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ORIGINAL ARTICLE

Salivary-free fluoride ion concentration measured using a flow-injection analysis device and oral environment in 4–6-year-old children

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Abstract Although fluoride (F) products are widely used for caries prevention, the safest and most effective modes of application, in particular for young children, remain to be elucidated. The limitations associated with the detection of ultra-low F ion concentrations are the major obstacles in accurately assessing the salivary F ion concentrations in children. This study aimed to measure accurate salivary-free F ion concentrations in children using a flow-injection analysis device and highlight the conditions or substances that influence changes in salivary content. Subjects were 4–6-year-old children, and we statistically compared the data involving the number of decayed, missing, or filled surfaces (dmfs), the levels of Mutans streptococci (MS) and Lactobacilli (LB) cariogenic bacteria, and oral hygiene habits. The information on the latter was obtained using a parent/guardian questionnaire. The average free F ion concentration measured was 0.421 ± 0.158 µmol/L (0.008 ± 0.003 ppm), which was considerably lower than that obtained in previous studies using the conventional F electrode method. No significantly different correlations were seen between salivary-free F ion concentrations and dmfs, MS and LB levels. With regard to salivary-free F ion concentrations and oral hygiene habits, only finishing brush of subjects' teeth by guardians showed a significant difference. In summary, the frequency of brushing was shown to correlate with free F ion concentration in saliva of children. Further studies are needed to circumstantially evaluate some other substances in saliva and oral hygiene habits.

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Introduction

Fluoride (F)-containing products, such as dentifrices, have long been accepted for their efficacy in caries prevention. In particular, regular use of a F dentifrice has consistently resulted in a significant reduction in caries [1–5] and has therefore been widely used to prevent dental caries in early childhood. Nevertheless, several factors regarding salivary F ion concentrations remain unclear, particularly for young children; therefore, the proper use of F-containing products needs be addressed in this age group. It has been reported that the anticaries properties of F are effective when there is a constant presence of a low concentration of free F ions in saliva. An enhancement of remineralization and an inhibition of demineralization have been reported at salivary-free F ion concentrations between 0.737 and 1.052 µmol/L (0.014 and 0.02 ppm) [6–8], measured using conventional F electrode methods. A flow-injection analysis device can measure ultra-low F ion concentrations, even in a small amount of sample [9], and it has three major advantages: speed, convenience, and small sample volume requirement. Furthermore, the measurement limit for F ion concentration is 0.016 µmol/L (0.0003 ppm). The device has been previously used to measure serum F ion concentrations [9].

Saliva clearance rates and some salivary components can contribute to a reduction in salivary F ion concentration. However, both fluoridation and appropriate F-containing product usage should be assessed for safer and more effective results, particularly for children [4]. To develop a proper F-containing product usage protocol, the factors affecting salivary F ion concentrations should be properly assessed.

This study aimed to confirm accurate salivary-free F ion concentrations in stimulated saliva of children using the flow-injection analysis device and compare the data obtained with oral environment factors and oral hygiene habits. With these results, the conditions and/or substances influencing salivary F ion concentrations in children could be assessed.

Materials and methods

Panel of subjects and study design

The trial subjects were 68 children (26 boys and 42 girls; age, 4–6 years; mean age, 5.6 ± 0.78 years) from two nursery schools in Yokohama, Japan. In one of the schools (39 children), F-mouth rinsing (11.84 mmol/L, 225 ppm) was performed after lunch in order to investigate long-term changes in salivary-free F ion concentrations. However, there was no special difference about their caries status of the subjects from two different schools. The results obtained from both schools were included in the same analysis because it was considered that various F-containing products were routinely used by the subjects both at home and under professional care. The trial was performed with the cooperation of the school teachers. Parental/guardian consent to collect the children's saliva and questionnaire responses were obtained prior to the commencement of the study.

Oral examinations took place in naturally lit rooms at the respective schools using dental mirrors and were performed by a single dentist in order to ensure that there was no handling-related bias, and the number of dmfs was counted. The process was conducted along with WHO standard methods [10].

