

***In vitro* demineralization of artificial large-scale hydroxyapatite single crystal: Implications of fluoridized tooth enamel for acid resistance**

Yuri Shiga^{*1}, Hideaki Narusawa², Shouji Hironaka³,
Yo Shibata² and Takahiro Funatsu¹

Received: 5 April 2021 / Accepted: 20 May 2021

Abstract

Fluoride treatment has been well known to improve acid resistance of tooth enamel, leading to the widespread use of fluoride therapies in dentistry such as fluoride rinse and fluoride-releasing dental materials. The effect of fluoridation is likely based on the chemical properties of fluoridized hydroxyapatite; however, nanoscale tiny hydroxyapatite crystals in tooth enamel make it difficult to observe the demineralization pattern of fluoridated enamel versus the untreated enamel at a single crystal level. The purpose of this study was to mimic the demineralization patterns of fluoridated and untreated enamel by means of the newly developed hydrothermally enlarged enamel-like hydroxyapatite single crystals at Showa University. Large-scale hydroxyapatite single crystals were hydrothermally produced from polyphosphoric acid and calcium oxide. The crystals were stored in sodium fluoride solutions with four different concentrations for 30 days, then exposed to 3% citric acid solution for up to 60 min. The degradation patterns of each sample were observed using a conventional scanning electron microscope and were analyzed using F K-edge X-ray absorption near edge structure spectra. The surfaces of the hydroxyapatite crystals treated by relatively low concentrations of fluoride showed degradation in citric acid as well as in the untreated control. The hydroxyapatite crystals treated with 1,500 and 9,000 ppm F revealed that the fluoridated outer pillar of the crystals remained mostly intact which made calcium fluoride detectable. Fluoridization of tooth enamel might be preventive of an early acid erosion at a crystal level with a caution of sufficient fluoride concentration and exposure over time. The remaining substantial portion of the crystal pillar implies of highly fluoridized crystals, and a possible remineralization occurs as long as saliva provides minerals required for enamel remineralization by *in vivo* regulation.

Key words :hydroxyapatite, hydroxyapatite single crystal, fluorine, caries prevention, acid resistance, F K-edge XANES

Introduction

Enamel is the outer covering of the teeth which

* Corresponding author

✉ Yuri Shiga
yuri-miyama@dent.showa-u.ac.jp

¹ Department of Pediatric Dentistry, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.

² Department of Conservative Dentistry, Division of Biomaterials and Engineering, Showa University School of Dentistry.

³ Department of Special Needs Dentistry, Division of Hygiene and Oral Health, Showa University School of Dentistry.

allows for essential load-bearing functions of teeth. During enamel development, ameloblast cells undergo apoptosis together with tissue maturation¹, so enamel cannot be repaired biologically, in contrast with other hard tissues with engineering protocols that enable them to regenerate.

Acidic erosion, called dental caries, is one of the most widespread and costly dental diseases that needs to be cured^{2,3}. Tooth enamel undergoes continuous balanced demineralization or remineralization. If remineralization is disrupted, demineralization will progress, causing further deterioration of the tooth structure. As such, the development of strategies to prevent early enamel erosion is long overdue.

The highly precise regulation of the bio-mineralization procedure during amelogenesis results in a prominent inorganic structure. This contains, on average, 95% inorganic hydroxyapatite (HAP), 4% water, and 1% organic substances by weight; or 87% inorganic components, 11% water, and 2% organic components by volume⁴. The most common form of HAP has a hexagonal arrangement, having a $P6_3/m$ space group symmetry, with lattice parameters of $a=b=9.432 \text{ \AA}$, $c=6.881 \text{ \AA}$, and $\gamma=120^\circ$ (a , b , c , and γ are defined by the lattice parameter)⁵. The partially non-stoichiometric HAP is the foundational structural unit of tooth enamel that exploits ion-exchange properties. Fluoride ions are believed to replace the OH^- in the lattice and form fluorapatite which, like enamel apatite, has the hexagonal space group, $P6_3/m$. These two apatite varieties have different chemical and physical properties, which affects the chemical reactivity and stability of enamel. In general, fluoridated apatite has better resistance to acid dissolution than non-fluoridated HAP, which is the major reason for the widespread use of fluoride in oral health care⁶. Although several previous studies have proven the effect of fluoride treatment on tooth enamel^{7,8}, the majority of these studies used empirical evidence and did not conduct a crystallographic study of fluoridized HAP in tooth enamel. This was because of the extremely tiny crystal scale of HAP in enamel that causes its demineralization patterns cannot readily be observed at a single crystal level⁹.

