



Biomimetic Mechanism of Action of Fluoridated Toothpaste Containing Proprietary REFIX Technology on the Remineralization and Repair of Demineralized Dental Tissues: An *In Vitro* Study

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Abstract

Objectives This *in vitro* study aimed to characterize the mineral content and surface and cross-sectional morphology of enamel and dentin tissues treated with a 1450 ppm fluoride-containing toothpaste with REFIX technology.

Materials and Methods Bovine enamel blocks ($n = 5$) were obtained ($4 \times 4 \times 6$ mm), demineralized (artificial caries lesion), and treated (pH cycling and brushing with the toothpaste). During the pH cycling, which lasted for 7 days (demineralization and remineralization took 6 and 18 hours, respectively), the enamel was brushed for 5 minutes using an electric toothbrush before being immersed in a remineralizing solution. The dentin blocks were acid-etched for 2 minutes (0.05 M citric acid, pH 1.8) to expose the dentinal tubules ($n = 5$). Morphological analysis of the dentin was performed immediately and after 7 days of brushing with the dentifrice, and compared with the control group. The specimens were then cross-sectioned. The surface and cross-sectional micromorphology were assessed using scanning electron microscopy (SEM). The elemental analyses (weight%) were determined with an energy-dispersive X-ray spectroscopy (EDS).

Results The toothpaste with REFIX technology remineralized and repaired the surface enamel effectively. The elemental analysis also demonstrated that treating the enamel with the toothpaste formed a silicon-enriched mineral layer on the enamel surface. Elemental analysis of the enamel cross-sections showed that the toothpaste induced a mineral change. The results were also consistent in the dentin, where the dentinal tubules were progressively occluded until there was complete occlusion after 7 days.

Conclusions We prove the biomimetic mechanism of action of fluoridated toothpaste containing proprietary REFIX technology for obtaining silicon-enriched, remineralized and repaired dental tissues.

Keywords

- ▶ enamel
- ▶ dentin
- ▶ tooth remineralization
- ▶ toothpaste
- ▶ microscopy
- ▶ electron
- ▶ scanning

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Introduction

Although there are toothpastes with specific indications on the market, multipurpose toothpastes have been launched with different therapeutic effects.¹ In spite of the varied reasons for choosing a toothpaste (brand name, taste, price, package, easy to buy, dentist's indication, among others), consumers are prone to choosing an all-in-one product to reduce dental hypersensitivity and promote enamel remineralization.¹ The effectiveness of these toothpastes in promoting dentin tubule occlusion and remineralization depends not only on the fluoride content but also on the presence of other active ingredients.² The addition of different components or supplements in combination with fluoride, known as the biomimetic approach, represents an alternative mechanism that reproduces the natural process of dental tissue mineralization.^{3,4} In this manner, several commercially available oral care products, containing different active ingredients associated with fluoride, claim to boost the remineralization and regeneration potential of these formulations.^{5,6}

The remineralization process can be enhanced by the substitution of small levels of ionic species in the different sites of the hydroxyapatite molecule.⁷ Each ionic grouping of the hydroxyapatite molecule can be replaced by another ionic group of the same or different valence.⁸ Changes in the hydroxyapatite solubility may occur, depending on the substitution at the different sites (calcium, phosphate, and hydroxyl sites).⁸ Fluorine ions (F⁻) can replace hydroxyl ions (OH⁻), and this substitution tends to reduce hydroxyapatite solubility.⁹ Substituting silicon, or silicate, into the remineralizing hydroxyapatite enhances the bioactivity and apatite-forming ability of hydroxyapatite.^{10,11} Although it is claimed that these changes occur mainly at the hydroxyapatite surface,¹² it seems to be the reason for retaining fluoride in the composition, even at lower concentrations, against the risk of fluorosis.¹³ In this manner, balanced compositions in these all-in-one formulations are of imperative importance.