To avoid bias, no specific F-containing product or techniques related to oral care and hygiene were recommended. Furthermore, the subjects were asked to perform their normal daily activities prior to the collection of saliva, although they were asked not to eat/drink anything and/or brush their teeth for 2 h prior to saliva collection. Given these restrictions, the collection time was set at 10 am.

The study was approved by the Ethical Committee of The Nippon Dental University School of Life Dentistry at Tokyo and adhered to the principles of the Declaration of Helsinki.

Saliva samples

Stimulated saliva samples (5 mL) were collected in plastic tubes after the subjects chewed on paraffin wax gum for 5 min. A live demonstration showed the subjects how to chew the gum and spit out their saliva, and they were allowed to practice several times on the day prior to collection. Collection was performed only when it was confirmed that all subjects could provide samples. For a special purpose, 0.2/5 mL of collected saliva in each plastic tube was immediately applied for an assessment of cariogenic bacteria. The rest of samples were transferred to the hospital at 4 °C and kept frozen until just before use when they were thawed at room temperature. Following centrifugation at $1062 \times g$ for 20 min, the supernatant liquid was used as the measurement sample. In addition, we set them as the samples for measuring salivary-free F ion concentrations, but they were supposed to be included a small amount of fluoride in plaque and on soft tissue in the subjects' mouth.

It is noteworthy that the F ion concentration of the drinking water in the Yokohama area was 3.158 μmol/L (0.06 ppm) on average and was defined to be <42.105 μmol/L (0.8 ppm) on the basis of the water quality standards of the Japan Waterworks Law. Water fluoridation is prohibited in Japan.

Measurement of free F ion concentrations

A flow-injection analysis device (FAU2200, manufactured by Yamato Denshi, Kyoto, Japan) was connected to an F electrode cell (Model 94-09, Orion) and was used to measure the free F ion concentration (Fig. 1). The device can distinctly measure lower free F ion concentration rapidly isolating F ion with a closed system not to be affected by outside contamination. It is possible to stably measure lower free F ion concentration compared with when F electrode is used alone. The inner diameter of the teflon-coated tube was 0.5 mm, and a plunger pump (P) was used to pump out the buffer solution and carrier. The buffer solution was pumped at a rate of 0.8 mL/min to stabilize the F electrode cell (FE), and a sample of 0.2 mL was subsequently injected into the sample injection equipment (S) until the electrode became completely stable. Ultrapure water (Milli Q, Millipore, Tokyo, Japan) was used as a carrier at a rate of 1.2 mL/min. The sample was then mixed with the buffer solution in the mixing coil (M) and run through the FE. The peak values of electric potential differences were measured using the device, and a standard calibration curve was prepared. F ion concentrations were measured according to the peak height.

All reagents used in this study were manufactured by Wako Pure Chemical Industries Ltd, Tokyo, Japan, and ultrapure water (Milli Q) was used to prepare the buffer and reagent solutions. The composition of buffer solution was as follows: 900 mL of ultrapure water, 136 g of sodium acetate trihydrate, 117 g of sodium chloride, 170 g of sodium nitrate, 3.6 g of sodium dihydrogen phosphate, 2-hydrate,

