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ORIGINAL ARTICLE

Salivary fluoride levels after daily brushing with 5000 ppm fluoride toothpaste: A randomised, controlled clinical trial

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Abstract

This study explored salivary fluoride levels following toothbrushing with 5000 and 1450 ppm fluoride toothpaste and determined the decline in salivary fluoride levels following the return from 5000 to 1450 ppm fluoride toothpaste. The study was a randomised, controlled double-blind parallel clinical trial ($n = 24$/group) measuring salivary fluoride five times during a 3-week trial phase involving 2×/day use of 5000 or 1450 ppm fluoride toothpaste, and five times during an ensuing 2-week wash-out phase where all participants used 1450 ppm toothpaste. Salivary fluoride was measured using a fluoride electrode and data were analysed using multilevel mixed-effects linear regression. Baseline salivary fluoride geometric means were 0.014 and 0.016 ppm for the 1450 and 5000 ppm groups, while the values at the end of the trial phase were 0.023 and 0.044 ppm, respectively. During the trial phase, except at baseline, differences between groups were statistically significant. The salivary fluoride levels for the 5000 ppm group remained statistically significantly higher than for the 1450 ppm group only at the first measurement in the wash-out phase (≈30 h after the last 5000 ppm brushing), indicating that higher salivary fluoride levels resulting from use of 5000 ppm are sustained only as long as the brushing habit continues.

KEYWORDS
dental caries, fluorides, humans, saliva, toothbrushing, topical

INTRODUCTION

Toothbrushing with fluoride toothpaste, typically containing up to 1500 ppm fluoride, remains the most widespread and significant caries-controlling intervention in contemporary populations [1]. However, the use of toothpaste with even higher fluoride contents (e.g., 2500–5000 ppm; high-dosage fluoride toothpaste) has been launched for caries-active individuals [2], and clinicians have embraced the use of high-dosage fluoride toothpaste for particularly high-caries risk groups [3].

While there is ample high-quality evidence to support the use of fluoridated toothpaste in concentrations up to 1500 ppm to control caries in the permanent dentition of children and adolescents, the evidence is less certain for the permanent dentition of adults [1], and approaches the anecdotal when it comes to the use of high-dosage fluoride toothpaste for high caries-risk adolescent, adult and elderly patient groups [4–9].

Caries controlling effects of high-concentration fluoride gels (1.1 % NaF, ≃5000 ppm fluoride) applied in custom-fitted carriers have been demonstrated among children [10] and among adult irradiated cancer patients with hyposalivation [4]. Neither study employed a control group using regular concentration fluoride products. Others have demonstrated a root caries controlling effect of the use of 5000 ppm fluoride toothpaste among adults and elderly [5–8], while the use of 5000 ppm fluoride toothpaste among caries-active adolescents was found to reduce enamel caries lesion progression on radiographs [9]. However, the caries incidence was only reduced following the use of 5000 ppm fluoride toothpaste among those adolescents who were irregular users of toothpaste or did not brush twice daily [9].

The fluoride level in the oral fluids, including saliva, is considered a proxy variable for caries controlling effect of fluoride applications, including fluoride toothpaste [11]. Most published studies on high-dosage fluoride toothpaste have reported the salivary fluoride concentration after a single or very few toothbrushing sessions and/or only during the first few hours after exposure(s) [12–20]. These studies show that a single or few exposures to high-fluoride toothpaste result in higher salivary fluoride levels during the first few hours after exposure than seen after the use of toothpaste with up to 1500 ppm fluoride. Two studies have reported on salivary fluoride levels resulting from a prolonged period of use of 5000 ppm fluoride toothpaste [21, 22]. Thrice daily use of 5000 ppm fluoride toothpaste over 2 weeks resulted in higher salivary fluoride levels among older volunteers than the use of 1450 ppm fluoride toothpaste [21]. A key observation was the huge variation between participants in their salivary fluoride levels, which in part could be ascribed to circadian variation owing to haphazard sampling times, and lack of standardisation of the time between brushing and salivary sampling [21]. Thrice daily brushing with 5000 ppm fluoride toothpaste for 10 days resulted in rather high salivary fluoride levels when assessed in the morning 12 h after last exposure [22]. Unfortunately, neither study addressed the duration of the elevated salivary fluoride levels.

Hence, little is known if regular use of high-dosage fluoride toothpaste may lead to a prolonged and sustained elevation of the salivary fluoride levels, leading to elevated steady-state ambient salivary fluoride levels important for caries control [11]. The results of a small study aiming to determine the relevant wash-out period following thrice daily exposure to 5000 ppm fluoride toothpaste for 4 days indicate that salivary fluoride levels return to baseline values in about 2 days [23]. Use of 5000 ppm fluoride toothpaste may be expected to lead to more extensive formation of calcium-based fluoride deposits on tooth [24, 25] and mucosal surfaces [17, 26, 27], in the biofilm [28, 29], and particularly in porous carious lesions [24, 25], and these deposits might contribute to elevated and sustained salivary fluoride levels.

