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Fluoride and Functionalised β-tricalcium Phosphate (fTCP) Fluoride Toothpaste Affect the Primary Dentin Caries Surface: A Comparison by Estimation Statistics

Trieu Kim Ly, Thoai Quoc Kieu, Nam Cong-Nhat Huynh*, Lan Thi Quynh Ngo

Department of Dental Basic Sciences, Faculty of Odonto-Stomatology, University of Medicine and Pharmacy at Ho Chi Minh City, 652 Nguyen Trai, Ward 11, District 5, Ho Chi Minh City 749000, Vietnam

* Corresponding author: namhuynh@ump.edu.vn

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ABSTRACT

This study aims to compare the effect of fTCP-contained toothpaste in combination with 950 ppm fluoride on primary dentin caries surface to ordinary 1100 ppm fluoride toothpaste by using estimation statistics. Dental caries with deep lesion and dentin exposure from nine primary teeth were cut into two equal parts and randomly divided into two groups in a before-after study. Each group was brushed with one type of toothpaste two times per day for 28 days and rested in artificial saliva. SEM images of cavity bottom surfaces and energy-dispersive X-ray spectrometry (EDX) were used to determine the percentage of calcium (Ca), phosphorus (P), fluoride (F) and oxygen (O) at six continuous measured areas from the surface of the cavity bottom into 45 µm depth before and after brushing. About 95% confidence interval of the mean difference was calculated by performing bootstrap resampling with 5000 resamples followed by comparison analysis. The percentage of Ca, P, F, O was shifted after treatment. There was no difference between the two groups. The SEM images reflected a similar illustration of EDX data. The combination of fTCP and 950 ppm fluoride in toothpaste showed equivalent effectiveness to the 1100 ppm fluoride toothpaste in primary dentin caries.

Keywords: *Fluoride; fTCP toothpaste; primary dentin caries; remineralization*

INTRODUCTION

Early childhood caries (ECC) is a serious public health challenge in both developing and developed countries. In Southeast Asia countries, recent studies reported a much higher prevalence of ECC than those in developed countries due to the high-frequency sugar intake, inappropriate sweetened feeding bottles, the lack of oral hygiene and low socioeconomic status (Karlinsey *et al.*, 2009; Khanh *et al.*, 2015). ECC progresses rapidly and often be untreated, affecting not only the general health but also the life quality of children. As a chronic infectious disease, bacteria, mainly *Streptococcus mutans* and *Streptococcus sobrinus*, cause a tooth decay lesion by acids from bacterial metabolism, changing from local environmental conditions to an acidic environment and resulting in demineralisation of dental hard tissues. Remineralisation, on the other hand, is a natural repair process occurring on the non-cavitated carious lesions. It rebuilds a new surface containing remineralised crystals which are acid-resistant. Therefore, increasing remineralization is key to ECC management and prevention (Sumikawa *et al.*, 1999).

The most effective method to prevent tooth demineralization and enhance the remineralization is topical fluoride application therapy, such as toothpaste and varnish (ten Cate, 2013). Fluoride ions speed up the remineralisation process, together with calcium and phosphate ions, diffuse into the tooth and form fluorapatite, which is much less soluble than carbonated hydroxyapatite and more difficult to dissolve by organic acids from bacterial metabolism. However, to form one unit of fluorapatite [$Ca_{10}(PO_4)_6F_2$], every two fluoride ions require ten calcium ions and six phosphate ions. Therefore, even the existence of an appropriate amount of fluoride ions, the remineralisation process can be restricted by the inadequate availability of calcium and phosphate ions. Functionalized β-tricalcium phosphate (fTCP) and fluoride-containing toothpaste have been developed for this reason (Karlinsey & Pfarrer, 2012). fTCP works in synergy with fluoride to form a more acid-resistant mineral incorporation to that attainable with fluoride or fTCP alone by the encouragement of greater calcium, phosphate, and fluoride uptake in demineralised areas, thus preventing demineralisation and enhancing remineralisation. Recently, numerous studies have demonstrated anticarcinogenic activity and remineralisation effect of fTCP/fluoride (F-fTCP) toothpaste. However, most studies were in vitro experiments on artificial caries like enamel lesions (demineralized by acidic solutions such as Ca(Ca $(NO_3)_2$, PO_4 (KH_2PO_4) and acetate) or in situ experiments using bovine root dentin blocks (Oliveira *et al.*, 2016; Kamath *et al.*, 2017; Alhamed *et al.*, 2020).