Recently, a proprietary technology named REFIX was developed. It comprises a fluoride-containing toothpaste in association with phosphates and silica. According to the manufacturer, this association favors the formation of a fluoridated apatite and the deposition of silicate, which is also incorporated deep into the hydroxyapatite and onto the open dentinal tubules. A previous *in vitro* study¹⁴ reported that the REFIX-containing toothpaste could remineralize within the subsurface carious lesion effectively when compared with other commercial 1450 ppm fluoride-containing toothpastes. A clinical trial evaluated the efficacy of a toothpaste containing REFIX technology against dentin hypersensitivity.¹⁵ The authors proved that this product was effective against dentin hypersensitivity, achieving the result after 1 week of consistent use.¹⁵

To date, the mechanism with which the REFIX technology mineralizes enamel and reduces dentin hypersensitivity is unclear. The purpose of this *in vitro* study was to characterize the mineral content and surface morphology of dental tissues treated with fluoridated toothpaste containing REFIX

technology to understand the mechanism of action of this oral care product.

Materials and Methods

Specimen Preparation of Dental Enamel

Enamel blocks (4 × 4 × 6 mm) were obtained from bovine incisors using a diamond-impregnated disc (Extec, Enfield, CT, USA) under water lubrication in a cutting machine (IsoMet 1000; Buehler, Lake Bluff, IL, USA). The enamel blocks ($n = 5$) were then embedded in epoxy resin (EpoxiCure Epoxy Resin and Hardener, Buehler, Lake Bluff, IL, USA). Surfaces were then wet-polished with 600-grit SiC paper at low speed and with 1,200-grit SiC paper at high speed using a polishing machine (Single Grinder Polisher, Buehler, Lake Bluff, IL, USA). The final polishing was performed with 1 μm diamond paste and wet felt wheels (Buehler, Lake Bluff, IL, USA).

The enamel blocks were immersed in 30 mL of 50 mM acetate buffer solution containing 3 mM CaCl₂·H₂O, 3 mM KH₂PO₄, 50 mM lactic buffer, and 6 μM methylhydroxydiphosphate (MHDP), at pH 5.0 for 5 days to demineralize the enamel and produce caries-like lesions.¹⁶ After demineralization, the enamel blocks underwent pH cycling by interchanging a demineralization solution (1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 50 mM acid lactic buffer, pH 5.0, 8 hour) and remineralization solution (5 mM CaCl₂, 0.9 mM KH₂PO₄, 130 mM KCl, 20 mM HEPES, 5 mM NaN₃, pH 7.0, 16 hour) for 7 days.¹⁷ During the pH cycling, the enamel surfaces were treated with 30 mL toothpaste slurry (3:1 weight ratio of deionized water to toothpaste) with an electric toothbrush (Oral-B Vitality Precision Clean rechargeable toothbrush; Procter & Gamble, Cuajimalpa, Mexico). Brushing was performed between the incubation with the remineralization and demineralizing solutions. The enamel blocks were brushed with the toothpaste slurry (2 mL/block) using the electric toothbrush for 5 minutes (120 cycles per minute, 22°C)¹⁴ three times a day for 1 week. The toothbrushes were fixed in a device that allowed the heads to be aligned parallel to the specimens' surfaces.¹⁸ The specimens were brushed in linear movements with an axial load of 200 g. Between the steps, the specimens were water rinsed with deionized water for 5 seconds.

Specimen Preparation of Dental Dentin

Bovine dentin blocks were obtained ($n = 5$) as described earlier. The dentin blocks were embedded in epoxy resin, and the surfaces were wet-polished with 600-grit SiC paper at low speed and with 1,200-grit SiC paper at high speed using a polishing machine. The specimens were acid-etched for 2 minutes (0.05 M citric acid, pH 1.8) to remove the smear layer and expose the dentinal tubules.¹⁹ The experimental groups for the dentin analysis were as follows: I, control, untreated group; II, morphological analysis immediately after brushing with the dentifrice; III, morphological analysis after 7 days of brushing with the dentifrice.¹⁵