It should be borne in mind that widespread use of high-dosage fluoride toothpaste poses a risk for adverse effects necessitating a trade-off between the desired caries controlling effects and the risk to the patient emerging from inadvertent ingestion of fluoride. We therefore designed this randomised, controlled double-blind parallel group clinical trial to explore, during a 3-week period, the extent to which twice daily toothbrushing with 5000 ppm fluoride toothpaste would lead to a sustained elevated salivary fluoride level compared to toothbrushing with 1450 ppm fluoride toothpaste. A secondary aim was to estimate the occurrence of adverse effects associated with the regular use of these toothpastes.

MATERIAL AND METHODS

Trial design

The study was designed as a two-phased (trial and wash-out) randomised, controlled double-blind parallel group clinical trial with an allocation ratio of 1:1. The trial phase comprised three weeks of twice daily use of a high-dosage fluoride toothpaste (5000 ppm fluoride, test group) or of an ordinary fluoride toothpaste (1450 ppm fluoride, control group) (Figure 1). In the wash-out phase, all participants used 1450 ppm fluoride toothpaste twice daily (Figure 1). Participants were unaware of the group to which they belonged just as they were unaware of the type of experimental toothpaste received during the experimental period.

The toothpastes used comprised an ordinary fluoride toothpaste containing 1450 ppm fluoride (Fresh Gel; Colgate-Palmolive) and a high-dosage fluoride toothpaste containing 5000 ppm fluoride (Duraphat 5000 ppm Fluoride Toothpaste; Colgate-Palmolive). In Denmark, 1450 ppm fluoride toothpaste is considered a cosmetic product and therefore available over the counter, whereas 5000 ppm fluoride toothpaste is regarded as a medicine and therefore by prescription only. Both toothpastes contain sodium fluoride as the fluoride source, silica as the abrasive system and both have a comparable blue colour and an almost similar taste. The two trial toothpastes came in identical (blinded) tubes. When handing over the trial toothpastes, participants were told that toothpaste from different tubes could differ slightly in colour and taste owing to their origin in different batches and that they should not worry.

Sample size considerations

The sample size for the study was determined based on data provided by Pessan et al. [22]. They reported that exposure to a 1000 ppm fluoride toothpaste during toothbrushing three times daily resulted in a salivary fluoride concentration of

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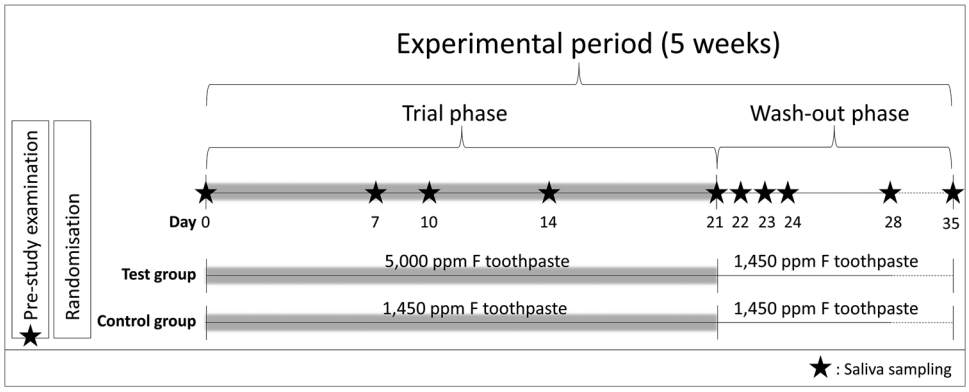


FIGURE 1 Outline of the study design. Participants brushed with the allocated toothpaste 2×/day (morning and evening). F, fluoride.

0.082 μg/mL (SE 0.016, $n = 14$) at 12 h after last brushing on day 10, while the similar use of 5000 ppm fluoride toothpaste resulted in a salivary fluoride concentration of 0.253 (SE = 0.044, $n = 14$) at 12 h after last brushing at day 10. Based on these data we calculated the necessary sample size on the basis of an expected salivary fluoride concentration of 0.10 μg/mL (SD 0.10) following use of 1450 ppm fluoride toothpaste for 3 weeks, and a salivary fluoride concentration of 0.25 μg/mL (SD 0.17) following use of 5000 ppm fluoride toothpaste for 3 weeks. The estimated sample size needed to detect a difference between groups of 0.15 μg fluoride/mL in saliva at day 21 of the trial phase was 20 persons per group at $\alpha = 0.05$ and 1-$\beta = 0.90$. To compensate for a possible drop-out it was decided to enroll 24 persons per group.