Following up on those insights, our study for the first time evaluated the effects of fluoridated (NaF, 1100 ppm F) and fTCP/fluoride-containing toothpaste (950 ppm F) on primary dentin caries surface by using energy-dispersive X-ray spectroscopy (EDX) and scanning electron microscope (SEM). In this study, we applied estimation statistics for the comparison analysis. The limitations of null-hypothesis significance testing have been discussed while the use of *P* values can lead to biased analyses because false positives may be overhyped, and some real effects are ignored. Estimation methods that estimate effect sizes and their uncertainty have great potential to shift the current data analysis away from dichotomous thinking and toward quantitative reasoning (Efron, 1992; Amrhein *et al.*, 2019; Wasserstein *et al.*, 2019). This study could add new evidence to the effect of fTCP/fluoride-containing toothpaste and fluoride toothpaste.

MATERIALS AND METHODS

Specimen Preparation

Nine primary teeth extracted because of their mobility and time for exfoliation from nine patients from 6 to 12-year-old at the Dental Treatment Center of the University of Medicine and Pharmacy at Ho Chi Minh City were used as specimens in a before-after study. The teeth exhibited dentin caries involving the dentin of the buccal surface with a caries lesion size larger than 2 × 2 mm without hypoplastic areas. The teeth were cleaned to remove calculus and remaining soft tissue. The specimens were then stored in distilled water until use. The use of discarded tissues for research purposes was approved by the Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City as a required part of graduate study (Ref. No.: 8720501).

The teeth were de-identified and analysed anonymously. Caries lesion in buccal surfaces was perpendicularly separated into two blocks using a water-cooled diamondimpregnated low-speed saw (Fig. 1A). Two blocks of the sample were obtained from each tooth. The cutting edge of the specimens was then polished using a 1 μm diamond polishing suspension with a polishing cloth. To prevent unexpected contact with the demineralising and remineralising agents, the enamel surfaces were painted with acid-resistant varnish, except for a window of 2 × 1 mm of caries lesion.

Experimental Design

Eighteen blocks of the sample were randomly and equally divided into two groups: group F - Colgate® Total Clean Mint (1100 ppm F as NaF; Colgate-Palmolive, New York, NY, USA) and group T (F-fTCP) - Clinpro™ Tooth Crème (fTCP + 950 ppm F as NaF; 3M, St Paul, MN, USA) (Fig. 1B). A standardised volume (0.15 mL) of the agent was applied to each sample for two minutes, twice a day for 28 days by orthodontics toothbrushes using brushing action. The specimens were rinsed with 10 mL distilled water and then soaked in the artificial saliva (pH = 7.1) solution in a 37°C incubator (Yamaguchi *et al.*, 2006). The artificial saliva solution was changed daily.

SEM and EDX Analysis

To evaluate and compare the remineralisation effect of each kind of toothpaste, SEM and EDX measurements of individual specimens were recorded and analysed before and after treatments. For SEM analysis, the specimens were dehydrated in increasing grades of ethanol (30%, 50%, 70%, 90%, 96% and 100%) for 10 minutes each grade and then freeze-dried at −30°C in a vacuum desiccator (de Azevedo *et al.*, 2014). The dried specimens were then mounted on to a SEM stub without coating. After that, tooth surfaces were analysed under a SEM-EDX spectroscopy (JSM - IT100, JEOL, Tokyo, Japan) at ×30, ×250, ×1000 and ×2000 magnifications using biological sample measurement setting. SEM photomicrographs were captured at 1000× magnification (20 kV voltage, low vacuum-45 Pa).

Following the SEM examination, EDX analysis was performed with the same SEM-EDX spectroscopy to determine weight percentages of calcium (Ca), phosphorus (P), oxygen (O) and fluoride (F) content of each specimen. The representative measurement areas were selected under 1000× magnification. The chemical elements analysis was performed on the surface of the cross-sections, starting at the outer layer of tooth surface moving pulp-wards (Fig. 2A). In total, five measurements (at 0–5, 5–10, 10–15, 15–20, 20–25 μm) with an area of 5 × 50 μm were taken every 5 μm per line. The final measurement section (at 25 to 45 μm) was parallel crossed (20 × 50 μm) in the dentinal tubule area. Distributions and chemical elements in each specimen were examined under the same conditions at 20 kV voltage, 10 mm working distance and under low pressure (40 Pa). For each specimen, the average of weight percentages of each element from 0–25 μm and 0–45 μm were calculated. Finally, Ca/P stoichiometric ratios and the percentage gain of Ca/P ratio after treatment were also valued.

