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# Effect of nanometric β-calcium glycerophosphate supplementation in conventional toothpaste on enamel demineralization: An *in vitro* study

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ABSTRACT

The aim of this study was to evaluate the effects of supplementing toothpastes containing 1100 ppm F with micrometric or nanometric [beta]-calcium glycerophosphate (β-CaGPm/β-CaGPn) on artificial enamel demineralization, using a pH cycling model. Bovine enamel blocks (4 mm × 4 mm, n = 120) selected using initial surface hardness were randomly allocated to ten toothpaste groups (n = 12): without fluoride or β-CaGPm or β-CaGPn (Negative control), 1100 ppm F (1100 F), and 1100 ppm F plus 0.125%, 0.25%, 0.5%, and 1.0% of β-CaGPm or β-CaGPn. Blocks were treated two times per day with toothpaste slurry and subjected to five pH cycles (demineralizing and remineralizing solutions) at 37 °C. The final surface hardness, percentage of surface hardness loss (%SH), cross-sectional hardness (ΔKHN), and profile analysis and lesion depth subsurface were analysed using polarized light microscopy (PLM). Fluoride (F), calcium (Ca), and phosphorus (P) concentrations were also measured. Data were analysed using ANOVA and Student-Newman-Keuls tests ([alpha] = 0.001). Blocks treated with 1100 F toothpaste containing 0.5%β-CaGPm or 0.25%β-CaGPn showed with reduced %SH values when compared with those treated with 1100 F alone ($p < 0.001$). Reduced lesion depths (ΔKHN and PLM) were observed for the slurry made up of 1100 F and 0.25%β-CaGPn ($p < 0.001$). The addition of β-CaGPm and β-CaGPn did not influence the enamel F concentration, with the 1100 F/0.25%β-CaGPn group exhibiting the highest Ca and P enamel concentrations ($p < 0.001$). Based on the findings of this *in vitro* study, we can conclude that the fluoride toothpaste produced a superior effect when combined at an appropriate β-CaGP molar ratio. This effect was achieved with a lower proportion of β-CaGP in the form of nanometric particles.

## 1. Introduction

Dental caries is a multifactorial disease affected by biological, behavioral, psychosocial, and environmental factors (Pitts et al., 2017; Machiulskiene et al., 2020). In the last years, there has been a significant reduction in dental caries incidence assigned to the fluoride products, as toothpastes; considered the major nonprofessional intervention against dental caries (Walsh et al., 2019). However, dental caries still affects approximately 600 million preschoolers worldwide, reducing their quality of life and their families (Pitts et al., 2017). The addition of calcium (Ca) and phosphate (P) in fluoride toothpastes has been studied since, in the presence of a greater quantity of these ions in the biofilm, fluoride would be more effective during the cariogenic challenge (Souza et al., 2016).

Alternatively, products containing other Ca and P salts, have been studied (Danelon et al., 2019). Evidence has demonstrated that

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nano-hydroxyapatite most biocompatible and bioactive materials and its nano-sized particles have similarity to the apatite crystal of tooth enamel in similar morphology, size, crystalline structure, solubility and biocompatibility morphology. However, in a recent study (Kranz et al., 2022) that evaluated the effect of a zinc carbonate hydroxyapatite (biorepair®) containing toothpaste on artificially demineralized enamel and dentin surfaces showed that treatment with biorepair® did not affect enamel surfaces. Therefore, the authors emphasize the need for caution when making assumptions about the effectiveness of toothpastes containing nano hydroxyapatite in remineralizing hard tissue defects compared to fluorides.

Other option is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and CPP-ACP-containing fluoride (CPP-ACFP) (Gonçalves et al., 2021; de Oliveira et al., 2022). The binding of the CPP-ACP complex is pH-dependent and breaks when there is a decrease in the pH of the oral environment. ACP has the ability to release Ca and P ions in the dental biofilm to maintain a supersaturated state, thus reducing the demineralization process. The study conducted by Gonçalves et al. (2021) showed that the products formed on the enamel surface by the CPP-ACP and CPP-ACPF complex have great potential to reduce mineral loss on the surface. In addition, to promoting great remineralization of the enamel surface, obliterating the enamel pores, reducing the diffusion of Ca and P ions into the deep areas of the subsurface lesion. Therefore, although *in situ* studies and randomized clinical trials showed that CPP-ACP and ACP-CPPF can interfere with the DES-RE process, it is not significantly different when compared to F (Yengopal and Mickenautsch, 2009; Li et al., 2014). There is no clear scientific evidence of the benefit of applying CPP-ACP and CPP-ACPF in systematic reviews (Chen et al., 2013; Li et al., 2014; Indrapriyadharshini et al., 2018).