We have developed a new method for growing single, hexagonal crystals of HAP with lengths on the order of hundreds of microns and widths on the order of tens of microns¹⁰. Like enamel apatite crystals, they contain a few percent of impurities, which indicates that these large-scale single crystals can serve as suitable representatives to assess the effects of fluoridization on enamel at the crystal (material) level. In this study, we exposed these large-scale HAP single crystals to several fluoridization conditions. Subsequently, the crystals were exposed to acid, and demineralization patterns were observed.

Materials and methods

1. Preparation of HAP crystals

Figure 1 depicts a flow chart of this study's methodology. The HAP crystals used in this study were prepared according to the report of Narusawa¹⁰ as follows: First, 85% orthophosphate (Tosoh Nikkemi Co., Japan) was stored in a silicon carbide container at 210°C for 2 weeks and was then used to produce polyphosphate. Next, 0.26 g of polyphosphate and

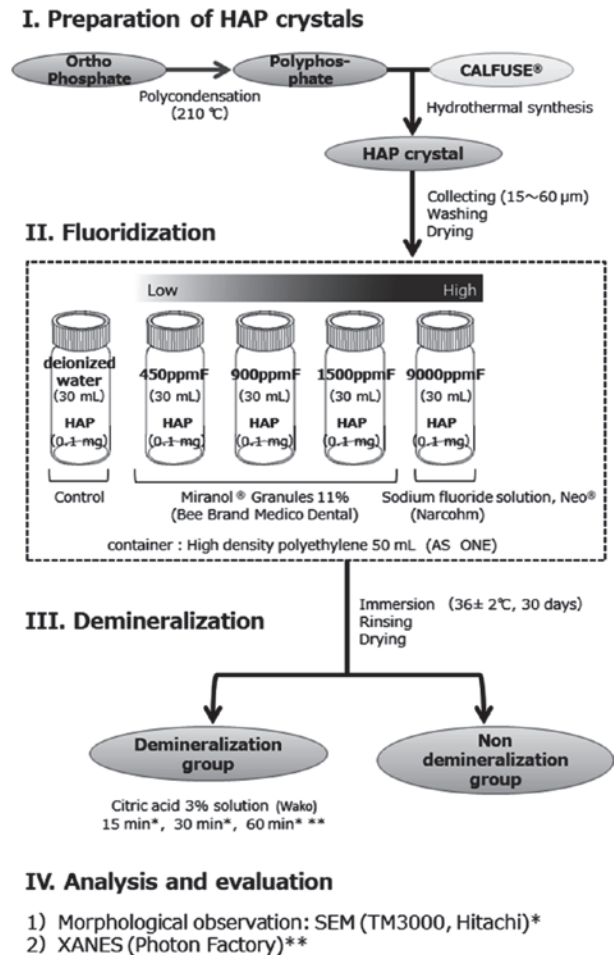


Fig. 1. Schematic illustration of the methods in this study

0.4 g of electro-fused calcium oxide (CALFUSE[®], Tateho Chemical, Hyogo, Japan) were added to a pressure vessel (HU-25, SAN-AI Kagaku, Japan) and mixed, followed by the addition of 12 ml of deionized water, and then stored at 210°C in an electrofurnace for 4 days. Then, the chamber was cooled, and the slurry was dried. The crystals with a diameter of just above 15 microns were collected, washed five times with deionized water, and then dried (Figure 2).

2. Fluoridization

Table 1 summarizes the sodium fluoride (NaF) solutions used in this study. Solutions with concentrations of 450, 900, and 1,500 ppm F were prepared by mixing one package (1.8 g) of the Miranol[®] Granules 11% (Bee Brand Medico Dental, Japan) with 200, 100, and 60 ml of deionized water, respectively. The 9,000 ppm F NaF solution was obtained as a stock liquid from Neo[®] (Narcohm, Japan).