Characterization of the enamel and dentin surfaces and cross-sections by scanning electron microscopy (SEM) imaging observation and energy-dispersive X-ray spectroscopy (EDS)

The morphological analysis of the specimens was performed using SEM (TESCAN VEGA3; LMU, Kohoutovice, the Czech Republic) operating at 15 kV. The blocks were first sputter-coated with gold in a vacuum evaporator (MED 010; Balzers, Balzers, Liechtenstein), and then microscopically analyzed to obtain photomicrographs of the superficial morphology of the treated specimens ($\times 1,000$ magnification). Representative images of selected regions of the specimens were obtained for characterizing the morphological aspect of the surface. The EDS point analysis (80 mm², SDD [silicon drift detector], Oxford Instruments, Concord, MA, USA) was performed to determine a qualitative elemental analysis of the specimens, operating in high-vacuum mode and an accelerating voltage of 15 kV. Five points per sample were randomly selected (300 μm^2 per point), and the mean values were calculated.

For the subsurface analysis, cross-sections of the bovine blocks were obtained by longitudinally sectioning the specimens under water cooling. Both half-blocks were used for the SEM and elemental analyses. The halves were dehydrated in silica gel for 3 hours. The specimens were then gold-sputtered and evaluated using SEM coupled with EDS.

Results

►Fig. 1 shows the representative scanning electron micrographs of the enamel. ►Fig. 1A demonstrates the clearly different areas, separated by the dotted line: the area above the line represents the control demineralized area; the area below the line represents the remineralized treated area.

►Fig. 1B and 1C show the representative scanning electron micrographs at higher magnification of both areas. The ability of the REFIX-containing toothpaste to remineralize and regenerate the highlighted area was clearly demonstrated (►Fig. 1C) in comparison to that observed in the demineralized area (►Fig. 1B). The treated regenerated enamel had smoother surface morphology in comparison to the demineralized area, which presented depressions induced by the caries-like lesions. ►Fig. 1D shows a cross-sectional area of the control demineralized enamel; for comparison, ►Fig. 1E shows a representative scanning electron micrograph of the remineralized cross-sectional area. In the same way, the morphological differences between these two areas are clearly demonstrated, in which a mineralized layer formed on the enamel surface after pH cycling interspersed with the REFIX toothpaste treatment (►Fig. 1E). This layer was absent in the control demineralized area (►Fig. 1D).

►Fig. 2 shows the representative scanning electron micrographs of the enamel cross-sections and the respective elemental mapping at different depths (from the superficial area A to the deepest area D). EDS detected stronger silicon signals at the surface areas A and B (0.56 weight%), which increased right below the mineralized layer formed at the enamel surface (C, 0.77 weight%), and then decreased in the deepest area (D, 0.24 weight%). The amounts of sodium, phosphorus, and calcium also varied as a function of the enamel depths.

►Fig. 3 shows scanning electron micrographs of the morphological analysis of the superficial and cross-sectional areas of the dentin. ►Fig. 3A shows a scanning electron micrograph of the demineralized area with opened dentin tubules.