Toothpaste containing CaGP proved to be an alternative to achieve the effect of 1100 ppm F against demineralization and favoring the remineralization of enamel using half of its concentration (do Amaral et al., 2013; Zaze et al., 2014a; Zaze et al., 2014b). CaGP is an organic source of calcium and phosphate and can be found in three isomeric forms: β-calcium glycerophosphate (calcium; 1,3-dihydroxypropan-2-yl phosphate), l (−) α-calcium glycerophosphate ([(2 S)-2,3-dihydroxypropyl] dihydrogen phosphate) e d (+) α-calcium glycerophosphate ([(2 R)-2,3-dihydroxypropyl] dihydrogen phosphate). β-CaGP is the isomer that favors the greater release of Ca and P (Inoue et al., 1992), increasing the concentration in the biofilm and interaction with the enamel, modifying the bacterial metabolism and buffering the pH of the biofilm (Lynch and ten Cate, 2006; Tenuta et al., 2009). Relevant finding is that β-CaGP has two binding sites (Emerenciano et al., 2023), showing a greater ability to be adsorbed to enamel. Thus, when it is adsorbed into the enamel surface, practically all the phosphate groups are neutralized by calcium and hydroxyl ions from the hydroxyapatite. Ca ion in the β-CaGP is released and made available to the enamel. For this reason, it is expected that there will be less mineral loss. In an *in situ* study, the addition of CaGP 0.25% wt (50% α-CaGP and 50% β-CaGP) to low-fluoride toothpaste showed superior remineralizing effect when compared to its counterpart without CaGP; but with no difference from 1100 ppm F (Zaze et al., 2014a). Emerenciano et al. (2023) demonstrated the addition of 0.5%β-CaGPm into 1100 F formulation increased the bioavailability of calcium and phosphate, promoting a higher remineralizing effect when compared to 1100 F alone.

Another strategy to improve the effects of fluoride toothpastes on caries control includes the use of phosphates in the form of nanometric (Danelon et al., 2017). This phosphates when added in nanometric particles exhibit greater area and surface chemistry reactivity when applied to the enamel (Danelon et al., 2017). Danelon et al. (2015) and Danelon et al. (2019) evaluated the *in vitro* and *in situ* effects of 1100 ppm F toothpastes containing sodium trimetaphosphate and sodium hexametaphosphate nanometric. The authors concluded that fluoride toothpastes with nanometric metaphosphates were more efficient in promoting remineralization and reducing dental demineralization than the 1100 ppm F toothpastes.

Given this and the absence of studies evaluating the effects of various concentrations of β-CaGP in toothpaste, the aim of this study was to evaluate the effect of 1100 ppm F toothpastes supplemented with micrometric or nanometric β-CaGP (β-CaGPm/β-CaGPn) on artificial enamel demineralization, using a pH cycling model. The null hypothesis was that fluoride toothpastes containing micrometric or nanometric β-CaGP would have no difference in reducing enamel demineralization when compared to their counterparts without β-CaGP.

## 2. Materials and methods

### 2.1. Preparation of enamel blocks

Enamel blocks (4 × 4 × 2 mm, n = 120) were obtained from buccal-cervical region of bovine incisors, dentin was adjusted (thickness ± 2 mm) for achievement of parallel surfaces between enamel and dentin. Then, enamel surfaces were sequentially polished using 600 (20 s), 800 (20 s), and 1200 (20 s) grade water cooled silicon carbide paper disks (Buehler, Lake Bluff, IL, USA), with a final polish using a felt disk (Polishing Cloth 40–7618, Buehler) moistened with a 1-μm diamond polishing suspension (Extec Corp., Enfield, CT, USA) with 20 lbs load (outer enamel removed ~120 μm). Hereafter, initial surface hardness (SHi; 320.0–380.0 kg/mm$^2$) was evaluated and the enamel blocks were randomly divided into ten experimental toothpaste groups (n = 12 each): without fluoride or β-CaGPm or β-CaGPn (Negative control), 1100 ppm F (1100 F), 1100 ppm F plus 0.125%; 0.25%; 0.5% and 1.0% of β-CaGPm or β-CaGPn.

### 2.2. Processing and characterization of nanometric β-CaGP

Nanometric β-CaGP (β-CaGPn) was processed using the methods described by Danelon et al. (2015, 2017). β-CaGPn was obtained from commercial CaGPm (80% β-isomer and 20% rac-α-isomer, CAS 58409-70-4 (G6626), Sigma Chemical Co, St Louis, Missouri, USA) was ball milled using 500 g of zirconia spheres (diameter of 2 mm). Commercial material (CaGPm: 70 g) was then added to a polyethylene bottle containing sintered zirconia spheres (2 mm in diameter) in isopropyl alcohol at one third of its volume. The bottles with CaGPm were sealed and carried to a ball mill for milling the CaGPm for 48 h. Then, the material was filtered and sealed with aluminum foil, and the vials were dried at 85 °C to evaporate the isopropyl alcohol (Merck KGaA, Darmstadt, Germany). X-ray diffraction (XRD) was used to identify the crystalline structures and estimate the crystallographic coherency domains of the CaGP (β-CaGPn). X-ray diffractograms were obtained from samples in powder form using XRD 6000 (Shimadzu Corp., Kyoto, Japan) a CuKα radiation source (λ = 1.54056 Å) at a voltage of 30 kV and current of 30 mA and with step scan of 0.02° and scan speed of 2° min$^{-1}$. Measurements were made continuously in the range of $5^\circ \leq 2\theta \leq 80^\circ$, a scanning rate of 2°/min. The structural identification of the samples was done by comparing the diffraction patterns obtained with tabulated patterns available in databases "Joint Committee on Powder Diffraction Standards—Powder Diffraction File (JCPDS—PDF)". The particle morphologies and size of the β-CaGPm and β-CaGPn suspensions were analysed using SEM. The SEM images were collected using an MEV-PHILIPS operating at 10–25 keV and spot 3 (XL-30 FEG, Philips, Amsterdam, Netherlands). The samples were prepared by placing one drop of the dispersion in isopropyl alchohol deposited on a silicon metal plate and oven dried at 40 °C for 12 h. The contacts between the sample and the support were carried out with conductive silver ink (Degussa). Peak broadening is resulted from the size effect and it was evaluated using the Scherrer equation (Camargo et al., 2001). After, histograms were constructed using the public domain ImageJ image processing software (version 1.50, National Institute of Health, Bethesda, MD, USA).