Then, the HAP crystals (0.1 g) were divided into five groups. In the first four groups, the crystals

were immersed in 450, 900, 1,500, or 9,000 ppm F NaF solutions at 36°C for 30 days. All crystals were washed with deionized water 10 times and were subsequently dried. In the fifth group, the crystals were immersed in deionized water as the control.

3. Demineralization conditions

Because the degradation rate of HAP depends on the pH level and not on the type of the acid¹¹, citric acid is an appropriate candidate for reproducing *in situ* demineralization of HAP single crystals. Dissolution is slow when the acid concentration is also low, and when the acid is highly concentrated, it is difficult to detect a dissolution difference. For these reasons, a 3% solution of citric acid (pH 1.5) was prepared using anhydrous citric acid (Wako, Japan) and deionized water. The five HAP crystal groups were treated with citric acid solution for 15, 30, and 60 min, rinsed 30 times with deionized water, and then dried.

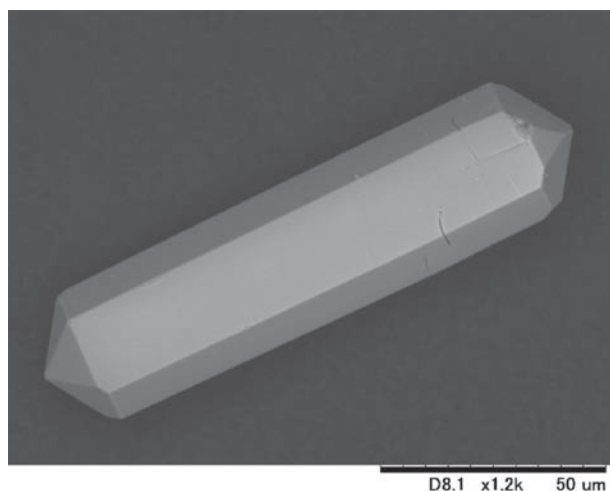


Fig. 2. Synthesized hydroxyapatite (HAP) crystal ($\times 1,2000$)

4. Analytical techniques

The degradation features of crystal structures were observed using scanning electron microscopy (SEM; TM3000: Hitachi, Japan) at $\times 1,000$ magnification. The X-ray absorption near edge structure (XANES) measurement was used on the five HAP crystal groups before and after demineralization. The F K-edge XANES spectra of each group were assessed in the soft X-ray spectroscopy station BL-16A at the Photon Factory (Tsukuba, Japan). The HAP crystals were then irradiated with soft X-ray beams approximately 50 microns \times 200 microns (horizontal \times vertical). The surfaces of fluorine molecules were measured selectively using the partial fluorescence yield method from 687 to 698 energies. Calcium fluoride (CaF₂) and fluorapatite (FAP) were used as the standard reference materials.

Results

1. SEM evaluation

Figure 3 displays representative SEM images of each HAP single crystal. Almost entirely smooth surfaces were observed before demineralization. However, after demineralization, the surface was disrupted by a large number of smaller defects. In addition, many small pores appeared on the surfaces of the control HAP crystals.

The HAP crystals exposed to 450 and 900 ppm F conditions showed similar defects with those in the controls. The surfaces of those samples were observed to be degradable on the surface and at depth.

The samples stored in 1,500 ppm F retained an intact surface even after 15 min of demineralization. Furthermore, crack initiation was observed after 30 min of demineralization. Then, the destruction of the outer shell partly occurred following the degradation of the HAP core portion after 60 min of demineralization. Finally, no considerable degradation

Table 1. Sodium fluoride solutions used in this study

Product name	Manufacturer	Lot number	Composition
Miranol [®] Granules 11%	Bee Brand Medico Dental	712RA	Sodium fluoride, D-mannitol, xylitol, macrogol 6000, sodium dihydrogen phosphate dihydrate, cetylpyridinium chloride, ethyl parahydroxybenzoate, propyl parahydroxybenzoate, hydroxypropylcellulose, cinnamon oil, l-menthol, perfume
Sodium fluoride solution, Neo [®]	Narcohm	A8G2	Sodium fluoride, distilled water