Participants

The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki. The study was independently reviewed and approved by the Central Denmark Region Committees on Health Research Ethics (no. 1-10-72-37-20) and the Danish Medicines Agency, and registered with the European Union Drug Regulating Authorities Clinical Trials Database (https://www.clinicaltrialsregister.eu, Eudra-CT no. 2020-000213-33). Further, the study was registered with the General Data Protection Regulation (GDPR) list at Aarhus University, Denmark and complied at all times with the European Union General Data Protection Regulation legislation. The study was undertaken with the understanding and written consent of each participant based on verbal as well as written information about the details of the study, according to the above-mentioned principles.

Eligible participants were identified among students from Aarhus University who volunteered based on posters in different campus canteens during the period from August 2020 to January 2021. Participants were required to be adult (≥18 years), to retain at least 20 teeth, to be non-pregnant and non-nursing, and to present an unstimulated salivary flow rate of at least 1 mL/5 min (0.2 mL/min). This was determined at a pre-study examination during which candidate participants were asked to drool into a tube (Falcon tube; Sarstedt) for 5 min. At this pre-study visit, a note was also made of the brand of toothpaste currently used by the participant (for the purpose of obtaining its labelled fluoride concentration), the time (hours) since their last toothbrushing, and the type of toothbrush used (manual or electric). Female participants were required to undergo a pregnancy test (in vitro hCG test (≥25 mIE/mL), Nantong Egens Biotechnology), to ensure that no pregnant women were included, in accordance with the recommendation by the Danish Medicines Agency and the medicine label insert for the high-fluoride toothpaste (Duraphat 5000 ppm Fluoride Toothpaste; Colgate-Palmolive). Female participants were required to take precautions to avoid becoming pregnant during the study period.

Denmark does not apply water fluoridation. The mean natural fluoride concentration in drinking water in the municipality of Aarhus, reported by the supplier, was 0.30 and 0.26 ppm in 2020 and 2021, respectively.

A total of 50 volunteers were evaluated and found eligible to participate in the study. Neither pregnancy nor too low salivary flow rate excluded any potential participants. Two participants screened for inclusion dropped-out before commencing the experimental part of the study due to COVID-19-related obstacles. Forty-eight individuals took active part in the study and completed both the trial and the wash-out phase (Figure 2).

Interventions

All participants were shown photos of both a manual and an electric toothbrush holding 1 g of toothpaste and instructed

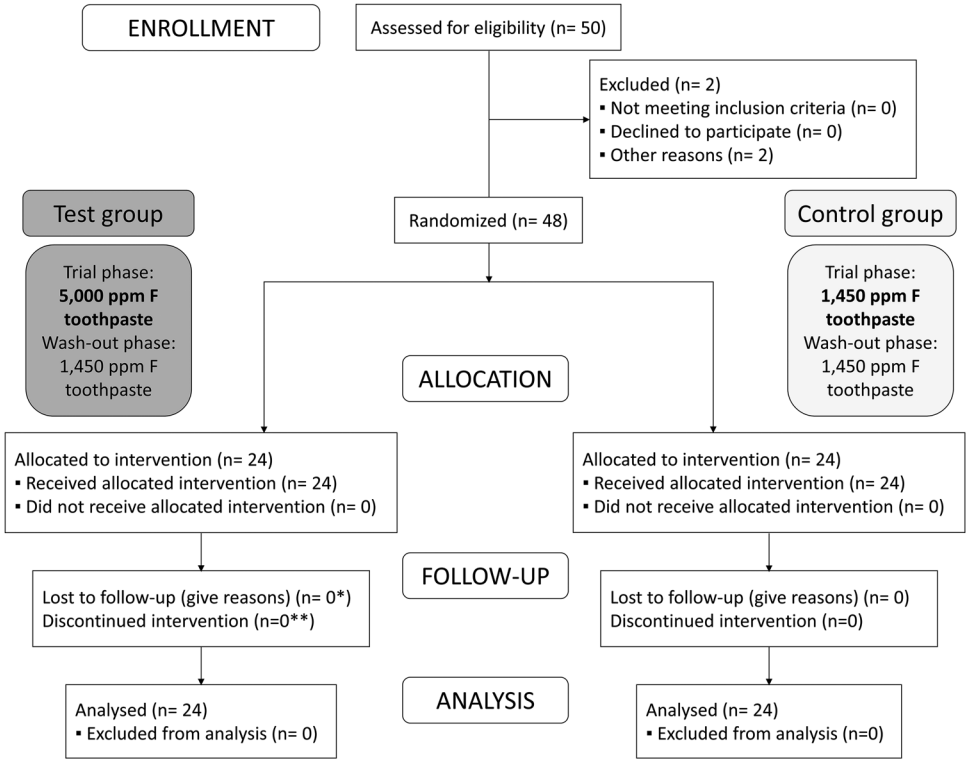


FIGURE 2 Flow chart of the trial. F, fluoride. * Two saliva samples were missing. The two participants in question were not excluded from the analysis. ** A single participant deviated from the protocol for four successive brushings after which the participant returned to the trial medicine. The participant was not excluded from the analysis.