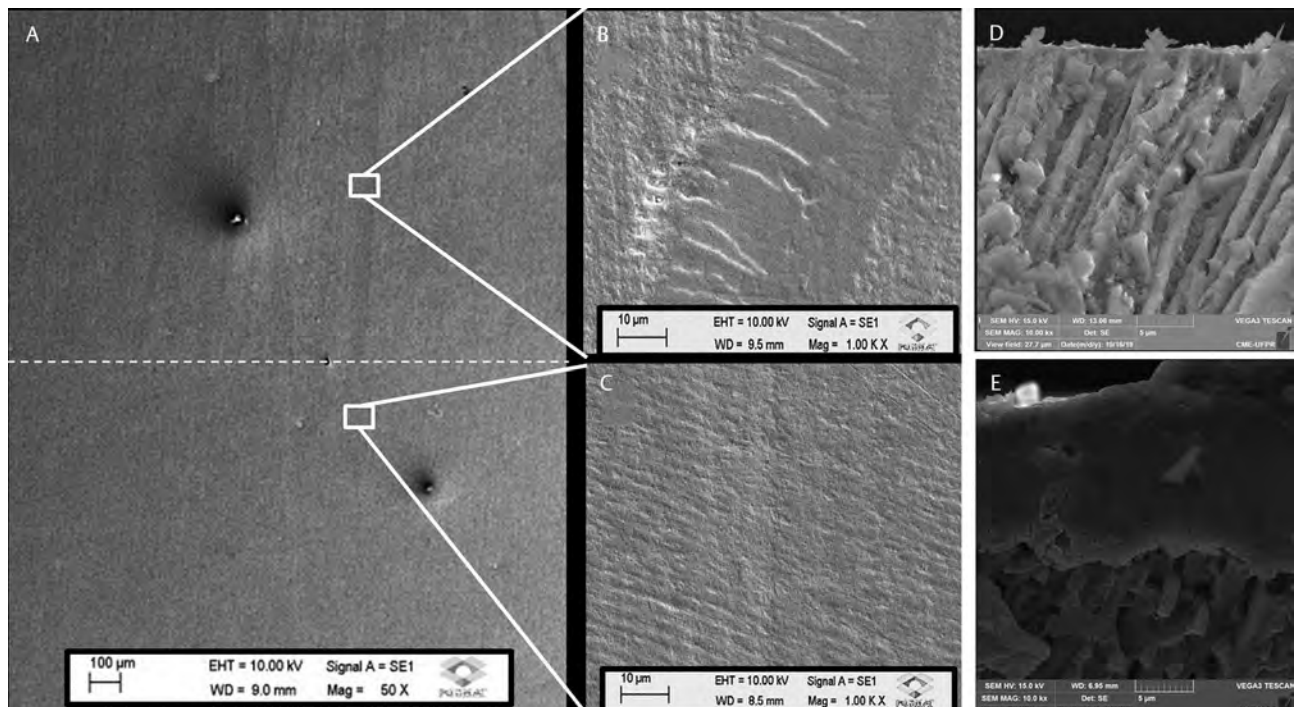


Fig. 1 (A) shows a representative scanning electron micrograph of the enamel surface (magnification 50x); above the line represents the control demineralized area; the area below the line represents the remineralized treated area. (B) shows a representative area of the demineralized enamel. (C) shows a representative area in which the enamel was remineralized and regenerated. (D) shows the cross-sectional area of the control, demineralized enamel half. (E) shows a representative scanning electron microscope (SEM) photomicrograph image the cross-section of enamel block in which a mineralized layer is formed at the treated enamel surface.

►Fig. 3B shows that a remineralized area is formed immediately after brushing with the toothpaste, exhibiting partially occluded dentin tubules. Conversely, ►Fig. 3C shows an area of the dentin surface in perspective, in which a mineralized layer was formed after 7 days of brushing; a protective layer

on the dentin surface of up to 3 µm was noted after 7-day brushing. ►Fig. 3D shows the same treated dentin area after 1 week of treatment, with a majority of occluded tubules. ►Fig. 3E shows a scanning electron micrograph of the mineralized layer formed at the dentin surface associated with the elemental analysis, which demonstrated the presence of Si (6.70 weight%), F (0.90 weight%), Ca (1.60 weight%), and P (0.46 weight%).