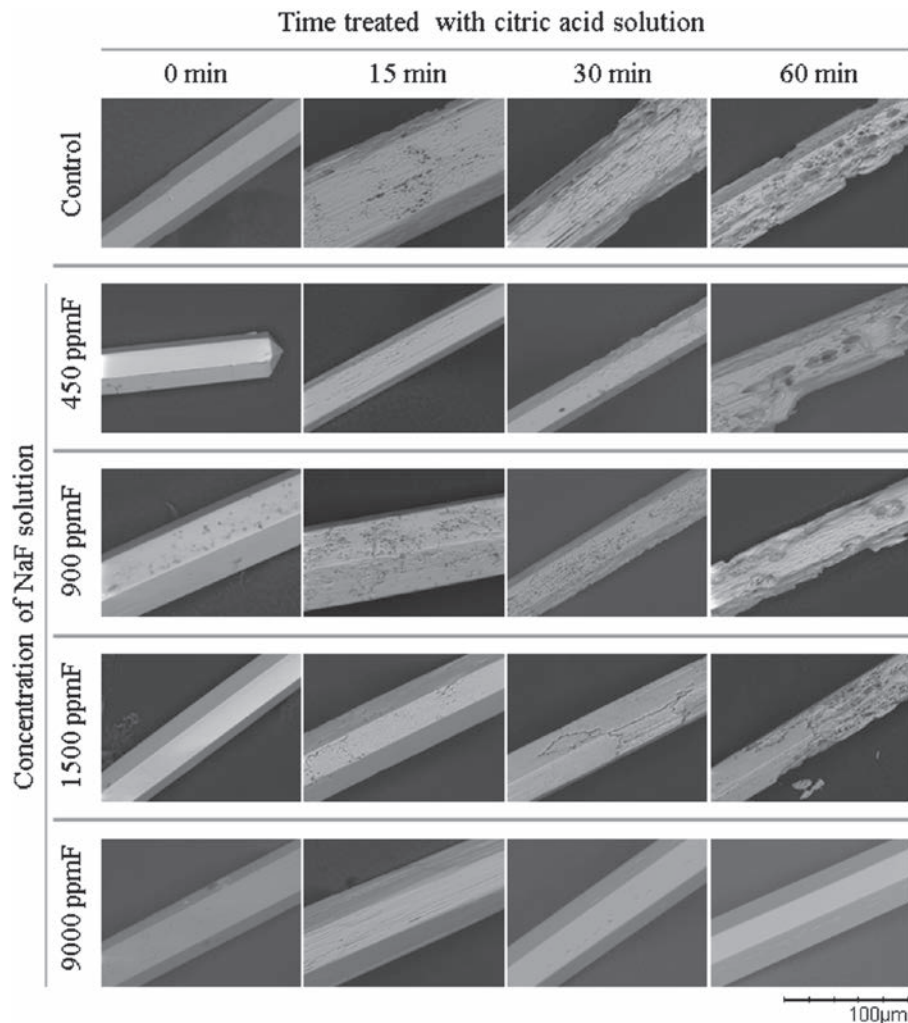


Fig. 3. SEM images of each demineralization sample for 0 min, 15 min, 30 min, and 60 min. The SEM images are evaluated at $\times 1,000$ magnification.

was observed for the samples stored in 9,000 ppm F over time.

2. XANES

Figure 4 shows the normalized spectra of the F K-edge XANES on the samples and standard reference materials.

FAP and CaF_2 exhibited two distinct peaks at 689 and 689.5 eV, respectively. Additionally, the pre-edge of CaF_2 differed from that of FAP (FAP 687 eV, CaF_2 688 eV). The near edge structure of the sample treated with 9,000 ppm F was attributable to CaF_2 both before (Figure 4a) and after (Figure 4b) demineralization. On the other hand, the samples treated with 1,500 ppm F might have shown an integrated peak of FAP and CaF_2 (Figure 4a), yet the spectrum shifted nearly to CaF_2 after demineralization (Figure 4b).