### 2.3. Assessment of the fluoride content and pH of toothpaste formulations

Toothpastes were produced with the following components: 0.5 g titanium dioxide (Sigma-Aldrich Co., St. Louis, MO, USA), 1.7 g carboxymethyl cellulose (Sigma-Aldrich Co., St. Louis, MO, USA), 0.08 g of methyl p-hydroxybenzoate sodium (Sigma-Aldrich Co., St. Louis, MO, USA), 0.1 g saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), 0.5 g mint oil, 26.6 g glycerin (Sigma-Aldrich Co., St. Louis, MO, USA), 10 g abrasive silica (Tixosil 73®, Rhodia, São Paulo, Brazil), 1.7 g sodium lauryl sulfate (Sigma-Aldrich Co., St. Louis, MO, USA), and adjusted with deionized water to 100 g. Toothpastes containing β-CaGPm and β-CaGPn (Sigma Chemical Co, St Louis, Missouri, USA) were prepared using concentrations of 0.25%, 0.5%, and 1.0% and 1100 ppm F in the form of NaF (Merck, CAS 7681-49-4, Germany) (Zaze et al., 2014a, 2014b). A toothpaste without F and β-CaGPm and β-CaGPn (Negative control) and a toothpaste with 1100 ppm F were also prepared as positive control. The toothpastes used in this study were stored at room temperature and kept properly closed to prevent any change in the samples (i.e: water loss and/or contamination with external residues). Total and ionic fluoride concentrations were determined in triplicate using a fluoride-specific electrode (Orion 9609-BN; Orion Research Inc., Beverly, MA, USA) connected to an ion analyzer (Orion 720 $A^{+}$; Orion Research Inc.) (Delbem et al., 2009), and values express in ppm F. The pH of each toothpaste was assessed in triplicate using a pH electrode (2A09E, Analyzer, São Paulo, Brazil) calibrated using solutions with pH 7.0 and 4.0 (Danelon et al., 2019).

### 2.4. Toothpaste treatment and pH cycling

Each of the enamel blocks was subjected to five pH cycles over a 7-day period at a constant temperature of 37 °C (Vieira et al., 2005). These blocks were immersed, under constant agitation on the shaker table-100 rpm (SK300; Jeio Tech, Seoul, Republic of Korea), 2 × /day, for 1 min, in toothpaste suspensions in deionized water (1: 3 by mass); then, they were placed in a demineralizing solution (DE) for 6 h (Ca and P 2.0 mmol $L^{-1}$ in acetate buffer 0.075 mol $L^{-1}$, 0.04 μg F/mL at pH 4.7–2.2 mL/$mm^2$) and then in a remineralizing solution (RE) for 18 h (Ca 1.5 mmol $L^{-1}$, P 0.9 mmol $L^{-1}$, 0.15 mol $L^{-1}$ KCl in 0.02 mol $L^{-1}$ sodium cacodylate buffer, 0.05 μg F/mL at pH 7.0–1.1 mL/$mm^2$) for each cycle. Deionized water rinses were performed between each step, and the blocks were placed in a fresh remineralizing solution for the final 2 days.

### 2.5. Analysis of enamel hardness

Surface hardness was determined using a hardness tester (Micromet 5114, Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations (baseline: SHi), spaced 100 μm from each other, were made in the center of the enamel block. After the pH cycling, five other indentations were performed (SHf) with a distance of 100 μm from the SHi. The %SH (%SH = [(SHf-SHi)/SHi] *100) was then calculated. The blocks were sectioned at the center (with diamond discs) and one half being immersed in acrylic resin and gradually polished using 320, 600, 800, and 1200-grade water cooled silicon carbide paper disks (Buehler, Lake Bluff, IL, USA), with a final polish using a felt disk (Polishing Cloth 40–7618, Buehler) moistened with a 1/4-μm diamond polishing suspension (Extec Corp., Enfield, CT, USA), until the enamel was totally exposed. Fourteen indentations at various distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 μm) were made from the surface of the enamel in the central region. The integrated area hardness (kg/$mm^2$ × μm) of the enamel lesion was calculated using the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated area of the hardness of the sound enamel, thus yielding the cross-sectional hardness (ΔKHN; kg/$mm^2$ × μm) (do Amaral et al., 2013; Zaze et al., 2014a; Zaze et al., 2014b).