Data Analysis

Data analysis was performed in RStudio (Version 1.2.1335, RStudio, Inc., Boston, MA, USA). Heatmaps of all data were generated by *pheatmap* package (Version 1.0.12). Estimation statistics and graphics were performed by DABEST (data analysis with bootstrap-coupled estimation) package (Version 0.2.2) as previously (Ho *et al.*, 2019). About 95% confidence interval (95% CI) of the mean difference was calculated by performing bootstrap resampling with 5000 resamples (Efron, 1992). Gardner-Altman estimation plot performed two features: Firstly, all data points were presented as a swarmplot and Tufte slopegraph, which orders each measure to display the underlying distribution. Secondly, the effect size was presented as a bootstrap 95% CI on separate but aligned axes. When the 95% CI bootstrap of difference includes the 0 value of effect size, there is no difference between the two groups (Gardner & Altman, 1986).

RESULTS

SEM Revealed the Mineral-Deposition in Dentin Tubular after 28 Days of Tooth Brushing

Differences were observed between the dentin surfaces obtained before and after treating with two types of toothpaste (Fig. 1C). SEM analysis of exposed dentin surface before treatment of both groups showed typical dentinal tubules and intertubular dentin structure (Fig. 1D). Moreover, remaining dentin layers were characterised by an irregular and rough surface with some smear layer covered on the surface and filled the dentinal tubules (Fig. 1E). Some enlarged dentinal tubules were recorded.

After treatments, the surfaces of demineralised dentin were partially covered by a layer of mineral particles. Mineral particles deposited within the dentinal tubules (Fig. 1F). The partial opening of dentinal tubules with a residual mineral deposit was also demonstrated (Fig. 1G). The well of dentinal tubules was fully occluded by mineral particles (Fig. 1H).

EDX and Estimation Statistics Indicated the Alteration of Element Compositions in Primary Dentin Caries Surface

Heatmap of all weight percentages of Ca, P, F and O from EDX measurement (at 0–5, 5–10, 10–15, 15–20, 20–25 μm) and the average of weight percentages of each element from 0–25 μm and 0–45 μm were visualised in Fig. 2B. In general, oxygen took the highest percentage in all measurements, followed by calcium, phosphorus respectively while fluoride was lowest.

Different groups being compared included observations from the same specimen (at two time-points, before and after brushing), we used a slopegraph to depict each pair of repeated observations (Fig. 3). The lines in these plots were able to illustrate the presence of any trend between the two time-points. The calcium weight percentages of 1100 ppm F toothpaste group were strongly decreased after 28 days in all sections from 0–45 μm (Fig. 3A). In reverse, 950 ppm F-fTCP toothpaste group showed a slightly decreased calcium percentage at the outer layers of the specimens (0–20 μm) while the change in 20–45 μm areas was undetermined (Fig. 3A). Phosphorus weight percentages were also in the same manner. Estimation analysis indicated that in 1100 ppm F toothpaste group, phosphorus percentages reduced after 28 days of brushing at 0–25 μm sections (Fig. 3B). In 950 ppm F-fTCP toothpaste brushed dentin, phosphorus weight percentages did not shift after treatment (Fig. 3B).

In contrast, fluoride weight percentages were increased at 15–20 μm and 20–25 μm sections in 1100 ppm F toothpaste group and at 0–5 μm section in 950 ppm F-fTCP toothpaste group after 28 days of brushing (Fig. 3C). Additionally, oxygen percentages shifted at all sectional areas in 1100 ppm F toothpaste group and at only one area (10–15 μm) in 950 ppm F-fTCP toothpaste group (Fig. 3D).

Compared to two types of toothbrush groups, there was no difference in weight percentages of any elements (C, P, F, O) between two groups at two time-points (data not shown). Likewise, Ca/P stoichiometric ratios were also not different in each type of toothpaste before and after brushing or between two groups at two time-points (Fig. 4A, 4B). Finally, the percentage gain of Ca/P ratio after treatment of two kinds of toothpaste was comparable (Fig. 4C).