The near edge structures of the samples treated

with 450 and 900 ppm F treatments were not distinctive but were more likely attributable to CaF_2 . The peak on the control was almost undetectable.

Discussion

This study embodied the demineralization patterns of fluoridated and untreated large-scale HAP single crystals, on behalf of the genuine extremely tiny-scale HAP crystals in tooth enamel. To this end, we reproduced fluoridation and demineralization patterns of tooth enamel in a laboratory setting at an observable scale, though the demineralization processes did not always reproduce the features as referenced in Arends *et al.*¹¹. The significance of the above differences will be considered later in this section.

The hydrothermally grown HAP crystals appeared in theoretical hexagonal pilaster forms, similar with case of the common crystal form observed in tooth enamel, which suggests that the large-scale

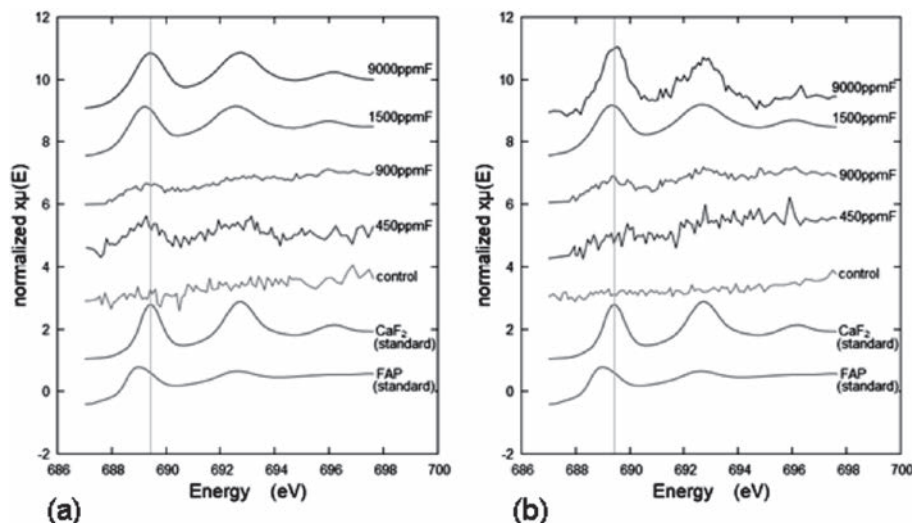


Fig. 4. F K-edge XANES spectra of hydroxyapatite single crystals treated with various concentrations of sodium fluoride solutions.

(a) Before demineralization

(b) Demineralization for 60 min

HAP single crystal used in this study was a good representative of the tiny single crystals embedded in tooth enamel. Moreover, the untreated control and HAPs subjected to a lower concentration of NaF showed remarkable surface dissolution in comparison with the samples subjected to a higher concentration which exhibited greater resistance to citric acid.

The near edge structure observed on the sample interface under relatively higher concentrations of NaF indicated the presence of fluorine compounds. Accordingly, higher acid tolerance on these samples was likely associated with fluoridization of HAPs. Taking these into account, *in situ* fluoride treatments of tooth enamel could be practically advised, taking into consideration the appropriate concentration and treatment duration.

According to the World Health Organization, mouth-rinse containing fluoride is recommended for people living in countries and territories where water fluoridation is less. Because of its simplicity, cost-effectiveness, and reliability¹²⁻¹⁴, mouth-rinse with fluoride is widely used in approximately 1 million people worldwide^{15,16}. The suggested daily and monthly routines for mouth-rinse are 450 ppm F and 900 ppm F, respectively^{17,18}. However, in this study, both 450 and 900 ppm F allowed only a very small amount of fluorine compound in the HAP crystals. As a consequence, no significant acid resistance was observed. Hence, the fluorine concentration found in mouth-rinse products suggested by the World Health Organization is unlikely to be efficient to improve the acid resistance of tooth enamel at the crystal level.