#### 2.5.1. Analysis of the profile and depth of subsurface lesions using polarized light microscopy

After cross-sectional hardness analysis, the enamel blocks embebed in acrilyc resin were sectioned to obtain slices of 300 μm and ground to a thickness of ~100 μm using 400 grit paper (Paper Discs CARBIMET, 30-5108-320, Buehler) at grinder polisher (Phoenix Beta with Vector Powerhead, Buehler, Lake Bluff, Illinois, USA), under constant water refrigeration (24.5 °C). Then, the enamel slices were manually polished in a sequence of sandpaper (600, 800, and 1200 grit sandpaper, Buehler) and deionized water, and mounted on glass slides with deionized water and covered with a coverslip glass, the edges of which were sealed with synthetic resin (Entellan, Merck, Darmstadt, Germany). The presence and thickness (μm) of surface layer of the enamel and depth of artificial demineralization (μm) were measured at three areas from the central region of the slices at × 40 magnification in polarized light microscopy (PLM) (AxioPhot, Zeiss, Oberkochen, Germany) (Danelon et al., 2014).

### 2.6. Analysis of F, Ca and P concentrations in the enamel

The other half of the longitudinally sectioned blocks was sectioned again to obtain 2 mm × 2 mm blocks. One of these blocks was fixed with glue to a mandrel coupled to a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, Athol, MA, USA) to measure enamel wear. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of a polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of enamel (~50 μm) each was removed from the enamel blocks by grinding the enamel surface against the polishing discs, in circular movements (Danelon et al., 2014, 2019). We then added 0.5 mL HCl 1.0 mol $L^{-1}$ to the enamel powder and kept the flasks kept under constant stirring for 1 h. For F analysis, a specific electrode 9409BN (Thermo Scientific, Beverly, USA) and microelectrode reference (Analyzer, São Paulo, Brazil) coupled to an ion analyzer (Orion 720 $A^{+}$, Thermo Scientific, Beverly, USA) were utilized. The electrodes were calibrated using standards ranging from 0.25 to 4.00 ppm F (100 ppm F, Orion 940,907) the same conditions as the samples. Readings were conducted using 0.250 mL of the sample (of 0.5 mL HCl 1.0 mol $L^{-1}$) supplemented with the same volume of TISAB II modified with 20 g NaOH/L. Ca analysis was performed using the Arsenazo III colorimetric method (Vogel et al., 1983). Absorbance readings were recorded at 650 nm with a plate reader (PowerWave 340, Biotek, Winooski, USA) while P was measured using the Fiske and Subbarow (1925) method, with absorbance readings recorded at 660 nm. All results of these assays were expressed in μg/$mm^3$.

### 2.7. Statistical analysis

The statistical program Sigmaplot® version 12.0 (version 12.0, Systat Software Inc., San Jose, CA, USA) was used to evaluate all data, and a critical α value of 0.05% was used in all analyses. The %SH, ΔKHN, PLM, F, Ca, and P values for each enamel sample were treated as variables; the percentage of β-CaGP and particle size were the variation factor. The variables presented normal (Shapiro-Wilk test) and homogeneous distribution (Cochran test), respectively: % SH ($p > 0.519$ and $p > 0.417$), ΔKHN ($p > 0.064$ and $p > 0.746$), F ($p > 0.075$ and $p > 0.175$), Ca ($p > 0.296$ and $p > 0.684$), and P ($p > 0.323$ and $p > 0.216$) in the enamel. The data were submitted to two-way analysis of variance and Student-Newman-Keuls post hoc test was performed for multiple comparisons.

## 3. Results

Fig. 1A depicts the SEM images of β-CaGPm with large aggregates and particles of smaller sizes (estimate average size of 1140 ± 400 nm). Fig. 1B portrays the SEM images of β-CaGPm particles with low size distribution and an estimate average size of 220 ± 117 nm. The milling