to brush their teeth with 1 g of toothpaste twice daily (morning and evening) for 2 min during the experimental study period, while at the same time refraining from the use of other fluoride-containing dental products. The choice of water rinsing after brushing was made at each participant's discretion. Also, no attempt was made to control participants' food consumption. Trial toothpaste tubes containing 51 g toothpaste were handed over to study participants at the beginning of the trial phase (day 0) and at the beginning of the wash-out phase (at the end of day 21 of the trial phase) (Figure 1). On day 21 of the trial phase and after completion of the experiment (day 14 of the wash-out phase), participants returned the used trial toothpaste tubes and the tubes were weighed to record the toothpaste use.

Randomisation procedures

Participants were randomly allocated 1:1 to the experimental arms using minimisation (MiminPy Program 0.3, http://minimpy.sourceforge.net). Minimisation is a method of adaptive randomisation to groups to ensure that the groups will not differ too much at the outset of a trial in terms of predefined covariates that could influence results [30]. In the present trial, minimisation was based on the salivary fluoride concentration observed at the pre-study examination. Briefly, the first participant is randomly allocated to one of the treatment groups. Whenever a new participant is to be randomly allocated to one of the groups, an imbalance score is calculated for each potential allocation of the participant (in the present case there were two options, test or control group) based on the level of their pre-study salivary fluoride concentration. The two imbalance scores will be compared, and the person allocated to the group resulting in the lower imbalance, with a certain probability, in this study 90%, to maintain the element of randomisation.

The batches of toothpaste kits were prepared and labelled by authorised pharmaceutical staff at the Hospital Pharmacy Central Denmark Region who had transferred the ordinary fluoride toothpaste to tubes identical to the tubes containing the high-dosage fluoride test toothpaste. The pharmacy kept a sealed list (AB-list; batch list) that explained which toothpaste kits were of the same contents (but not which the contents were), as well as a sealed randomisation list. The principal investigator was allowed to break the AB-list upon commencing the data analysis. The sealed randomisation list was not to be broken until after the data analysis had been completed. However, sealed individual randomisation envelopes were kept in case of events indicating potential adverse effects, which would necessitate action. However, none of the individual randomisation envelopes had to be broken.

Outcomes

The primary outcome was the fluoride concentration in saliva recorded at the 10 predetermined days during the trial (Figure 1). The secondary outcome was the estimated occurrence of adverse effects associated with the regular use of the two toothpastes.

Saliva sampling

Saliva samples were collected from the participants in the afternoon at 10 different days, corresponding to day 0, 7, 10, 14, 21, 22, 23, 24, 28 and 35 of the 5-week duration of the experiment (Figure 1). Accordingly, the first five samplings covered the trial phase (day 0, 7, 10, 14 and 21), and the fifth sampling (day 21) plus the last five samplings (day 22, 23, 24, 28 and 35) covered the wash-out phase (Figure 1). All saliva samples were collected by letting the participant drool for 5 min into a tube (Falcon tube; Sarstedt). The collections took place in a quiet room and participants were instructed to sit slightly stooped with feet apart. Gustatory stimulation affecting salivary flow [31] was avoided by instructing participants to refrain from eating and drinking for 1 h before sampling. The time (hours) since the last toothbrushing was recorded at each sampling session. All samples were stored in a freezer (−18˚C) until analysis for fluoride content.

Covid19-related restrictions necessitated that 59 (12% of the total) of the saliva samples had to be obtained at the participants' homes. Each sample was collected in a Falcon tube and immediately put in the participant's freezer (−18˚C) and an appointment was arranged for delivery of the frozen sample to the study centre. All concerned participants were instructed to keep the samples frozen during transportation by keeping them on ice and wrapping them thoroughly.

The principal investigator of the clinical trial, LSL, provided all information to participants, conducted the pre-study eligibility examination and managed all saliva sampling appointments. At every appointment, each participant was asked about occurring events, incidents and health-issues since last appointment, as is a mandatory part of the study setup following the regulations of the European Union Drug Regulating Authorities. All appointments took place at the Research Laboratories at the Department of Dentistry and Oral Health, Aarhus University, Denmark.

Fluoride analyses

All fluoride analyses were performed using a combination fluoride electrode (Orion 9609BNWP, ionplus Sure-Flow Fluoride; Thermo Fisher) connected to a potentiometer (PHM210, STANDARD pH METER; MeterLab). Five hundred microliters of sample was buffered with 50 μL TISAB III (Orion 940,911; Thermo Fisher) according to the manufacturer's instruction. Calibration included 10 standard fluoride solutions covering the range from 0.01 to 10 ppm fluoride. The frozen saliva samples were analysed for fluoride content in batches which comprised samples collected on different days of the experiment in order to eliminate a distinct pattern considering the different sampling days.