At the 1,500 ppm F concentration, an integrated peak attributable to both FAP and CaF₂ was observed, which in turn shifted to a likely pure CaF₂ phase after acid treatment. FAP has been considered to be more stable and more acid tolerant than pure HAP¹⁹. However, in the case of citric acid, FAP was found to be more soluble than the CaF₂ that formed on the surface of the HAPs under high fluoridization conditions at 1,500 ppm F and 9,000 ppm F in this study. In particular, at 9,000 ppm F, no substantial degradation was observed on the HAP crystal under SEM observation. At least in this *in vitro* study using citric acid, CaF₂ was more resistant than FAP. Hence, future studies using more varied acidic conditions, hopefully mimicking a human oral cavity, would be useful in exploring results.

The overall solubility of enamel in the oral environment decreases with distance from the enamel surface to the enamel-dentine junction (EDJ). The fluoride concentration has been found to be highest near the enamel surface (<100 μm), and it decreases dramatically toward the EDJ²⁰. Accordingly, the untreated control HAP could mimic the chemical properties of genuine enamel HAP near the EDJ. Hence, we could anticipate a close similarity between the outermost enamel and the experimental large-scale HAP single crystals that were subjected to higher concentrations of NaF. Indeed, the demineralization patterns of the single crystal fluoridated at 1,500 ppm F might follow the rule advocated by Arends *et al.*¹¹, that is, the primary degradation of HAP core portion occurs while the

outer shell is stable during the initial demineralization (caries) stage. A latest study also revealed that the enamel single crystals are constituted by an outer shell and a core portion which causes high acid tolerance and high solubility, respectively²¹. Moreover, as long as HAP single crystals are preserved as hollow pillars with a fluoridated outer shell, it may enable remineralization of enamel nanocrystals, resulting in a tooth that could survive for a longer time getting rid of dental caries.

In practical conditions, various complications that diminish the effect of fluoridization exist, such as loss of the topical fluoride by salivation or deglutition²². More studies to explore the production of fluoride levels in saliva are required. Furthermore, a follow-up study could further examine the properties of the fluoridated layer or HAP crystallinity or the arrangement of atoms at various depths in enamel by measuring, for example, Ca L_{2,3}-edge X-ray Absorption Fine Structure (XAFS). Additionally, it is necessary to perform a more detailed inspection of the effects of pH and pure reagents to obtain more detailed information about fluoride tooth surface treatment agents. Moreover, because the teeth repeatedly undergo demineralization and remineralization²³⁻²⁶ and because fluorine is thought to be involved in such processes²⁷⁻³⁰, further research should replicate the remineralization of HAP crystals and the influence of fluoride phases.

Conclusions

Fluoridization of tooth enamel might prevent early acid at the crystal level, given sufficient fluoride concentration and exposure duration. Acid resistance of fluoridated enamel could be partly associated with the chemical property of each HAP single crystal. Our newly developed larger-scale enamel-like HAP single crystals are useful for assessing enamel characteristics of single crystals.

Acknowledgments

The authors would like to thank Kenta Amemiya, Inter-University Research Institute Corporation High Energy Accelerator Research Organization (KEK).

Declaration of Competing Interest

The authors declare no conflicts of interest associated with this manuscript.