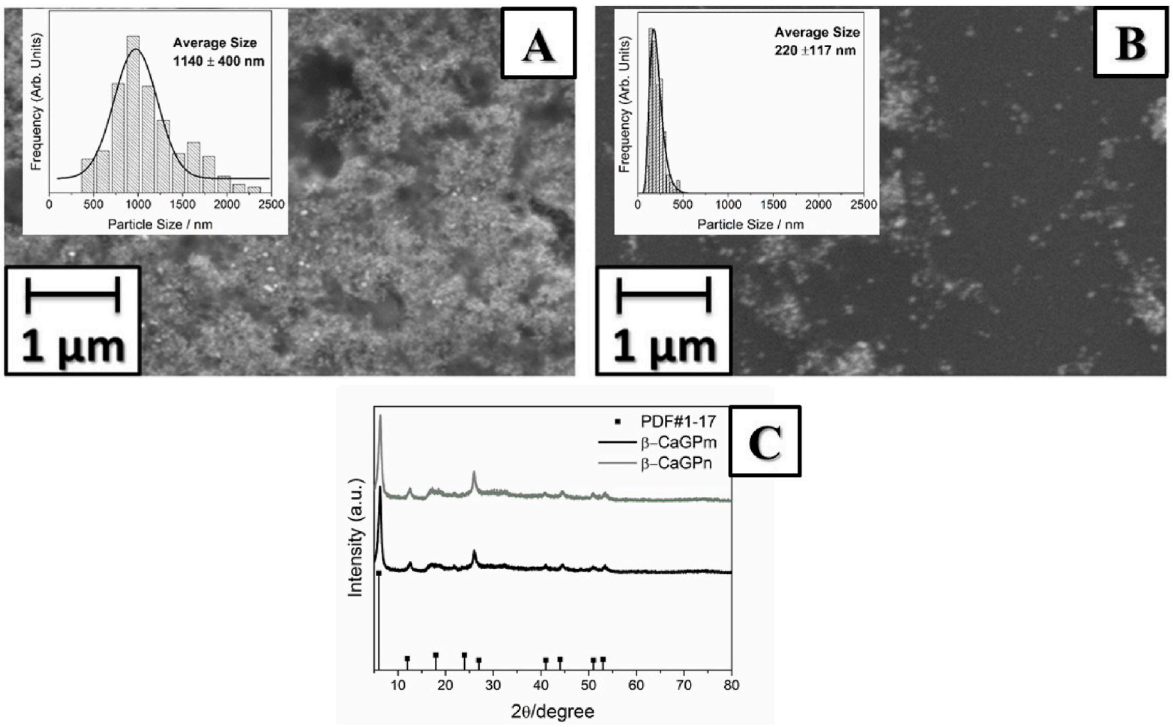


**Fig. 1.** A–B: Scanning electron microscopy images and average size (histograms) and C: X-ray patterns of the β-CaGPm and β-CaGPn after milling for 48 h. Magnification: 20,000×.

process reduced the particle size of the β-CaGPn powder without affecting its crystalline structure. The data shows diffraction peaks at $2\theta$ = 6.2°, 12.5°, 18.4°, 24.1°, 27.3°, 41.4°, 44.0°, 50.9° and 53.2° which corresponds to the crystallographic form of β-CaGPm salt (PDF Nº 1–17; Fig. 1C).

All toothpastes showed neutral pH, ranging from 7.2 to 7.6 (Table 1). The amount of ionic fluoride was less than the total fluoride in fluoride toothpastes, without influence of the concentration and particle size of the β-CaGP (micro or nano). The variation in fluoride concentration in the formulations was at a limit of 9% based on the proposed for toothpaste (Table 1).

The mean (SD) SHi for the blocks was 368.4 (0.2) KHN, and no significant differences in SHi were observed between the groups after random allocation ($p = 0.104$). Treatment with toothpaste containing 1100 F associated with 0.5%β-CaGPm showed lower value for %SH (~ 30%) compared to 1100 F toothpaste ($p < 0.001$). The 1100 F associated 0.125%β-CaGPm and 0.25%β-CaGPm toothpastes was similar to 1100 F toothpaste ($p > 0.001$). The highest %SH was observed for 1100 F/1.0%β-CaGPm ($p < 0.001$). For β-CaGPm, the treatment with toothpaste 1100 F/0.25% reduced the %SH in ~ 32% when compared to 1100 F ($p < 0.001$). 1100 F/0.5%β-CaGP and 1100 F/1.0%β-CaGP treatment resulted in the highest %SH ($p < 0.001$) (Table 2).

The subsurface lesion (ΔKH) was ~ 36% lower for the 1100 F/0.5%β-CaGPm treatment ($p < 0.001$). For 1100 F/0.125%β-CaGPm and 1100 F/0.25%β-CaGPm toothpaste the ΔKHN was similar ($p > 0.001$). The ΔKHN value for 1100 F/0.25% β-CaGPn was ~43% and ~10% lower than those for 1100 F and 1100F-0.5%β-CaGPm, respectively ($p < 0.001$) (Table 2 and Fig. 2).

β-CaGPm and β-CaGPn supplementation did not influence the concentration of F in the enamel, making its effects similar to that of 1100 F alone; only the Negative control group demonstrated a significant reduction in F content ($p < 0.001$).

Treatment with 1100 F/0.5%β-CaGPm increased the enamel concentration of Ca by ~21% when compared to treatment with 1100 F ($p < 0.001$). The treatment with 1100 F/1.0%β-CaGPm resulted in the lowest concentrations of Ca ($p < 0.001$). After treatment with toothpaste

**Table 1**
Mean values (SD) of total fluoride (TF), ionic fluoride (IF) and pH from toothpastes, and fluoride (F), calcium (Ca) and phosphorus (P) in the enamel according to the particle size and β-CaGP concentration in the toothpastes.