Data analysis

The raw data consisted of mV-values recorded in saliva samples from the initial pre-study examination plus the 10 saliva sampling days (Figure 1) for each of the 48 participants. Reference curves were generated using standard fluoride solutions as described by Staun Larsen et al. [17]. Briefly, for every measurement day procedure fp (earlier fracpoly) of STATA Release 17 (StataCorp) was used to determine the best-fitting fractional polynomial regression model describing the relationship between the mV values and the log-transformed ($\log_{10}$) fluoride concentrations (ppm) of the standard solutions. The log-transformation was carried out in order to remove skewness and normalise the data. The resulting mathematical functions were used to convert the observed mV-values into log fluoride concentrations ($\log_{10}$(ppm)). Two missing data points were estimated using unit imputation, that is, by averaging the values obtained immediately before and immediately after the missing data point. The pre-study examination saliva sample data were only used for the purpose of allocating the participants to the experimental groups and were not included in the statistical analysis described below. The $\log_{10}$(ppm) fluoride concentrations were subsequently converted to ln(ppm) fluoride concentrations for the purpose of being able to estimate contrasts of marginal linear predictions following mixed-effects linear regression analysis.

As the data comprised 10 repeat fluoride concentration (ln(ppm)) estimates per participant, a hierarchical model was used in the analysis. A mixed-effects linear regression with an unstructured covariance structure was fitted using trial experimental regimen (test group vs. control group, Figure 1), and day of sampling as the predictors. An interaction term was added, between the experimental regimen and day of sampling. The geometric mean salivary fluoride concentrations and their 95% confidence intervals were estimated by back-transforming the ln-transformed values for each of the ten sampling days, day 0 (baseline), 7, 10, 14, 21, 22, 23, 24, 28 and 35 for both experimental study arms. Likewise, the fluoride concentration differences (in percentage of the control) including their 95% confidence intervals between the two groups were estimated for each of the 10 sampling days. Model validation was carried out by inspecting the standardised residuals against the fitted values and inspecting the QQ-plot for the standardised

TABLE 1 Baseline demographic and clinical characteristics. Estimates are mean values (SD) or counts (%).

Group	Control ($n = 24$)	Test ($n = 24$)
Sex (n, % female)	16 (67 %)	19 (79 %)
Age (years)	23.5 (2.2)	22.4 (1.5)
Salivary flow rate (mL/5 min) at the pre-study clinical examination	2.1 (1.7)	2.3 (1.6)
Using manual toothbrush[a]	13 (54 %)	13 (54 %)
Fluoride concentration (ppm) in toothpaste used prior to study start	1450 ($n = 24$)	1250 ($n = 1$) 1450 ($n = 22$) 1490 ($n = 1$)
Time since brushing (h) at the pre-study examination	5.8 (1.4)	6.2 (1.5)
Time since brushing (h) at baseline; trial phase, day 0	7.7 (2.3)	7.7 (2.5)

[a]Five individuals, reporting to use alternately manual and electric toothbrush, were allocated to the group using electric toothbrush.

residuals; and the assumptions of equal standard deviations and correlations in the two experimental groups were found reasonable.

The amount of toothpaste (g) used per brushing was estimated for each of the two groups (test and control) along with the difference between groups.

RESULTS

The baseline (before randomisation to groups) geometric mean salivary fluoride level in the present study, at day 0 of the trial phase, was 0.015 ppm (95% CI [0.012; 0.018]), and the post-randomisation values were 0.014 ppm (95% CI [0.011; 0.019]) and 0.016 ppm (95% CI [0.012; 0.021]) for the control and test group, respectively. Baseline demographic and clinical characteristics across groups are described in Table 1.

During the study, two saliva samples could not be obtained according to plan, leaving 478 saliva samples analysed for fluoride. The two missing data points were estimated using unit imputation. Substitution values were obtained as the mean of the value immediately before and after each missing value for each of the two participants in question. All saliva samples collected during the trial and wash-out phase were collected at a minimum of 4 h after the last toothbrushing with a mean of 7.2 h in both groups and SD values of 1.3 and 1.4 for the control and test group, respectively.

The salivary fluoride concentration increased markedly in the 5000 ppm fluoride group during the trial phase (Figure 3). The salivary fluoride concentration for the 1450 ppm fluoride group apparently also increased during the trial phase, but to a much lesser extent than the increase shown in the test group (Figure 3). The difference in mean salivary fluoride concentration for the test group and the control group, accumulated during the trial phase, reduced promptly (Figure 3), remaining statistically significantly higher only at day 22 (the first day of the wash-out phase), but tended to remain higher. Notably, the 95% confidence intervals for the geometric mean salivary fluoride concentrations (Figure 3) indicate a very large variation in the salivary fluoride concentrations at all time points in both experimental groups.

