of the main causes of breath odor, has no relation with BR. Moreover, TC is related to SL and DE in the elderly. It is suggested that one of the main odor emission sources of breath odor may be the dentures.

Key words : Breath odor, Elderly, Electronic nose, Bad breath

Introduction

Organoleptic judgments and measuring concentration of particular substances known to be the causes of odors have been applied to measurement of odors. It is well known that organoleptic judgment and measurement of volatile sulfur compounds (VSC) are the most standardized method for breath odor evaluation at this time^{1,2)}.

An electronic nose (e-nose) developed recently mimics the human nose sensing mechanism, which discriminates various odors by pattern-recognition of the responses in olfactory receptor³⁾. The e-nose used in this study (Odor Discrimination

Analyzer FF-1, Shimadzu, Japan) is equipped with MOS (metal-oxide-semiconductor) sensors as alternatives to olfactory receptors, and the e-nose recognizes the sensor patterns for discriminating odors¹⁾. Thus, the e-nose system enables objective assessment of the odor discrimination of the different types of odors.

The aim of this study is to compare breath odors and odor emission sources (saliva, tongue coat, and dentures in current use) of the elderly and to define the relationships among the odors.

Materials and Methods

1. Subjects

All of the subjects were recruited among the patients visiting the outpatient clinic, Tokyo Medical and Dental University Geriatric Dentistry (15 patients : male = 9, female = 6 : mean age 73.9 ± 4.1 yrs). The inclusion criterion was to have

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communication skills for understanding instructions. The exclusion criterion was to have communication problems. All subjects signed a written informed consent document. Permission for this study was obtained from the Ethical Committee for Clinical Research of Tokyo Medical and Dental University (No. 66).

2. Methods

At the initial visit, the patients' chief complaint and medical history were obtained. Periodontal examinations and dental examinations were performed. On the second visit, breath odor was assessed by both organoleptic judgment and by e-nose measurement. Odor samples of the dentate patients were taken from 1) the breath, 2) the saliva, and 3) the tongue coat. Denture wearers provided an additional odor sample from 4) the surface of the denture (8 patients : male = 4, female = 4).

2-1. Organoleptic judgment of the breath odor

Prior to the odor assessment, the subjects were instructed to abstain from oral activities such as eating and drinking, from oral hygiene such as brushing and mouth rinsing, and from smoking on the morning of the test day⁵⁾. Odor assessment was performed between 9 : 00 and 10 : 30 AM¹⁾. The subjects were instructed to keep the mouth closed and to breathe through the nose for a minute. Then, the subjects were requested to exhale slowly facing the odor judge. One trained panel performed the organoleptic judgment. Organoleptic results were recorded on a scale of 0 to 5 as follows : 0 : no appreciable odor ; 1 : barely noticeable odor ; 2 : slight, but clearly noticeable odor ; 3 : moderate odor ; 4 : strong odor ; 5 : extremely unpleasant odor⁶⁾.

2-2. Measurements by electronic nose

1) Odor sample of the breath odor (BR)

Again, subject was instructed to keep the mouth closed and to breathe through the nose for a minute. Subject was then requested to exhale into a three-liter polyester bag (Flek-Sampler[®]

NIOIBUKURO, OMI ODORAIR SERVICE Co. Ltd., Japan). The duration of the exhaling was two to three seconds to prevent dilution of mouth odor with lung air. This procedure was repeated 10 times.

2) Odor sample of the saliva (SL)

After the breath odor assessment, each subject donated a sample of whole saliva, which was collected by chewing paraffin for five minutes. The collected saliva was injected into a three-liter polyester bag, and then the bag was filled with pure nitrogen. The saliva was kept in the polyester bag for an hour in room air (mean temperature 24.1°C, mean humidity 53.3%). The gas evaporated from the saliva was transferred into another polyester bag for the e-nose measurement.