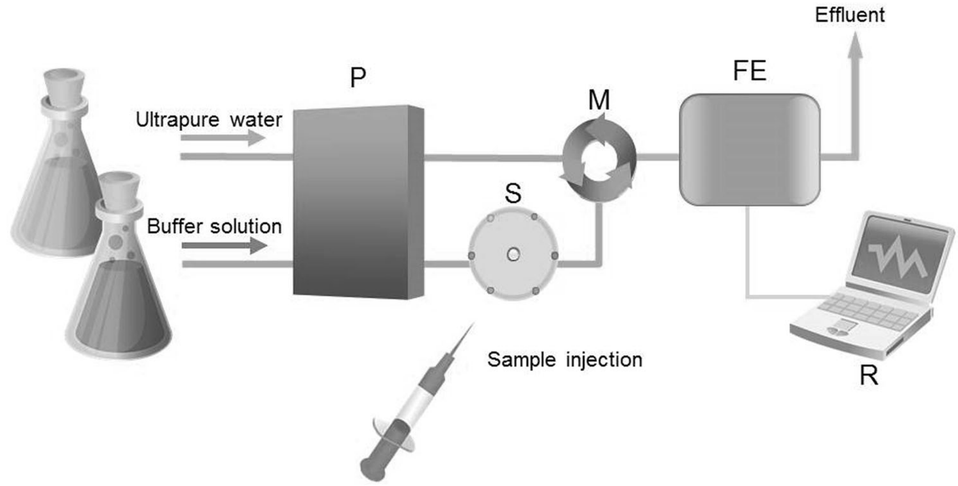

Fig. 1 Flow-injection analysis device that can measure ultra-low fluoride ion concentration. *P* plunger pump, *I* sample injection device, *M* mixed coil, *FE* fluoride electrode cell, *R* ion meter, system integrator and recorder output

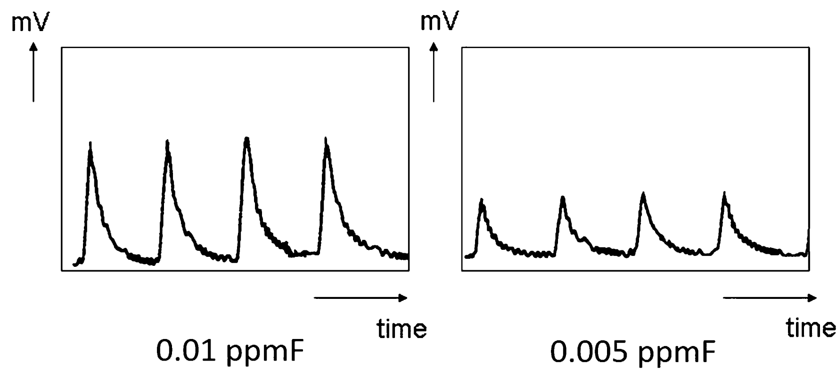

Fig. 2 Examples of waveforms obtained by a recording of the flow-injection analysis device that measures ultra-low fluoride ion concentrations

and 28 g of EDTA·4Na. Furthermore, it was adjusted to pH 5.4 ± 0.1 with hydrochloric acid and 0.02 mL of 52.632 mmol/L (1000 ppm) F solution was added. The solution was diluted with ultrapure water to 1000 ml. Finally, 1 g of Triton-X 100 was added after filtration with a 0.45-μm membrane filter. Solutions with F ion concentrations of 0.053, 0.263, 0.526 and 1.052 μmol/L (0.001, 0.005, 0.01, and 0.02 ppm) were prepared as the F standardization solutions by dissolving sodium fluoride powder in ultrapure water. The measurement of standard solutions was performed in triplicate for each concentration, and the average was calculated. In the same manner, the measurement of saliva samples (0.2 mL each) was performed in triplicate for each subject, and the average was determined as the free F ion concentration score. Prior to this study, we confirmed the accuracy, stability, and consistency of this device and it exhibited a stable waveform (Fig. 2).

Level of cariogenic bacteria

The dental laboratory service (BML, Ltd. Tokyo, Japan) assisted in the biological assessment of the levels of MS and LB cariogenic bacteria. Swabs with 0.2 mL of absorbed saliva were collected, placed in plastic tubes containing 3.0 mL of phosphate buffered saline (PBS), and mixed to release the bacteria into the liquid. The liquid was inoculated on the selective agar medium for each bacterium: improved MSB culture medium for MS and Rogosa medium for LB. Bacterial numbers were calculated as colony forming units (CFU)/mL following 48 h of anaerobic culture.

Questionnaire

Questionnaires were given to the nursery school teachers who distributed them to the subjects' parents/guardians and collected them upon completion. The questionnaires consisted of information concerning brushing habits and F use, including F-containing product use.

Data analysis

Parametric comparisons were performed using analysis of variance (ANOVA) and Student's *t* test, and nonparametric multiple comparisons were performed using the Mann–Whitney *U* test and the Kruskal–Wallis test, considering the within-subjects and sample variables in the study.

Comparisons with the questionnaire items were performed using Spearman's rank correlation coefficient; the significance level was set at 5 % ($p < 0.05$). All analyses were performed using statistical calculation software SPSS 21.0 J for Windows (IBM, Tokyo, Japan).