The multilevel mixed-effects linear regression analysis showed statistically significant differences between groups for all time points during the trial phase, except at baseline (Figure 3). The salivary fluoride level for the test group remained statistically significantly elevated for 1 day during the wash-out phase where use of 1450 ppm fluoride toothpaste was reinstituted, but after this time, the difference between groups were not statistically significant (Figure 3).

Adverse effects

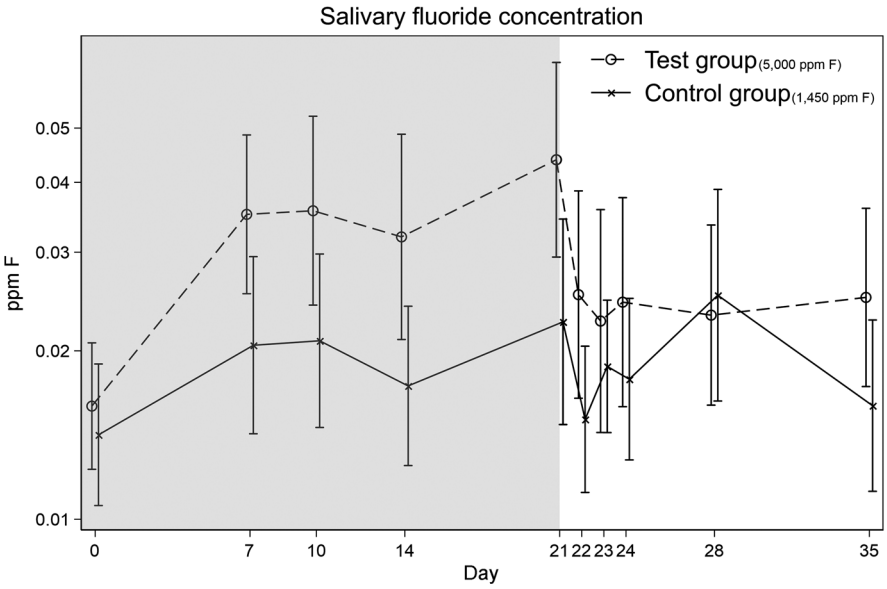
One participant, having no prior history of migraines, reported having had a migraine during day 12 and 13 of the trial phase. Another participant reported having developed a skin rash in the ear, neck and thorax areas 2 weeks into the experimental period. Both symptoms were registered as possible adverse reactions since it could not be ruled out that the trial toothpaste was the cause. The participants in question were later shown to belong to the test group. No other adverse effects or reactions were reported.

Compliance

A single participant, belonging to the test group, deviated from the protocol for four successive brushings during the trial phase, starting with the evening-brushing at day 17. This participant used a regular 1450 ppm fluoride toothpaste for these four brushings but returned to the trial toothpaste afterwards. The remaining 47 participants reported to have complied with the protocol. However, weighing of the used tubes showed that the amount of paste used per brushing varied considerably between participants. The amount of toothpaste used, 0.64 and 0.60 g/brushing for the control and test group, respectively, did not differ statistically significantly (diff. 0.04 g/brushing (95% CI [−0.05; 0.14])).

DISCUSSION

To the best of our knowledge, the present study is the first to show both the wash-in and the wash-out effect on the ambient



		Trial phase					Wash-out phase				
		Day 0	Day 7	Day 10	Day 14	Day 21	Day 22	Day 23	Day 24	Day 28	Day 35
Test group	GM (ppm)	0.016	0.035	0.036	0.032	0.044	0.025	0.023	0.024	0.023	0.025
	[95 % CI]	[0.01; 0.02]	[0.03; 0.05]	[0.02; 0.05]	[0.02; 0.05]	[0.03; 0.07]	[0.02; 0.04]	[0.01; 0.04]	[0.02; 0.04]	[0.02; 0.03]	[0.02; 0.04]
Control group	GM (ppm)	0.014	0.020	0.021	0.017	0.023	0.015	0.019	0.018	0.025	0.016
	[95 % CI]	[0.01; 0.02]	[0.01; 0.03]	[0.01; 0.03]	[0.01; 0.02]	[0.01; 0.03]	[0.01; 0.02]	[0.01; 0.02]	[0.01; 0.02]	[0.02; 0.04]	[0.01; 0.02]
Estimated difference between groups in salivary F in % of control [95 % CI]		13 [-23; 65]	71 [8; 172]	71 [3; 184]	85 [10; 210]	95 [11; 243]	67 [0; 78]	21 [-28; 102]	37 [-19; 133]	-8 [-47; 160]	56 [-5; 156]

FIGURE 3 Estimated geometric mean (GM) values and 95% confidence intervals for the salivary fluoride concentrations for the two experimental arms, test and control group at each time point. The table below the figure shows the estimated differences in the salivary fluoride concentrations (in %) and their 95% confidence intervals for each time point (test-control; in % of control). The shaded areas in the figure and the table represent the trial phase. F, fluoride. (n = 24/group).

salivary fluoride level following regular and prolonged use of 5000 ppm fluoride toothpaste. The findings show distinctly higher ambient salivary fluoride levels after commencement of brushing with 5000 ppm fluoride toothpaste than seen with continued use of 1450 ppm fluoride toothpaste. This elevated level of fluoride in saliva was reduced after only 1–2 days after switching back to toothbrushing with 1450 ppm fluoride toothpaste, indicating no durable salivary fluoride reservoir.