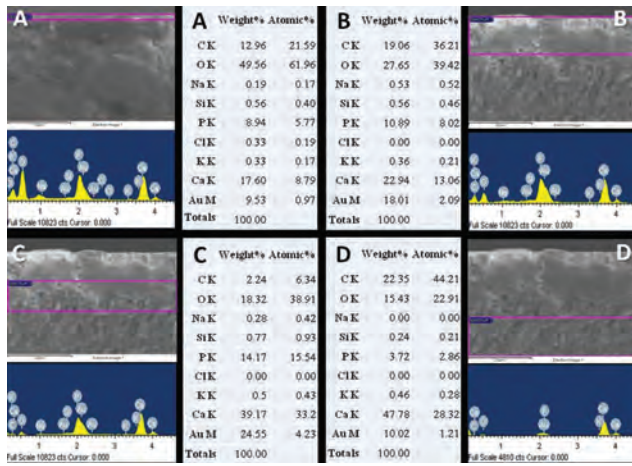


Fig. 2 Representative scanning electron micrographs of the enamel cross-sections and the elemental mapping at different depths (from surface area A to the deepest area D). EDS detected stronger silicon signals at the surface area (0.56 weight%), which increased right below the mineralized layer formed at the enamel surface (C, 0.77 weight%), and decreased in the deepest area (D, 0.24 weight%). The amounts of sodium, phosphorus, and calcium also varied as a function of the depth.

Discussion

The toothpaste tested in the present study contains proprietary REFIX technology, which according to the manufacturer, represents a novel multifunctional phosphate-based dental gel technology in an acidified stabilized phosphate/fluoride complex, which is established especially in saliva.¹⁴ This combination with saliva and dental tooth structures favors the generation of new minerals containing calcium/phosphate/fluorine, promoting the enamel surface and remineralizing within the subsurface carious lesion.¹⁴ This product presents an acid pH as a function of the proposed technology, whose action is possibly more effective due to the formation of calcium phosphate crystals in an acidic environment, which appears to be the main reason for its effectiveness.^{14,15}

The difference observed in the elemental mapping demonstrates that the REFIX toothpaste could modify the structure of the demineralized enamel. It was clearly demonstrated that a remineralized layer formed on the enamel