Results

Salivary-free F ion concentration

Salivary-free F ion concentrations ranged between 0.211 and 0.947 µmol/L (0.004 and 0.018 ppm), with an average of 0.421 ± 0.158 µmol/L (0.008 ± 0.003 ppm) (Table 1).

Comparison data between free F ion concentrations, dmfs, MS, and LB

The subjects' average dmfs score was 3.68 ± 7.882 (range 0–37), showing a complete polarization between subjects who had no cavities and those who had many (Table 1). In addition, 45 subjects (66.18 %) had a dmfs score of 0 and an average free F ion concentration of 0.421 ± 0.105 µmol/L (0.008 ± 0.002 ppm). The remaining 23 subjects (33.82 %) had a dmfs score of >1 and an average free F ion concentration of 0.421 ± 0.158 µmol/L (0.008 ± 0.003 ppm). Thus, no significant difference in free F ion concentration was observed between the groups.

Table 1 Salivary-free fluoride ion concentrations and caries experiences among Japanese school children (aged 4–6 years)

	Mean	SD	Minimum	Maximum
Age	5.6	0.78	4.1	6.9
Salivary-free F ion concentration (µmol/L)	0.421	0.158	0.211	0.947
dmfs	3.68	7.882	0	37

dmfs decayed, missing, or filled surfaces, *SD* standard deviation

Table 2 shows the results of MS and LB scores among the subjects. As expected, the higher the MS or LB scores, the higher the dmfs scores, indicating a significant correlation between them. However, no significant correlation was observed between MS or LB scores and free F ion concentrations.

Comparison data between questionnaire response variance and salivary-free F ion concentrations

Table 3 shows the parents/guardians questionnaire responses. A significant correlation was observed between brushing by the guardians after the children to inspect their teeth (Finishing brush) ≥2 times/day and salivary-free F ion concentrations. Several remarkable tendencies were observed regarding the frequency of brushing, the amount of dentifrice used on a toothbrush, and the subjects' salivary-free F ion concentrations. In some questionnaire responses, there were no significant differences in each intergroup even though there were items related to F product usage and experience (Table 4).

Regarding the timing of brushing the teeth, 18 guardians performed finishing brush ≥2 times/day, most of whom

Table 2 Comparative data between salivary-free fluoride ion concentrations and MS and LB levels in Japanese school children (aged 4–6 years)

	Score (CFU/mL)	*N* (68)	dmfs		F ion concentration (µmol/L)	
			Mean	SD	Mean	SD
MS	Undetected	41	0.46	1.42	0.421	0.158
	$<10^3$	8	4.12	6.6	0.474	0.158
	10^3–10^4	12	7.75	10.52	0.474	0.158
	$10^4<$	7	15.00	13.17	0.421	0.158
			MS score intergroup comparison with dmfs Kruskal–Wallis test, $p < 0.001$ and Spearman's rank correlation = 0.633 ($p < 0.01$)		No significant differences between MS score and F ion concentration	
LB	Undetected	48	1.79	4.52	0.421	0.158
	$<10^3$	8	3.00	4.38	0.421	0.053
	10^3–10^4	7	11.14	17.05	0.474	0.158
	$10^4<$	5	12.40	9.71	0.474	0.158
			LB score intergroup comparison with dmfs. Kruskal–Wallis test, $p < 0.05$ and Spearman's rank correlation = 0.374 ($p < 0.05$)		No significant differences between LB score and F ion concentration	

MS Mutans streptococci, *LB Lactobacilli*, *dmfs* decayed, missing, or filled surfaces, *SD* standard deviation

Table 3 Responses to the questions possibly associated with salivary-free fluoride ion concentration

Item	Grouping	*N* (68)	F ion concentration (μmol/L) Mean ± SD	*p* value	rs (*p* value)
Frequency of finishing brush by guardians	Two times per day*	18	0.526 ± 0.158	0.003*	0.276*
	One time per day	23	0.421 ± 0.105	(ANOVA)	(0.023)
	Sometimes	20	0.421 ± 0.158		
	Little	7	0.421 ± 0.105		
Frequency of brushing by themselves	Two times or more per day	43	0.474 ± 0.158	0.007	0.228
	One time or less per day	25	0.421 ± 0.105	(*t* test)	(0.061)
Coverage of toothbrush by dentifrice	>1/2	11	0.526 ± 0.211	0.074	0.195
	1/3–1/2	18	0.421 ± 0.158	(ANOVA)	(0.154)
	<1/3	26	0.421 ± 0.105		
	No response	13			