The observed changes in the salivary fluoride concentration over time for the group of participants assigned to 5000 ppm fluoride toothpaste corroborates the pattern described in a study by Duckworth and Morgan [32] who explored the salivary fluoride level after use of toothpastes with different fluoride concentrations. Use of 1500 ppm fluoride toothpaste resulted in salivary fluoride levels similar to baseline observations in the present study, whereas the use of 2500 ppm fluoride toothpaste resulted in salivary fluoride levels of approximately 0.017 ppm being reached after 1-4-weeks use (estimated from a graph) [32]. This was about half the level attained in the present study following the use of 5000 ppm fluoride toothpaste (0.035, 0.032 and 0.044 ppm fluoride after 1, 2 and 3 weeks, respectively). As in the present study, Duckworth and Morgan [32] also reported a rather prompt drop in salivary fluoride after ending high-fluoride exposure.

While it is intuitively easy to understand that fluoride in saliva increases with increasing fluoride in toothpaste, it is more challenging to explain the rapid decrease in salivary fluoride after discontinued use of high-fluoride toothpaste. The higher levels of ambient salivary fluoride recorded after 3 weeks of regular use of 5000 ppm fluoride toothpaste are presumed to result from fluoride retained in the oral cavity (fluoride reservoirs). These reservoirs mainly consist of calcium fluoride and phosphate-containing calcium fluoride deposits (calcium fluoride-like deposits) precipitated on dental hard tissue and in dental biofilm [33] and fluoride bound to mucosal epithelial cells [26] by extracellular calcium-bridging [27], although mucosal surfaces may only serve as a short-term reservoir [17]. Theoretically, calcium

fluoride-like deposits precipitate when the fluoride concentration in saliva increases above 100 ppm [34], and it is common perception that standard fluoride toothpaste (≤1500 ppm) does not lead to any notable calcium fluoride precipitation due to the immediate mixing of the paste with saliva [34]. However, brushing with high-dosage fluoride toothpaste is assumed to form a considerable amount of calcium fluoride/calcium fluoride-like deposits on tooth surfaces and in dental biofilm and particularly in porous carious lesions [16, 24, 33].

Calcium fluoride formed in caries lesion porosities are protected by phosphate [35]. These precipitates are therefore supposed to dissolve very slowly during weeks to months whereby they give rise to local elevated fluoride concentrations at the biofilm-lesion interface for a relatively long period of time [11, 24]. On the other hand, calcium fluoride deposits on clinically sound surfaces have been shown to dissolve quickly within a few hours [25, 36]. In the current study of healthy young university students with limited biofilm accumulation due to frequent brushing, tooth surfaces may therefore have been depleted of fluoride within a few hours. This may explain the rapid decrease in the concentration of fluoride in saliva after cessation of using 5000 ppm fluoride toothpaste. Our results support the assumption that the calcium fluoride deposits formed are not sufficiently large to sustain a long-lasting elevation of the whole mouth saliva fluoride level in individuals with good oral hygiene. The present findings thus indicate that the desired elevated day and night bioavailability of fluoride in whole mouth saliva can only be obtained by regular, as opposed to irregular, use of the 5000 ppm fluoride toothpaste. Even so, it cannot be ruled out that minute calcium fluoride reservoirs could still be present which might produce an effect in the immediate microenvironment, rather than in the 'global' oral environment represented by the whole saliva sample. It should be recognised that fluoride toothpaste is the major source of salivary fluoride, and it is therefore not entirely unexpected to find that the salivary fluoride levels, in persons with normal salivary flow and (presumably) a minimal number of active caries lesions, reduce quite soon after the transition from the use of a 5000 ppm fluoride toothpaste to a version containing 1450 ppm fluoride. This confirms a short-term study by Fernández et al. [23] reporting a rapid decrease of salivary fluoride levels following thrice daily use of 5000 ppm fluoride toothpaste.