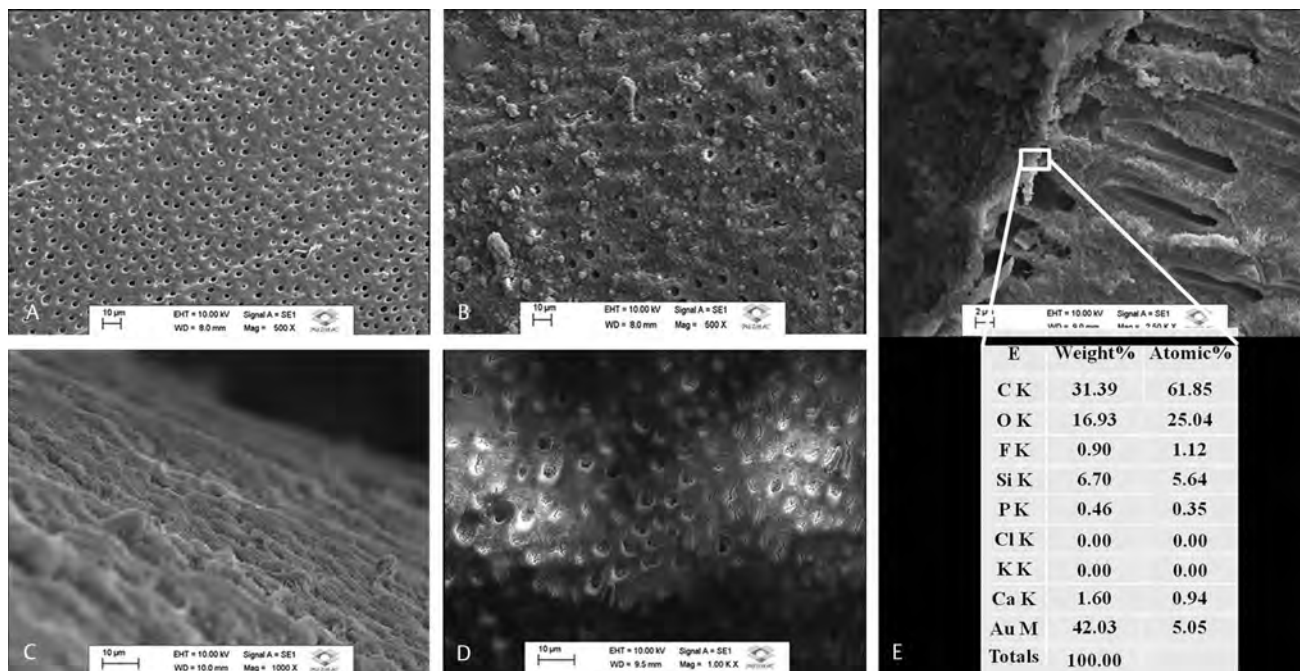


Fig. 3 Scanning electron micrographs of the morphological analysis of the surface and cross-sectional areas of the dentin: (A) shows a representative scanning electron micrograph of the demineralized dentin with open tubules. (B) shows a representative scanning electron micrograph of the dentin immediately after brushing with the toothpaste with occluded dentinal tubules. Figure 3 (C) shows the dentin surface in which a mineralized layer was formed after 7 days of treatment. (D) shows the scanning electron micrograph of the treated dentin with a majority of occluded tubules after 1 week of treatment. (E) shows a scanning electron micrograph of the mineralized layer formed at the dentin surface, demonstrating the presence of silicon (6.70 weight%), fluorine (0.90 weight%), calcium (1.60 weight%), and phosphorus (0.46 weight%) in the elemental analysis.

surface after pH cycling. The elemental mapping demonstrated the presence of silicon deeper in the enamel, with higher concentrations right below the mineralized layer formed on the enamel surface (►Fig. 1). This silicon-enriched hydroxyapatite present in the mineral layer formed on the enamel surface was induced by the REFIX-based toothpaste, which favored the formation of complexes of the bioactive particles calcium, phosphorus, and sodium, as demonstrated in ►Fig. 2. Bioactive glass is claimed to induce faster capitation of sodium, calcium, and phosphorus, thus boosting the precipitation of minerals for remineralizing the enamel surface or for reducing the effects of demineralization.²⁰ In this way, silicon is regarded as a component of bioactive glass, inducing the formation of nucleation sites for the precipitation of calcium and phosphate ions in the formation of hydroxyapatite.²¹ Calcium silicate is then formed, which is responsible for boosting the mineralization processes of saliva via hydroxyapatite nucleation and mineral formation.²² Silicon is claimed to promote more rapid release of Ca and P²³ and induce low negative zeta potential in the biological medium, which has important *in vivo* effects.²¹ As previously reported, substituting phosphate ions (PO₄) with silicate ions (SiO₄) in hydroxyapatite structures to form calcium silicate also confers a protective effect by inducing the formation of hydroxyapatite after its deposition onto the eroded surfaces.²⁴ This is particularly due to the induced bioactivity and apatite-forming ability of hydroxyapatite, which is enhanced by the substitution of silicon, or silicate, into the remineralizing hydroxyapatite.^{10,11}

Another component of the toothpaste, according to the manufacturer, is the tetrasodium pyrophosphate in association with sodium fluoride (1450 ppm).¹⁴ The aim of oversaturating the enamel surface with calcium and phosphate is to contribute to the decrease in ions on the previously demineralized enamel surface, consequently increasing the resistance to the cariogenic process in the presence of fluoride ions.²⁵ An *in vitro* study¹⁴ investigated the remineralization potential of 1450 ppm fluoride-containing toothpastes containing different active remineralization agents after cariogenic challenge with pH cycling. Although the REFIX toothpaste could not recover the hardness of the remineralized enamel surface completely in comparison to the intact enamel (94%), the product significantly reversed the lesion formed at the subsurface to a depth of around 60 µm. The mean lower hardness of the remineralized superficial enamel as compared with the intact enamel can be explained by the fact that replacing PO₄ with SiO₄ seems to lead to decreased crystallinity and thermal stability, and also to increased solubility of the hydroxyapatite structure.²⁶ Substituting PO₄ with SiO₄ is believed to affect the mechanical properties of the silicon-enriched hydroxyapatite in a dose-dependent manner, decreasing hardness and the elastic modulus.²⁷ Conversely, as previously noted, the silicon content in the toothpaste formulation in association with fluorine and phosphate groups induces increased bioactivity and apatite-forming ability of hydroxyapatite, which is enhanced by the substitution of silicon, or silicate, into the remineralizing hydroxyapatite.^{10,11}