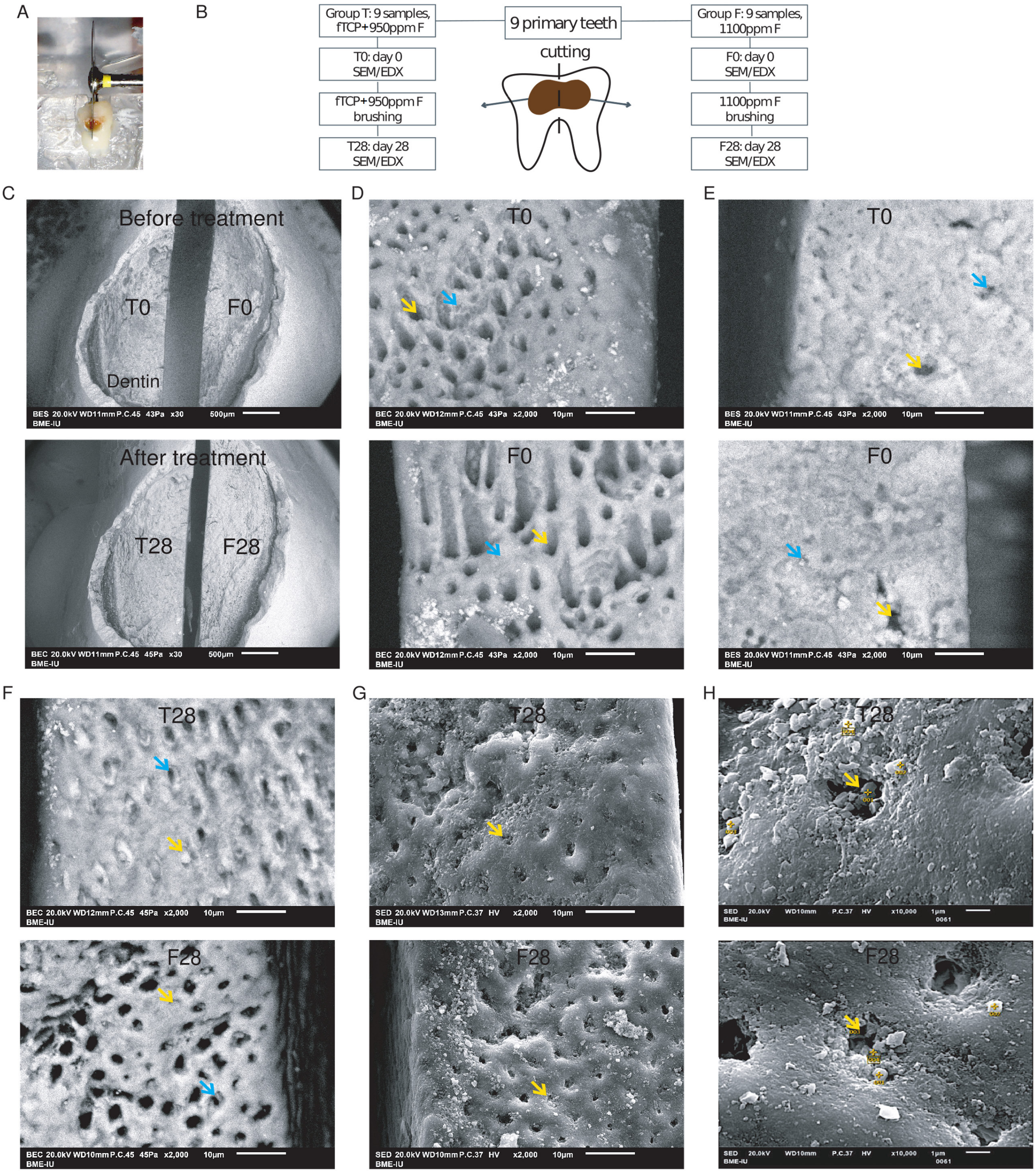

Fig. 1 **(A)** Caries lesion in buccal surfaces was perpendicularly separated into two blocks. **(B)** Framework of experimental design. **(C)** x30 non-coated SEM images of two specimens with the dentin caries lesion from the same tooth before and after treatment. **(D)** x2000 non-coated SEM images of two groups at day 0 (baseline), blue arrowhead: intertubular dentin, yellow arrowhead: dentinal tubule. **(E)** x2000 non-coated SEM images of two groups at day 0 (baseline) with an irregular and rough surface with some smear layer covered intertubular dentin and filled the dentinal tubules (blue arrowhead), yellow arrowhead: enlarged dental tubule. **(F)** x2000 non-coated SEM images of two groups at day 28, yellow arrowhead: mineral-deposited dentinal tubule, blue arrowhead: peritubular dentin. **(G)** x2000 and **(H)** x10000 gold-coated SEM images of two groups at day 28, yellow arrowhead: mineral-deposited dentinal tubule.