3) Odor sample of the tongue coat (TC)

The subjects were instructed to keep the mouth wide-open and to stick the tongue out. Then, saliva on the dorsal surface of the tongue was removed with a tissue (Kim Wipe, USA). The tongue coat was removed with a small spoon from the terminal sulcus to the apex of the tongue⁷⁾. The spoon used to scrape the tongue coat was put in a three-liter polyester bag, and then the bag was filled with pure nitrogen. The spoon used to scrape the tongue coat was kept in the polyester bag for an hour in room air (mean temperature 24.1°C, mean humidity 53.3%). The gas evaporated from the tongue coat was transferred into another polyester bag for the e-nose measurement.

4) Odor sample of the denture (DE)

The denture of the subject was put in a three-liter polyester bag, and then the bag was filled with pure nitrogen. The denture was kept in the polyester bag for an hour in room air (mean temperature 23.8°C, mean humidity 51.9%). The gas evaporated from the denture was transferred into another polyester bag for the e-nose measurement. The denture was returned to the subject immediately after the sampling gas was collected.

The operating sequence of the e-nose is as fol-

lows : (a) sampling : the gas sample is sucked into the preconcentrator (a trap tube to concentrate the sample gas). The flow rate is 165 cc/min with a the sampling time of 60 seconds, (b) top-note measurement mode : the trapped odor is driven to the sensor section and the odor is detected by the six MOS sensors, (c) drying : the gas sample trapped in the preconcentrator is dried with pure nitrogen, (d) deep-note measurement mode : the preconcentrator is heated from 40°C to 220°C, then the trapped odor is driven to the sensor section for the six MOS sensors to detect the odor, (e) cleaning : the preconcentrator is heated to 250°C in order to clean out the remaining odor. The top-note measurement mode of the sensors one to six is shown as channels one to six, and the deep-note measurement mode is shown as channels seven to twelve.

Each of the sample gases was measured seven times, and the first two responses were not used for data analysis because of the poor stability due to sensor performance. The means of the latter five responses were used for data analysis.

3. Statistical analysis

Principal component analysis (PCA) is a method of identifying patterns in data, and describing the data to highlight their similarities and differences. The data of the twelve sensor responses are identified their similarities and differences in terms of principal component (PC) by PCA. (SPSS, Ver11.0, USA). Therefore, the twelve sensor responses are summarized into two (PC1 + PC2) to three (PC1 + PC2 + PC3) dimensions in order to emphasize the similarities and differences of the data.

PC1 is an axis which describes the largest distribution of the data simplified by PCA. PC2 is the second largest axis of perpendicular to PC1, and PC3 is the third largest axis of perpendicular to PC2.

PC 1, 2, and 3 (Eigenvalues >1) were analyzed by one-way ANOVA with Scheffé's test for multiple comparisons. The mean distances between

the odors show the similarities and differences between the odors. The farther plots in the results of the PCA shows the different patterns in the data. The nearer plots in the PCA results show the similar patterns in the data. Significance was set at $p < 0.05$.

Results

The result of the organoleptic judgment of the breath odor is shown on Table 1. There are 11 subjects whose scores are 0 or 1 (no oral malodor), and there are four subjects whose scores are over 2 (presence of oral malodor).

BR and the other odors were separated. SL, TC, and DE were not clearly separated (Figure 1a). BR and the others are separated upon PC 1, but not separated on PC 2 (Figure 1b).

When data of BR and SL were extracted, two odors were clearly separated on PC 1, but not sep-

Table1. Results of the organoleptic judgement of the breath odor

scale		number of subjects
0	no appreciable odor	5
1	barely noticeable odor	6
2	slight, but clearly noticeable odor	3
3	moderate odor	0
4	strong odor	1
5	extremely unpleasant odor	0

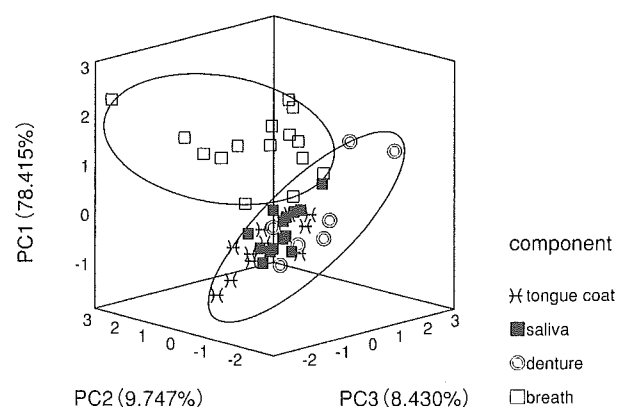


Figure 1a. Results of PCA among breath, saliva, tongue coat and denture. Y-axis : PC 1, X-axis : PC 2, Z-axis : PC 3. Contribution ratio% in parentheses. □breath ○denture ■saliva *tongue coat

PC 2. Unexpectedly, however, in this study, the nearest odor to BR was DE, and the farthest odor was TC.