* $p < 0.05$

ANOVA analysis of variance, *SD* standard deviation

Table 4 Exposure situation of F products on the responses to questionnaires

Item	Grouping	*N* (68)	Rate (%)
F mouth rinsing	Active	39	57.4
	Not active	29	42.6
Frequency of dentifrice usage	Every time	45	66.2
(no object who use)	Sometimes	10	14.7
	No use	13	19.1
Experience of F application	No experience	23	33.8
(9000 μg F/g of APF) at dental clinics	One–three times	26	38.2
	Four–five times	9	13.2
	Six times or more	9	13.2
	No response	1	1.5

brushed their children's teeth after breakfast and before bedtime. Among those who performed finishing brush once per day, 23 did so before bedtime. Regarding the relationship with list of frequency of dentifrice usage, 18 guardians (finishing brush ≥2 times/day) were divided into three groups of every time, sometimes, and no use as 15, 2, and 1, respectively, and 23 guardians (finishing brush once a day) as 12, 3, and 8.

Discussion

Salivary F ion concentrations

To date, there have been few reports concerning the investigation of salivary F ion concentrations in children. In addition, some recently available F measurement techniques themselves are not standardized and a universal standard method for F determination has not been established, although researchers have measured F ion concentrations in diverse ways [11]. Furthermore, collecting saliva samples from young children is problematic because of the requirement of parental/guardian consent and cooperation, as well as the practicalities involved in chewing wax gum and spitting into tubes. Thus, the investigation of salivary F ion concentration in children has been largely avoided. However, the safety and effectiveness of protocols for F-containing product usage in children cannot be guaranteed if F ion concentration studies are not conducted in the target population. Therefore, our novel study assessed free F ion concentrations in samples collected from 4- to 6-year-old children using the flow-injection analysis device that the accuracy, stability, and repeatability of this device have been previously verified [12].

It has been reported that the primary processes required to enhance remineralization and inhibit demineralization for caries prevention were observed when the salivary-free F ion concentration was between 0.737 and 1.052 μmol/L [6–8]. However, the average free F ion concentration observed in the present study was 0.421 ± 0.158 μmol/L. This value is lower than that required for caries prevention.

This indicates that salivary-free F ion concentrations in young children should be increased.

Several authors have reported that higher salivary F ion concentrations provide more effective caries prevention [2, 11, 13]. However, the risk of overdosing should be considered. Salivary F ion concentrations temporarily increase following the application of F-containing products because F attaches to both hard and soft tissues, such as teeth and plaque, respectively. Nevertheless, F ion concentrations tend to decrease with salivary clearance so their long-term maintenance remains a challenge. Over the past decades, several studies have evaluated salivary F ion concentrations [14–17]; however, these reports investigated the changes in F ion concentrations following the application of F-containing products. The evaluation of equilibrium concentrations of salivary F ion is important [18], even if lower levels are obtained as a result, as these equilibrium concentrations have a long-term influence on the oral environment. It was reported that the baseline F ion concentration in saliva increased with the frequency of F-containing product use, indicating that it is possible to achieve the expected F ion concentrations and maintain a favorable oral environment with regular use [13].

Comparison data between free F ion concentrations, dmfs, MS, and LB

F ions easily attach to both soft tissues of gums, tongues cheeks, and hard tissues as well as to other substances present in saliva as the reservoirs for F. Therefore, it was presumed that F ion attachment to MS, and LB could account for the reduction in salivary F ion concentration. However, as shown in the present study, no correlation of free F ion concentration with MS or LB was observed. It has been reported that MS is a high-impact caries-inducing risk factor that is significantly correlated with dmfs during the deciduous tooth period, although no difference appeared between the scores of dmfs and levels of MS in this study. Furthermore, cavity formation increases with salivary MS and LB levels [19] and it happens more readily in adulthood [20]. Therefore, it is necessary to control the status of MS and LB in the oral environment.