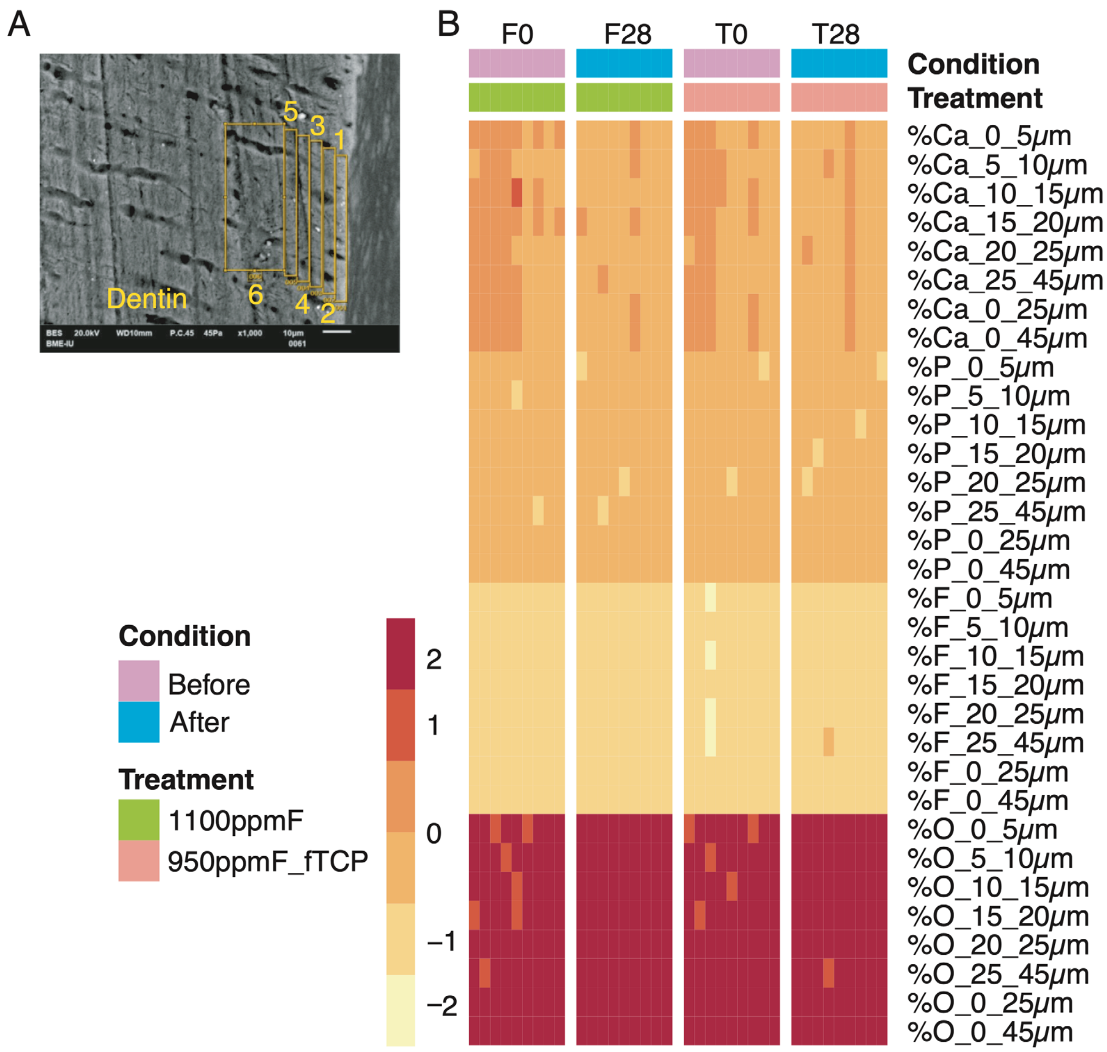

Fig. 2 **(A)** EDX measurement areas (1, 2, 3, 4, 5, 6: 0–5, 5–10, 10–15, 15–20, 20–25, 25–45 µm, respectively). **(B)** Heatmap of all weight percentages of Ca, P, F and O from EDX measurement (at 0–5, 5–10, 10–15, 15–20, 20–25 µm) and the average of weight percentages of each element from 0–25 µm and 0–45 µm. Data were scaled from actual values to scaled values (–2 to 2) for heatmap visualisation.