| Particle size | Toothpastes | Analysis in the toothpaste | | | Enamel analysis (μg/mm$^3$) | | |
|---|---|---|---|---|---|---|---|
| | | TF (ppm F) | IF (ppm F) | pH | F | Ca | P |
| – | **Negative control** | 24.4 (5.0) | 17.0 (0.4) | 7.5 (0.1) | 1.5$^{a}$ (0.2) | 126.4$^{a}$ (21.0) | 159.2$^{a}$ (32.2) |
| | **1100F** | 1168.8 (10.8) | 1061.0 (25.2) | 7.6 (0.2) | 2.5$^{b}$ (0.5) | 305.5$^{b}$ (64.0) | 248.2$^{b}$ (45.1) |
| **Micro** | **1100 F/0.125%β-CaGPm** | 1202.5 (12.7) | 1149.3 (12.2) | 7.4 (0.1) | 2.5$^{b}$ (1.0) | 271.7$^{b,d}$ (76.9) | 240.1$^{b}$ (56.9) |
| | **1100 F/0.25%β-CaGPm** | 1005.5 (9.5) | 1004.9 (11.1) | 7.3 (0.2) | 2.1$^{b}$ (0.6) | 276.8$^{b}$ (85.2) | 229.5$^{b,e}$ (46.5) |
| | **1100 F/0.5%β-CaGPm** | 1050.7 (11.1) | 1056.0 (10.4) | 7.4 (0.1) | 2.3$^{b}$ (0.5) | 368.5$^{c}$ (70.4) | 268.2$^{c}$ (50.6) |
| | **1100 F/1.0%β-CaGPm** | 1110.0 (14.4) | 1071.8 (9.4) | 7.5 (0.3) | 2.3$^{b}$ (0.7) | 224.2$^{d}$ (60.5) | 176.7$^{a}$ (50.8) |
| **Nano** | **1100 F/0.125%β-CaGPn** | 1178.4 (10.1) | 1176.0 (8.3) | 7.4 (0.2) | 2.4$^{b}$ (0.8) | 243.9$^{d}$ (46.4) | 197.9$^{a,e}$ (47.4) |
| | **1100 F/0.25%β-CaGPn** | 1137.7 (9.4) | 1058.1 (13.4) | 7.2 (0.4) | 2.5$^{b}$ (0.8) | 469.4$^{d}$ (78.2) | 319.1$^{d}$ (86.1) |
| | **1100 F/0.5%β-CaGPn** | 1070.2 (12.3) | 1041.8 (9.4) | 7.3 (0.1) | 2.1$^{b}$ (0.5) | 227.5$^{d}$ (63.4) | 216.5$^{e}$ (54.9) |
| | **1100 F/1.0%β-CaGPn** | 1127.6 (8.2) | 1048.4 (9.6) | 7.3 (0.1) | 2.4$^{b}$ (0.6) | 245.5$^{d}$ (49.6) | 224.8$^{e}$ (48.9) |

Distinct superscript letters indicate statistical significance among the treatment in each analysis (Two-way ANOVA, followed by Student–Newman–Keuls' test). Values represent means (standard deviations).

**Table 2**
Mean (SD) of the variables analysed in the enamel according to the particle size and concentration of β-CaGP in the toothpastes.

| Particle size | Toothpastes | %SH (kg/mm$^2$) | ΔKHN (kg/mm$^2$ × μm) | PLM Surface (μm) | PLM Depth (μm) |
|---|---|---|---|---|---|
| – | **Negative control** | −84.4$^a$ (5.6) | 7183.9$^a$ (1,167.5) | 0.6$^a$ (0,2) | 28.5$^a$ (4.8) |
| | **1100F** | −44.6$^b$ (3.2) | 4545.4$^b$ (682.5) | 5.0$^b$ (0.5) | 23.7$^b$ (5.6) |
| **Micro** | **1100 F/0.125%β-CaGPm** | −46.8$^b$ (4.7) | 5577.5$^c$ (504.9) | 4.6$^b$ (0.4) | 21.8$^b$ (3.4) |
| | **1100 F/0.25%β-CaGPm** | −46.0$^b$ (5.5) | 5508.7$^c$ (641.6) | 5.6$^{b,c}$ (1.1) | 13.3$^c$ (2.3) |
| | **1100 F/0.5%β-CaGPm** | −31.4$^d$ (1.9) | 2893.7$^d$ (492.9) | 6.4$^c$ (1.0) | 10.0$^d$ (4.7) |
| | **1100 F/1.0%β-CaGPm** | −53.0$^c$ (4.9) | 4740.6$^b$ (635.6) | 3.7$^d$ (0.9) | 17.6$^c$ (4.9) |
| **Nano** | **1100 F/0.125%β-CaGPn** | −53.7$^c$ (4.8) | 5337.2$^c$ (502.0) | 3.4$^d$ (0.7) | 18.1$^c$ (1.0) |
| | **1100 F/0.25%β-CaGPn** | −30.3$^d$ (4.0) | 2578.8$^d$ (372.1) | 11.6$^e$ (1.2) | 6.0$^d$ (1,6) |
| | **1100 F/0.5%β-CaGPn** | −58.2$^e$ (3.5) | 5027.3$^c$ (653.6) | 2.0$^f$ (0.4) | 17.7$^c$ (0.6) |
| | **1100 F/1.0%β-CaGPn** | −61.7$^e$ (4.8) | 5589.8$^c$ (975.1) | 2.5$^g$ (0.5) | 16.5$^c$ (0.9) |

Distinct superscript letters indicate statistical significance among the treatment in each analysis (Two-way ANOVA, followed Student–Newman–Keuls' test). Values represent means (standard deviations). %SH: percentage of surface hardness loss. ΔKHN: integrated subsurface hardness loss. PLM surface: thickness of the enamel surface layer. PLM depth: thickness of demineralization depth.

1100 F/0.25%β-CaGPn, the highest concentration of Ca was observed, being higher in ~ 54% in relation to 1100 F ($p < 0.001$).