This relation between the odors may be caused by characteristic of the denture. The subjects in this study were elderly persons (over 70 years old). It is reported by Hoad-Reddick¹⁷⁾ that only 40% of the dentures of the elderly are properly cleaned. In this study, the subjects' denture hygiene was not assessed, but they may have not cleaned their dentures properly. It is recommended that dentures should be cleaned mechanically and to use denture cleaner after mechanical cleaning. But even though dentures are cleaned properly, the sterilization of dentures are effective in only few denture cleaners¹⁸⁾.

Tanaka¹⁹⁾ reported that the responses of the e-nose, which was the same type as that used in this study had positive correlations with age, smoking habits, pocket depth, tongue coating status, and plaque score. VSC concentrations of the breath showed positive correlations with only tongue coating status and plaque score. Tanaka's findings indicate that the e-nose is capable of detecting of not only VSC but also other gases. It is suggested that gases beside VSC may affect the similarity of BR and DE, which comes from the PC 1 result.

Previous findings show that tongue coating is the main cause of breath odor^{2, 15, 16)}. However, there have been no reports referring to the characteristics of BR and TC due to a lack of an objective method for discriminating the odor characteristics. Moreover, odor characteristics determined by human judges may lack objectivity, and the outcome of the organoleptic judgment may not be reproducible.

This electronic nose system is regarded as the objective method for discriminating odor characteristics. In spite of the previous reports that BR and TC are similar, the results of PCA and of one-way ANOVA clearly separated the two odors.

From the present results, it cast doubt on the view that the tongue coat is the main cause of breath odor in the elderly patients. In previous studies^{11, 12)}, the age distribution of the subjects tends to be younger than the subjects in this study. The results of this study do not contradict the fact that the tongue coat is the main cause of breath odor in the younger population. However, in the elderly, denture odor can be assumed to have a closer relation to breath odor.

Conclusion

The tongue coat odor, previously named as the main cause of breath odor, had no similar characteristics to breath odor. In the elderly, odor from the denture could be insisted to have a closer relation to breath odor.

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高齢者の口臭と口腔内における発生源の関連

—電子嗅覚装置の測定結果より—

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抄録：高齢者の口臭と口腔内における発生源となる唾液、使用中の義歯、舌苔のにおいを比較し、相互のにおいの関係を明らかにすることを目的とした。我々の勤務する歯学部付属病院の外来受診患者を対象とした。対象患者の口臭、唾液、舌苔および義歯を採取し、各々の揮発ガスをサンプリングバッグに集めた。サンプルガスは電子嗅覚装置（におい識別装置FF-1、島津製作所）を用いて測定した。においのデータ解析には主成分分析を用いた。口臭と口腔内における発生源（唾液、舌苔、使用中の義歯）の比較には1元配置の分散分析を用いた。多重比較にはSchefféテストを行った。第一主成分で口臭と唾液、口臭と義歯、口臭と舌苔のにおいには有意な差が認められた ($p < 0.001$)。唾液と舌苔 ($p = 0.554$)、唾液と義歯 ($p = 0.526$)、舌苔と義歯 ($p = 0.074$) 間のおいには有意な差を認めなかった。口臭、唾液、舌苔、義歯、それぞれに対する平均値の差の比較では口臭が一番近いのは義歯、次いで唾液、最後に舌苔となっていた。本研究では高齢者においては舌苔のにおいはむしろ唾液のにおいと類似した傾向があることが明らかになった。高齢者の口臭のにおい発生源としては義歯が関連が深いことが明らかになった。

キーワード：口臭、高齢者、電子嗅覚装置

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