DISCUSSION

Within the scope of this study, we investigated the effects of fluoridated (NaF, 1100 ppm F) and fTCP/fluoride-containing toothpaste (950 ppm F) on primary dentin caries surface by using EDX and SEM, and applied estimation statistics for the comparison analysis. The study was designed to mimic the real situation where the primary teeth were brushed with different types of toothpaste to remineralise the dentin caries. Dentin samples were low conductivity materials and re-measured after 28 days. In our study model, we used biological sample measurement setting (low vacuum, 20 Kv) of SEM-EDX spectroscopy instead of high vacuum setting for uncoated dentin samples to avoid burning sample. About 20 kV voltage was also used effectively to measure elements with low atomic number such as fluoride (with nine protons) by increasing the detection capacity (Vicente *et al.*, 2017a; Vicente *et al.*, 2017b; Scimeca *et al.*, 2018). Moreover, the rehydration of human dentin sample could reverse the mechanical property changes due to dehydration process (Jameson *et al.*, 1993; Nalla *et al.*, 2005;

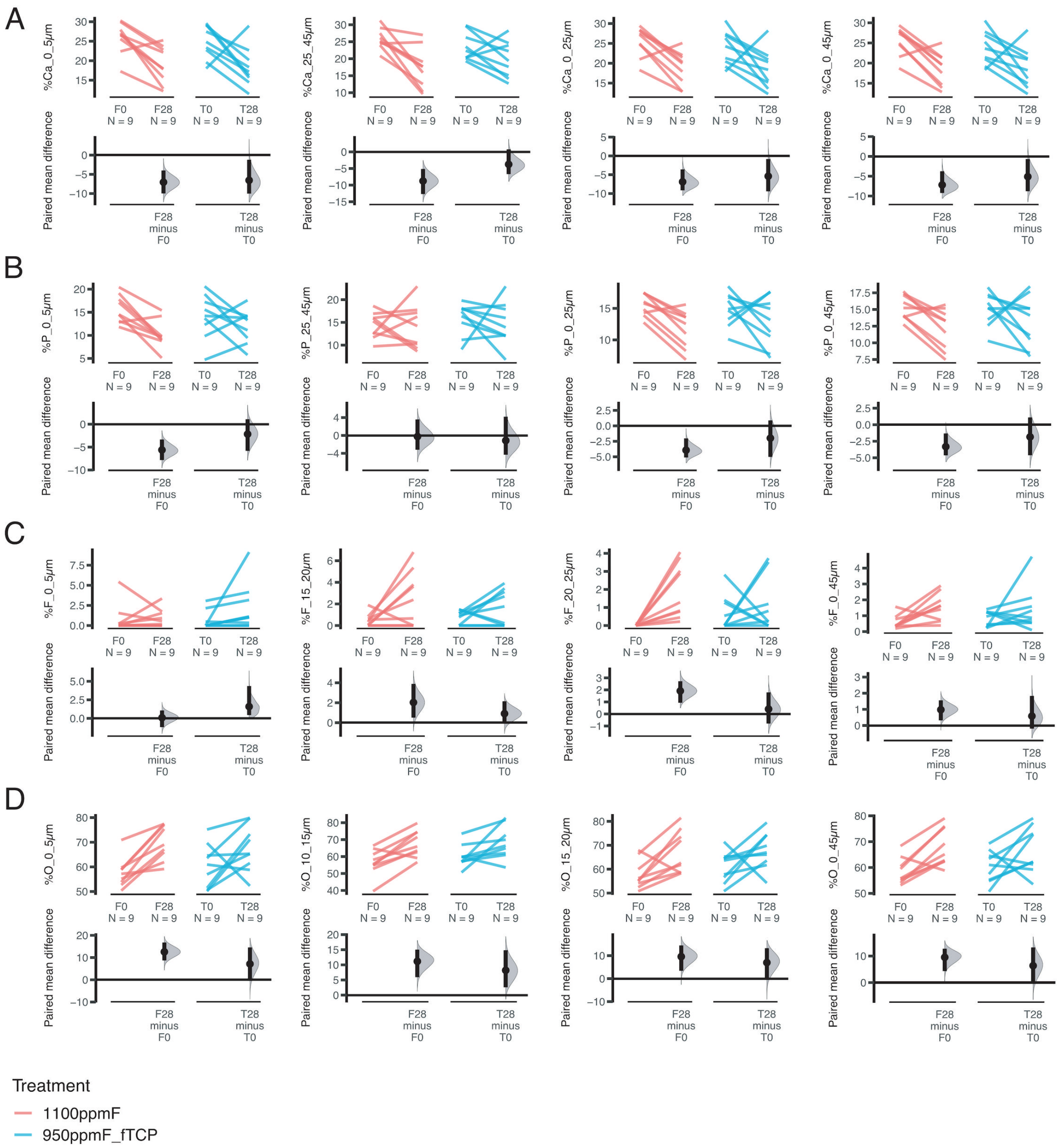

Fig. 3 Estimation comparison of two paired groups (at two time-points, before and after brushing) of each type of toothpaste groups. The weight percentages of **(A)** calcium (Ca), **(B)** phosphorus (P), **(C)** fluoride (F) and **(D)** oxygen (O) at 0–5, 5–10, 10–15, 15–20, 20–25 µm and the average of weight percentages of each element from 0–25 µm and 0–45 µm were illustrated. Some sections which were not different were not shown.

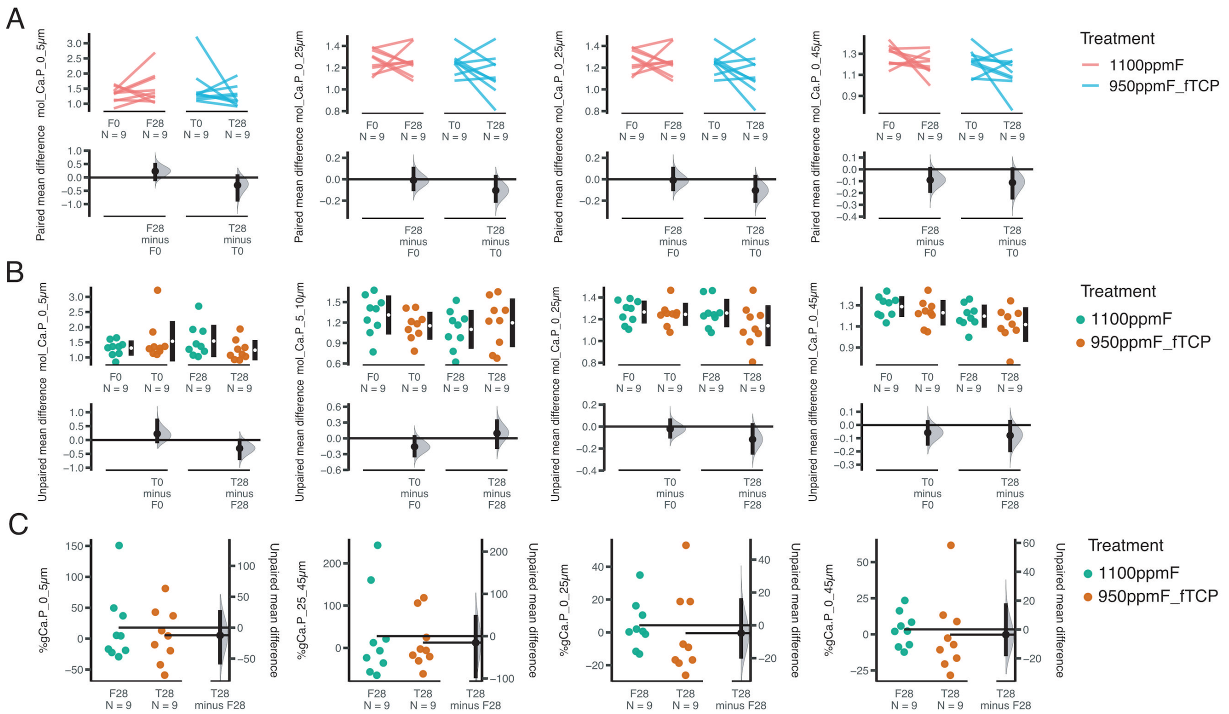

Fig. 4 **(A)** Estimation comparison of two paired groups (at two time-points, before and after brushing) of each type of toothpaste groups on Ca/P stoichiometric ratios. **(B)** Estimation comparison of two toothpaste groups of each time-points (day 0 and day 28) on Ca/P stoichiometric ratios. **(C)** Estimation comparison of two toothpaste groups on percentage gain of Ca/P ratio after 28 days. Some sections which were not different were not shown.