For the P content in the enamel, the blocks treated with 1100 F/1.0%β-CaGPm toothpaste showed lower values ($p > 0.001$), and 1100 F/0.5%β-CaGPm treatment showed high value (~ 8% in relation 1100 F) ($p < 0.001$). However, when treated with β-CaGPn, the P content of the enamel in the 1100 F/0.25% β-CaGPn group was higher than that in the other treatment groups ($p < 0.001$) and ~29% higher than that for the 1100 F group ($p < 0.001$) (Table 1).

## 4. Discussion/conclusion

Several therapies to control dental caries have been proposed in the literature, among them the use of F in association with Ca and P stands out, since the presence of these ions in the salivary and biofilm environment positively interfere in the process of remineralization and demineralization. Thus, different concentrations of micrometric and nanometric β-CaGP were added to 1100 ppm F toothpaste to improve the anticaries effect compared to toothpaste containing 1100 ppm F without β-CaGP. The results showed that addition of 0.5%β-CaGPm and 0.25%β-CaGPn confers superior protection to the dental enamel when compared to toothpaste containing only 1100 ppm F, leading to the rejection of the null hypothesis.

It is established that a toothpaste must maintain the fluoride soluble in the formulation to guarantee an anti-caries effect. Thus, to use a soluble calcium sources, sodium monofluorophosphate (SMFP) should be utilized to avoid the formation of a poorly soluble phase between calcium and fluoride (Shen et al., 2018), and to reduce the anti-caries effect (Tenuta et al., 2009; Zaze et al., 2014a). Notwithstanding, a previous study demonstrated that the addition of CaGP in toothpastes with 500 ppm F (NaF) did not reduce the soluble fluoride in the formulation after 8 months (do Amaral et al., 2013). Even assuming that there is some interaction between F and β-CaGP, during the 15 min need to prepare the toothpaste suspensions, this did not interfere with the uptake of fluoride into the enamel, regardless of the amount and size of β-CaGP added (Table 1). Similar results were observed in the studies by do Amaral et al. (2013) and Zaze et al. (2014a, 2014b). Moreover, the addition of β-CaGP led to higher presence of Ca and P in the enamel and lower enamel demineralization on surface and in depth (Table 2, Fig. 2) (do Amaral et al., 2013; Zaze et al., 2014a). A randomized clinical trial with an 18-month follow-up in children showed no difference in the anti-caries effect between 500 ppm F + 0.25%CaGP and 1100 ppm F (Freire et al., 2016).

Considering the previous study (Zaze et al., 2014b) and the present results, a critical factor to observe or not effect on enamel demineralization was the F:β-CaGP ratio. Failure to observe the effect on enamel

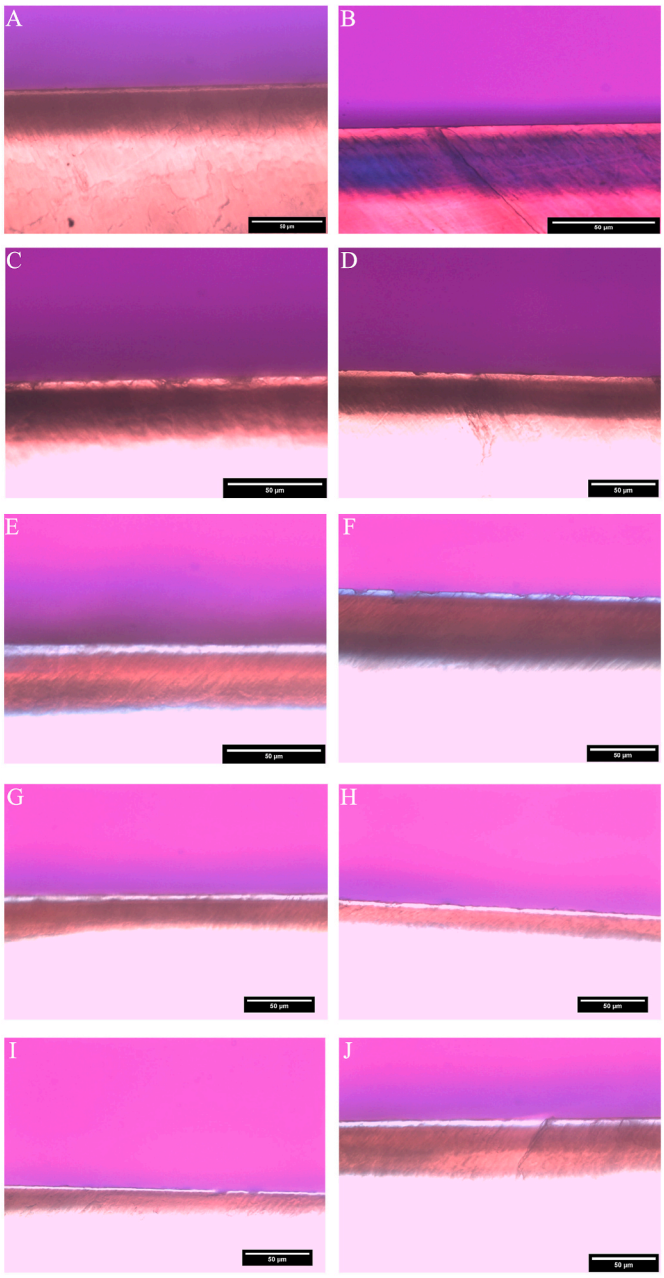


**Fig. 2.** Photomicrograph with polarized light of the lesion formed after treatment with (A) Negative control; (B) 1100 F; (C) 1100 F/0.125% β-CaGPm; (D) 1100 F/0.25% β-CaGPm; (E) 1100 F/0.5% β-CaGPm; (F) 1100 F/1.0% β-CaGPm; (G) 1100 F/0.125%β-CaGPn; (H) 1100 F/0.25% β-CaGPn; (I) 1100 F/0.5% β-CaGPn; (J) 1100 F/1.0% β-CaGPn. ( × 40).