Comparison data between free F ion concentrations and questionnaire response variance

In Japan, dentifrice penetration rate has reached over 90 %, and almost all commercial dentifrices include F for the purpose of caries prevention. To date, only F dentifrice [NaF, Na_2FPO_3 (SMFP) and SnF_2 up to 1000 μg F/g], F application (9000 μg F/g as APF and NaF) and F-mouth rinses (11.8, 23.7, and 47.4 mmol/L NaF; 225, 450, and 900 ppm F as NaF) are available for general pediatric use. In our study, given the high prominence of dentifrice penetration, it was assumed that most guardians knew of its benefits in caries prevention; however, some were not aware of whether the dentifrice they used contained F. Moreover, in our study, some guardians did not use dentifrice to brush their children's teeth, and 23 children had never received F application at a dental clinic.

Overall, although it was previously suggested that guardians were well aware of F product usage [21], the results of the questionnaire indicated otherwise despite this information being well engrained in the Japanese education system. In Japan, it is advised to have regular checkups, and health guidance is available during pregnancy and from birth to school-age for children, as determined by the Maternal and Child Health Act and School Health Act. However, adequate oral hygiene instructions are not being followed despite specialized lectures for pregnant women and mothers.

The dmfs results indicated that there were many children with no cavities. These children may not have had the need to see a dentist and may have had a few opportunities to obtain direct information about F products and receive professional F application. Considering that the attachment of MS in the mouth starts from the moment of eruption of a primary tooth for the first time, particularly if their guardians have high risks of caries [22], further instruction on effective F product usage should have been provided to parents/guardians much earlier given the average age of the current study group.

Despite a lack of guidance in oral care, there were 18 guardians who performed finishing brush of their children's teeth ≥2 times/day, commonly after breakfast and before bedtime. We did not request the data on the number of rinses after brushing, and it was unclear whether dentifrice was used at the time of finishing brush. It was presumed that the dentifrice used in the morning of the examination day influenced the subjects' salivary F ion concentration, thus revealing a significant difference. Nevertheless, even if the guardians' knowledge was unclear, dentifrice usage may have influenced the results because of the common oral care habits of the subjects. Some researchers suggested that concentrations are influenced by the method and number of oral rinses after brushing [1, 18], or by time spent brushing and dentifrice quantity [23]. Therefore, further studies are required to assess and question the guardians about the method and number of oral rinses after brushing, and the time spent in brushing considering the reduction in salivary F ion concentrations over time.

This study highlighted the established oral hygiene habits, the high frequency of finishing brush by guardians, and the frequency of dentifrice usage, proving that most guardians were interested in oral hygiene. On the other hand, it also showed that there was misinformation regarding the correct amount of dentifrice to be used (such as the recommended pea-sized amount), the effectiveness of F-containing products in caries prevention, and the appropriate method of mouth rinsing after brushing. An optimum, evidence-based method of oral hygiene should be established, and the guardians should be appropriately informed. The salivary environment has many complicated features, such as saliva clearance over time and the ease of F ion attachment to substances. However, further studies should be conducted to elucidate the substances and oral hygiene habits that may reduce salivary F ion concentration in children. Currently, we are investigating substances that influence F ion concentration and caries formation based on proteins and inorganic components present in saliva.

As mentioned above, in Japan, water fluoridation is not performed, and F ion concentrations in dentifrice can reach up to 1000 μg F/g for home use. Furthermore, fluoride therapy is performed under professional or public care at dental clinics, schools, or local communities. We suggest that more information on F product usage should be provided to the public and salivary F ion concentrations in children should be investigated to develop safer and more effective F product usage protocols.

Conclusions

In the present study, the salivary-free F ion concentrations varied between 0.211 and 0.947 μmol/L among the subjects, with an average of 0.421 ± 0.158 μmol/L. These values are considerably lower than the concentrations required for caries prevention. No correlations were observed between salivary-free F ion concentrations and dmfs, MS, and LB. Regarding the oral hygiene habits of children, only finishing brush by guardians resulted in a significant difference in salivary-free F ion concentration. To date, it has been suggested that we need to provide proper information about F-containing product usage for its advantage of caries prevention to guardians with defined evidence not only in a dental clinic but also in public.

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Conflict of interest The authors declare that they have no conflict of interest.

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