References

- Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med*. 1998;**9**:128-161.
- Stephan RM. Changes in hydrogen-ion concentration on tooth surfaces and in carious lesions. *J Am Dent Assoc*. 1940;**27**:718-723.
- Stephan RM. Intra-oral hydrogen-ion concentrations associated with dental caries activity. *J Dent Res*. 1944;**23**:257-266.
- He LH, Swain MV. Understanding the mechanical behaviour of human enamel from its structural and compositional characteristics. *J Mech Behav Biomed Mater*. 2008;**1**:18-29.
- Ma G, Liu XY. Hydroxyapatite: hexagonal or monoclinic? *Cryst Growth Des*. 2009;**9**:2991-2994.
- Jenkins GN. Theories on the mode of action of fluoride in reducing dental decay. *J Dent Res*. 1963;**42**:444-452.
- McDonagh MS, Whiting PF, Wilson PM, *et al.* Systematic review of water fluoridation. *BMJ*. 2000;**321**:855-859.
- Recommendations for using fluoride to prevent and control dental caries in the United States. Centers for disease control and prevention. *MMWR Recomm Rep*. 2001;**50 (RR-14)**:1-42.
- Kerebel B, Daculsi G, Kerebel LM. Ultrastructural studies of enamel crystallites. *J Dent Res*. 1979;**58 (Spec Issue B)**:844-851.
- Narusawa H. Growth of hydroxyapatite huge single crystals by hydrothermal decomposition of polyphosphate. *Nature Protocol Exchange*. 2018;**111**.
- Arends J, Jongebloed WL. Ultrastructural studies of synthetic apatite crystals. *J Dent Res*. 1979;**58 (Spec Issue B)**:837-843.
- Petersen PE, Lennon MA. Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach. *Commun Dent Oral Epidemiol*. 2004;**32**:319-321.
- Fluoride Recommendations Work Group. Centers for Disease Control and Prevention. Recommendations for using fluoride to prevent and control dental caries in the United States. *MMWR Recomm Rep*. 2001;**50 (RR-14)**:1-42.
- Twetman S, Petersson L, Axelsson S, *et al.* Caries-preventive effect of sodium fluoride mouthrinses: a systematic review of controlled clinical trials. *Acta Odontol Scand*. 2004;**62**:223-230.
- Marinho VCC, Chong LY, Worthington HV, *et al.* Fluoride mouthrinses for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev*. 2016;**7**:CD002284. (accessed 2021 Apr 20) Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6457869/pdf/CD002284.pdf>
- Rugg-Gunn A. Preventing the preventable - the enigma of dental caries. *Br Dent J*. 2001;**191**:478-488.
- WHO expert committee on oral health status and fluoride use. Fluoride mouth-rinsing. In *Fluorides and*

- oral health. WHO technical report series 846. Geneva: World Health Organization; 1994. pp32-33.
18. WHO Expert Committee. Mouth rinsing with dilute solutions of fluoride. In *Prevention methods and programmes for oral diseases: report of a WHO Expert Committee. World Health Organization Technical Report Series 713*. Geneva: World Health Organization; 1984. p17.
 19. Kay MI, Young RA, Posner AS. Crystal structure of hydroxyapatite. *Nature*. 1964;**204**:1050-1052.
 20. Weatherell JA, Deutsch D, Robinson C, et al. Assimilation of fluoride by enamel throughout the life of the tooth. *Caries Res*. 1977;**11 Suppl** 1:85-115.
 21. DeRocher KA, Smeets PJM, Goodge BH, et al. Chemical gradients in human enamel crystallites. *Nature*. 2020;**583**:66-71.
 22. Castioni NV, Baehni PC, Gurny R. Current status in oral fluoride pharmacokinetics and implications for the prophylaxis against dental caries. *Eur J Pharm Biopharm*. 1998;**45**:101-111.
 23. Fejerskov O, Larsen MJ. Demineralization and remineralization: the key to understanding clinical manifestations of dental caries. In *Fejerskov O, Nyvad B, Kidd EAM. Dental caries: the disease and its clinical management. 3rd ed*. Chichester, West Sussex, UK: John Wiley & Sons; 2015. pp155-170.
 24. Fontana M, Young DA, Wolff MS, et al. Defining dental caries for 2010 and beyond. *Dent Clin North Am*. 2010;**54**:423-440.
 25. Moreno EC, Zahradnik RT. Chemistry of enamel subsurface demineralization in vitro. *J Dent Res*. 1974;**53**:226-235.
 26. Larsen MJ. Chemically induced in vitro lesions in dental enamel. *Scand J Dent Res*. 1974;**82**:496-509.
 27. Varughese K, Moreno EC. Crystal growth of calcium apatites in dilute solutions containing fluoride. *Calcif Tissue Int*. 1981;**33**:431-439.
 28. Ten Cate JM, Arends J. Remineralization of artificial enamel lesions in vivo. III. A study of the deposition mechanism. *Caries Res*. 1980;**14**:351-358.
 29. Ten Cate JM, Featherstone JD. Mechanistic aspects of the interactions between fluoride and dental enamel. *Crit Rev Oral Biol Med*. 1991;**2**:283-296.
 30. Featherstone JD, Glena R, Shariati M, et al. Dependence of in vitro demineralization of apatite and remineralization of dental enamel on fluoride concentration. *J Dent Res*. 1990;**69**:620-625; discussion 634-636.