The REFIX-containing toothpaste could also remineralize the dentin tissue effectively by depositing a mineral layer, thus progressively occluding the dentinal tubules. Depositing phosphate and calcium ions at the surface of exposed tubules forms a protective layer against dentinal hypersensitivity,²⁸ which is an effective treatment.²⁹ A clinical trial that evaluated the effectiveness of the REFIX-based toothpaste in 53 participants found that the participants experienced a significant reduction in dentin hypersensitivity after 1 week of use.¹⁵ The REFIX-containing toothpaste reduced 88.3% of the dentin hypersensitivity after 1 week of use. The authors speculated that the reduction could have been due to the formation of enamel-like deposited crystals, which would reduce the functional area of the dentinal tubules by partially occluding the tubule openings, reducing the fluid flow and, consequently, the dentin hypersensitivity.¹⁵ In that study, the authors also hypothesized that phosphate-mediated calcium fluoride and calcium silicate crystals would be formed and then deposited on the dentin surface and/or inside the dentinal tubules. Its effect against dentin hypersensitivity was possible since the first use, which corroborates the findings of the present study. Here, a remineralized area formed immediately after brushing with the toothpaste, exhibiting partially occluded dentin tubules (►Fig. 3B). After 1 week of use, the majority of tubules were occluded (►Fig. 3D). The elemental analysis also demonstrated the presence of silicon (6.70 weight %) in the remineralized area. The elemental analysis also detected fluorine (0.90 weight%), calcium (1.60 weight%), and phosphorus (0.46 weight%) (►Fig. 3E).

An *in vitro* study²⁴ evaluated the remineralizing effects of calcium silicate deposition on the properties of acid-eroded dental surfaces. Calcium silicate could confer a protective effect by inducing the formation of silicon-enriched hydroxyapatite after its deposition onto the eroded surfaces.²⁴ Other studies have also demonstrated the promising effects of silica-based compounds for obliterating dentin tubules.^{30,31} These results are associated with the ability of silicon-containing oral care products to promote infiltration and subsequent remineralization in order to occlude the dentinal tubules.³² This is possible due to ability of silica to adhere and occlude tubules via hydroxyl groups, which aid silica binding with the calcium receptors on the dentin surface.^{31,32} This helps to explain the effectiveness of REFIX toothpaste for inducing dentinal tubule occlusion.

The present study has characterized the mineral content and surface morphology of enamel and dentin tissues treated with 1450 ppm fluoride-containing toothpaste along with REFIX technology. Considering its limitations, the present study could be performed with human teeth instead of bovine teeth. In addition, other toothpastes could be used as a gold standard for comparing the results. In spite of this concern, a control untreated group was used for comparison. Despite the limitations of the present *in vitro* study, the results of previous studies^{14,15} corroborate our results. Further research is needed to investigate the performance of toothpaste containing REFIX technology in *in situ* and *in vivo* studies on the effectiveness of the toothpaste on dental substrates.

Conclusion

The 1450 ppm fluoride-containing toothpaste with REFIX technology can remineralize and repair the surface enamel effectively. Elemental analysis demonstrated that treating the enamel with the toothpaste forms a silicon-enriched mineral layer on the enamel surface. In addition, elemental analysis of enamel cross-sections showed that the toothpaste could induce a mineral change. The results are consistent with that of the dentin specimens, where the dentinal tubules were progressively occluded until there was complete occlusion after 7 days of application, corroborating the results of previous studies. A silicon-enriched layer was also formed, occluding the dentinal tubule openings. Further studies are needed to evaluate the resistance of the layer formed on the dental tissue surface against acid challenges for maintaining oral health.

Conflict of Interest

None declared.

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