demineralization with the addition of 0.13% CaGP in the *in situ* study by Tenuta et al. (2009) may be related to an F:CaGP ratio that has no effect even at lower fluoride concentrations (Zaze et al., 2014b). The present data show that fluoridated toothpastes containing β-CaGP reduce the depth of the lesion and mineral loss compared to the Negative control group (Table 2). The higher values of Ca and P in the enamel of these groups collaborate to explain this effect (Table 1). For micrometric particles, a superior effect than 1100 ppm F (~36% for ΔKHN and ~58% for PLM Depth) is only obtained with 0.5% β-CaGP and can be supported by the greater presence of Ca and P in the enamel as demonstrated in previous studies (do Amaral et al., 2013; Zaze et al., 2014a). Even at concentrations below or above 0.5%, it was possible to verify a reduction in the extent of the lesion in depth when compared to treatment with 1100 ppm F. Probably, associating β-CaGP to fluoride, may help to minimize mineral loss in demineralization cycles, but it does not favor mineral gain in remineralization cycles. These groups present greater loss of hardness, less presence of Ca and P and less thick surface layer than 1100 ppm F.

The pH-cycling model should be able to simulate the demineralizing and remineralizing episodes, considering the composition of the different dental substrates, in order to create a subsurface mineral loss and not an erosive lesion at the end of the experiment. Although the model leads to the formation of a subsurface lesion in the enamel, the design of the model was created to verify how much an agent can reduce it. Thus, agents have to demonstrate two abilities to minimize the formation of the lesion: (1) reduce demineralization during the acid challenge and, (2) enhance mineral recovery during the remineralization phase. It is also important to note that the pH-cycling model used to evaluate the anticaries effect of experimental toothpastes, in this *in vitro* study, produces a subsurface lesion (Vieira et al., 2005), with a greater number and diameter of the pores. This type of lesion has greater superficial enamel permeability, which facilitates ionic changes due to large and numerous pores. When these pores are obliterated, ionic changes can occur, leading to less reduction in remineralization of demineralized areas in depth. It was found in this study that at concentrations above 0.5%β-CaGPm and 0.25%β-CaGPn, there was no improvement in mineral gain, results also found in the study by Zaze et al. [2014a].

In addition to the above-mentioned mechanisms, another factor that can interfere with the properties of the active agent is its dimensions; the use of nanometric phosphate has emerged as an innovative method to optimize the effect of fluoride toothpastes in the control of dental caries. By reducing the particle size, the proportion to obtain a superior effect is changed to a lower concentration of β-CaGP. All the superior effects verified with micrometric 0.5%β-CaGP were obtained with nanometric 0.25%β-CaGP; except for the greater thickness of the enamel surface layer, and the greater presence of Ca and P in the enamel produced by the nanometric particles (Tables 1 and 2; Fig. 2). However, reducing the size of a particle to became it more reactive can make it more liable to agglomerate. Nevertheless, nanometric particles exhibit extra colloidal stability responsible for their chemical reactivity, homogeneous distribution, and size stability over time in toothpaste. Moreover, low viscosity of medium and the complex composition with several substances (such as carboxymethyl cellulose, mint oil or glycerin) of toothpaste can act on the surface of nanometric particles stabilizing them, i.e., less propensity to agglomerate. The performance of nanometer-sized materials is related to their different and enhanced thermodynamic and kinetic properties only possessed by them. For instance, toothpastes containing same amount (in mass) of micrometric and nanometric particles can show quite different rheological properties that affect their general performance, which obviously cannot be analysed exclusively in terms of particle size. Increasing the reactivity of a particle may not have the expected effects on the artificial caries process, but it does reduce the amount of β-CaGP added, which facilitates its solubility and manipulation of toothpaste (Danelon et al., 2015, 2017).

Although it is known that pH cycling models are suitable for assessing the impact of new active agents in fluoridated toothpastes as well as their association with other anticaries treatments, *in vitro* protocols have limitations. They do not simulate all the complexities of an oral cariogenic environment *in vivo*, forcing researchers to disregard the impact of the saliva/biofilm on the enamel. Therefore, the results obtained in this study are only an initial evidence to support the further *in situ* and *in vivo* studies.

## 5. Conclusion

Based on the findings of this *in vitro* study, we can conclude that the fluoride toothpaste produced a superior effect when combined at an appropriate β-CaGP molar ratio. This effect was achieved with a lower proportion of β-CaGP in the form of nanometric particles.

## Author statement

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## Declaration of competing interest

The authors Marcelle Danelon, Alberto Carlos Botazzo Delbem and Emerson Rodrigues de Camargo hold a patent request for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmbbm.2023.106354.

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