Nalla *et al.*, 2006). In our study, we used artificial saliva to rehydrate dentin specimens in order to limit any harm during the sample preparing.

SEM is one of the most sensitive, qualitative techniques to evaluate the remineralisation of the dental caries lesions *in vitro* as reported in previous studies (Rodríguez-Vilchis *et al.*, 2011). Our SEM analysis indicates that both types of the toothpaste moderated the decayed dentin surface in primary teeth which were demonstrated as seen by the formation and deposition of mineral particles within the porous defects. The proposed mechanism could be fTCP fluoride toothpaste providing mineral ions to repair demineralised fluorapatite crystals in decay dentin rather than new crystal formation. However, no difference in the surface morphology was observed between two experimental groups. Noticeably, the deposited mineral crystals were not well oriented and different in nanosized particles in comparison to apatitic crystals of tooth enamel in morphology which suggested that the remineralisation layers were may not uniform HA crystals layer. Although it was not the same as the natural enamel crystals in structure, to some extent, the regenerated tissue was quite similar to the enamel when considering the similar composition and structure to the enamel and the similar protective functions to the dentin (Alhamed *et al.*, 2020; Vinod *et al.*, 2020).

Using a different approach from ordinary statistics methods, we calculated the 95% CI of the mean difference by performing bootstrap resampling. The bias-corrected and accelerated bootstrap is a simple but powerful technique that creates multiple resamples with replacement from a single set of observations and computes the effect size of interest on each of these resamples. There are two important benefits when using bootstrap: There is no need to assume that the observations, or the underlying populations, are normally distributed. Moreover, it is easy for the construction of the 95% CI from the resampling distribution (Ho *et al.*, 2019). EDX analysis showed that the calcium weight percentages of 1100 ppm F toothpaste group were clearly decreased in all sections. In reverse, 950 ppm F-fTCP toothpaste group showed a decreased calcium percentage at the outer layers of the specimens. Phosphorus weight percentages were also in the same manner while it did not shift after treatment. Moreover, the extended increase of oxygen and fluoride percentage may affect the percentage of other elements especially the Ca/P ratio and increasing Ca/P ratio (from average 1.4 to 1.8 at dentin) indicated the remineralization (Rythén *et al.*, 2010). In the fTCP group, we used 950 ppm sodium fluoride toothpaste containing fTCP, which, according to the manufacturer, is an innovative calcium-based additive that can help to remineralize enamel (Karlinsey *et al.*, 2009). We expected that the fTCP toothpaste comprising 950 ppm F showed a superior effect including the increase of Ca/P ratio. However, the outcome of treatment with fTCP and 1100 ppm F toothpaste was similar that may be explained by the short treatment time (28 days in artificial saliva extra-oral environment). As Ca/P ratio is one of dental tissue mineralisation indicators, our results contribute to the fact that different values for the Ca/P ratio in enamel have been found in several studies (Lakomaa & Rytömaa, 1977; Sasaki *et al.*, 1987; Rythén *et al.*, 2010; Paganelli *et al.*, 2015). Our study showed that fluoride toothpaste increased fluoride content of the primary dentin caries surface. However, within the short time of our experiments, there was no clear remineralisation enhancement evidence as the formation of fluorapatite in the mineral dental tissue is a long time-consuming process. In fact, it requires the presence of fluoride for a long period (Rølla & Saxegaard, 1990; Vicente *et al.*, 2017a). The diversity and complexity of primary dentin caries may help to explain the difficulty and complexity of remineralization by different types of toothpaste in daily practice (Chen *et al.*, 2021). Clinically, in the limit of this study, we propose that using 950 ppm fluoride fTCP toothpaste may moderate primary dentin caries and increase fluoride content. Further studies need to be performed with longer experimental duration to investigate the effects of F-fTCP toothpaste.

CONCLUSION

There was an alteration of dentin caries surface after treatment with two kinds of toothpaste for 28 days *in vitro*. The combination of fTCP and 950 ppm fluoride in toothpaste showed equivalent effectiveness to the 1100 ppm fluoride toothpaste in primary dentin caries.

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