The present study has been preceded by three studies evaluating the wash-in effect of brushing with 5000 ppm fluoride toothpaste on steady-state ambient salivary fluoride levels [21–23]. Fernández et al. [23] reported fluoride levels of 0.04 to 0.05 ppm (mean values) during thrice daily use of 5000 ppm fluoride toothpaste, and these results corroborate the findings of the present study. However, the salivary fluoride levels reported by both Pessan et al. [22] and Ekstrand et al. [21] were notably higher than observed in the present study. Though, these studies differ considerably in their design from the present study [21, 22], in terms of higher fluoride level in the drinking water [22], fluoride measurements based on stimulated saliva samples collected at standardised intervals after overnight fasting 12 h after brushing [22], participants being older community dwelling adults [21], use of prescription drugs possibly reducing salivary flow [21], unsupervised saliva sampling at time points varying from early morning to night [21] and thrice daily toothbrushing [21, 22]. An additional factor to consider is the caries-activity of the participants in the studies. It is well-known that active non-cavitated lesions constitute a considerable (possibly the most important) reservoir of calcium fluoride [34]. This factor could prove particularly significant considering individuals with reduced saliva secretion owing to the low salivary clearance rate. Unfortunately, the caries status, in particular with respect to the number of non-cavitated active lesions, was not assessed in any of the studies.

In this context, the large inter-individual variation in salivary fluoride levels deserves attention. As indicated above, many factors may affect the salivary fluoride levels in a way that would contribute to inter-individual variation, and such variation is a common finding in other studies, following both standard and high-dosage fluoride exposures [21, 22, 32, 37]. In the present study we sought to remove some of this variation by employing a minimisation-based randomisation-procedure based on salivary fluoride level. However, this clearly did not reduce the variation satisfactorily, and in hindsight it might have been a better approach to base the minimisation on salivary flow rate [38] and perhaps caries-activity assessment of the participants [39, 40].

In the present study, we tried to take into account the circadian rhythm in salivary flow [41] and salivary fluoride clearance [38] by standardising sampling to the afternoon, which was the most convenient time point for the participants owing to study obligations. While this might have resulted in additional variation in the salivary fluoride levels, the sampling point was approximately the same for all participants throughout the study. An additional source contributing to the considerable inter-individual variation in the salivary fluoride levels may relate to the fact that working near the limit of detection for the fluoride electrode may pose a risk for obtaining approximate rather than absolute data, even when being extremely meticulous during analyses [42]. It could be argued that we should have used a cross-over trial design rather than the present parallel group design. However, the risks to crossover trials are changes between the first and second trial period and carry-over effects. As we considered these factors to pose serious risks in a cross-over trial, possibly leading to one half of the trial being abandoned, we preferred the parallel group design. Finally, because the Covid-19

restrictions led to 12% of the samples being collected unsupervised in participants' homes we might suspect a greater variability in these samples.

During the trial phase, we observed a small but noticeable increase of the salivary fluoride levels in the control group using 1450 ppm fluoride toothpaste, even though the participants had used similar toothpaste prior to study start. The most plausible explanation for this observation is a Hawthorne effect [43], that is, a behavioural modification in response to the awareness of being observed. Hence, the many appointments at the trial site can be expected to have reminded the participants of the use of toothpaste, which may have led to a better 'brushing compliance' than before entering the study. The fact that all participants were individually instructed to brush carefully twice daily and use 1 g of toothpaste per brushing is also likely to have contributed to this behavioural modification.

Reporting of adverse events and reactions is an essential part of the mandatory rigorous regulations on clinical trials on medicinal products for human use. During the present study no serious adverse effects (SAEs), serious adverse reactions (SARs) nor suspected unexpected serious adverse reactions (SUSARs) were registered. Two incidents were registered as possible adverse reactions (ARs), since it could not be ruled out that the trial toothpaste (Duraphat) was the cause. One of the incidents covered a skin rash in the ear, neck and thorax area. This type of reaction, an allergic reaction, is mentioned in the label insert for Duraphat as a possible side effect. However, determining which component in the toothpaste might be responsible for this adverse event remains unknown, but we consider it unlikely that this adverse effect is due to the high fluoride concentration. The other incident, migraine, is beyond the known side effects of Duraphat.

In conclusion, the present study has shown that twice daily toothbrushing with 5000 ppm fluoride toothpaste increases the level of salivary fluoride, which—among young healthy university students—is sustained only as long as the brushing habit continues.

AUTHOR CONTRIBUTIONS

Conceptualization: Line Staun Larsen, Bente Nyvad, Vibeke Baelum; **Methodology**: Line Staun Larsen, Bente Nyvad, Vibeke Baelum; **Formal analysis**: Line Staun Larsen, Vibeke Baelum; **Investigation**: Line Staun Larsen; **Data Curation**: Line Staun Larsen; **Writing—original draft preparation**: Line Staun Larsen; **Writing—review and editing**: Line Staun Larsen, Bente Nyvad, Vibeke Baelum; **Visualization**: Line Staun Larsen, Vibeke Baelum; **Supervision**: Line Staun Larsen; **Project administration**: Line Staun Larsen; **Funding acquisition**: Line Staun Larsen, Bente Nyvad, Vibeke Baelum.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interests.

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