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Análise *in vitro* do potencial remineralizador de formulações dentifrícias contendo trimetafosfato de cálcio e sódio em lesões de cárie em dentina

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista (UNESP), como parte dos requisitos para a obtenção do título de Mestra em Ciências na Área de Concentração de Saúde Bucal da Criança.

Orientador: Prof. Dr. Alberto Carlos Botazzo Delbem

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“Os seus cargos são temporários, os títulos que tem são provisórios, mas a maneira como você trata as pessoas sempre será lembrada.”

Autor desconhecido

RESUMO

Poli, MCF. **Análise *in vitro* do potencial remineralizador de formulações dentifrícias contendo trimetafosfato de cálcio e sódio em lesões de cárie em dentina**. 2024. Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista (UNESP), Araçatuba, 2024.

O objetivo do presente estudo foi avaliar a capacidade de dentifrícios contendo apenas diferentes concentrações de trimetafosfato de cálcio e sódio (CaNaTMP) em promover a remineralização de lesões de cárie artificais em dentina em um modelo *in vitro*. Para tanto, foi feito inicialmente a síntese e caracterização do CaNaTMP e 60 blocos de dentina radicular bovina foram preparados nas dimensões 4 mm x 4 mm x 2 mm e selecionados por dureza de superfície e lesões de cárie artificial foram induzidas em dois terços da superfície por meio de um protocolo de 7 dias. Assim, os blocos com cárie artificial foram divididos aleatoriamente em 5 grupos/dentifrícios experimentais (n = 12): 1) sem F/NaCaTMP (Placebo-controle negativo); 2) contendo 1100 ppm F (controle positivo); 3) contendo 0,25% de CaNaTMP; 4) contendo 0,5% de CaNaTMP; e 5) contendo 1% de CaNaTMP. Para o tratamento, os blocos de dentina foram submetidos a ciclagem de pH de 7 dias e ao tratamento com suspensão aquosa dos dentifrícios experimentais durante 1 minuto, 2 vezes ao dia. A concentração mineral ($g_{Hap} \times cm^{-3}$) foi determinada por microtomografia computadorizada de raios-X. Os dados obtidos foram submetidos à ANOVA seguida do teste de Student-Newman-Keuls ($p<0,05$). A incorporação de 0,5% e 1% de CaNaTMP em dentifrícios promoveu efeito remineralizador superior ao dentifrício convencional, dose-dependente, em cárie dentinária artificial. O tratamento com 1% CaNaTMP levou a menor perda mineral e reduziu a profundidade das lesões quando comparado ao dentifrício fluoretado ($p < 0,001$). Dessa forma, conclui-se que dentifrícios contendo 0,5% e 1% de CaNaTMP mostraram maior capacidade remineralizadora de lesões artificiais de cárie em dentina, sugerindo que podem ser uma alternativa terapêutica eficaz para melhorar a saúde bucal de todos os indivíduos, especialmente aqueles com alto risco e/ou atividade de cárie e crianças em idade precoce.

Palavras-chave: Cárie Dentária; Dentifrícios; Remineralização Dentária; Dentina.

ABSTRACT

Poli, MCF. ***In vitro* analysis of the remineralizing potential of dentifrice formulations containing calcium and sodium trimetaphosphate in dentin caries lesions.** 2024. Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista (UNESP), Araçatuba, 2024.

The objective of the present study was to evaluate the ability of dentifrices containing only different concentrations of calcium and sodium trimetaphosphate (CaNaTMP) to promote remineralization in artificial caries lesions in dentin in an *in vitro* model. To this end, the synthesis and characterization of CaNaTMP was initially carried out and 60 blocks of bovine root dentin were prepared in dimensions 4 mm x 4 mm x 2 mm and selected by surface hardness and artificial caries lesions were induced on two thirds of the surface by through a 7-day protocol. Thus, the blocks with artificial caries were randomly divided into 5 groups/experimental toothpastes (n = 12): 1) without F/NaCaTMP (Placebo-negative control); 2) containing 1100 ppm F (positive control); 3) containing 0.25% CaNaTMP; 4) containing 0.5% CaNaTMP; and 5) containing 1% CaNaTMP. For treatment, the dentin blocks were subjected to pH cycling for 7 days and treatment with an aqueous suspension of the experimental dentifrices for 1 minute, twice daily. Mineral concentration ($g_{Hap} \times cm^{-3}$) was determined by X-ray microcomputed tomography. Data were subjected to ANOVA followed by Student-Newman-Keuls test ($p <0.05$). The incorporation of 0.5% and 1% of CaNaTMP in dentifrices promoted a remineralizing effect superior to conventional dentifrice, dose-dependently, in artificial dentin caries. Treatment with 1% CaNaTMP resulted in the lowest mineral loss and reduced lesion depth compared to fluoridated toothpaste ($p <0.001$). Therefore, it is concluded that dentifrices containing 0.5% and 1% CaNaTMP showed greater remineralizing capacity for artificial caries lesions in dentin, suggesting that they can be an effective therapeutic alternative to improve the oral health of all individuals, especially those with high risk and/or caries activity and children at an early age.

Keywords: Dental Caries; Toothpastes; Dental Remineralization; Dentin.

Sumário

1. Introduction

Dental caries is one of the highly prevalent diseases worldwide, with an estimated 600 million children affected by it, considered by the American Academy of Pediatric Dentistry as the most common oral disease in childhood (Moraes et al., 2021; Pitts et al., 2019). The development of caries is influenced by various factors, mediated by biofilm and modulated by diet, and, in molecular terms, it occurs due to the loss of hydroxyapatite structure. Specifically, the metabolic action of dietary carbohydrates by bacteria present in the oral cavity causes an initial decrease in the pH of the oral environment and promotes an imbalance between remineralization and demineralization processes, favoring the demineralization of dental structure (Díaz et al., 2024; O'Hagan-Wong et al., 2021).

In the treatment of caries lesions, the use of fluoride (F) is a well-established strategy to prevent and inhibit demineralization, as well as to favor the remineralization process, with effective responses in dental enamel (Choi et al., 2023; Caravalho et al., 2024), with the use of fluoride being considered the main reason for the reduction in caries prevalence observed in recent decades. However, fluoride as the sole means of prevention and remineralization does not provide a complete solution, as fluorides are limited only to the superficial remineralization of carious lesions and by the availability of calcium and phosphate ions (Gonçalves et al., 2018; O'Hagan-Wong et al., 2021; Nunes et al., 2022). Additionally, in dentin tissue, the complexity of lesion arrest and remineralization process is expanded by the heterogeneity and dynamics of dentin, where organic components such as matrix metalloproteinases and cysteine-cathepsins (Dias et al., 2023; Nunes et al., 2022) play an important mechanical and biological role (Gonçalves et al., 2018).

It is already known that when caries affects dentin, mineral loss, and collagen breakdown are events that lead to substrate alteration, even impacting adhesive restorative procedures. Therefore, strategies adopted in dentin substrates must allow for the arrest of demineralization and not interfere with the restorative process; they must also preserve and stabilize the dentin collagen matrix, which is essential for the remineralization process, as it provides support for mineral deposition in the presence of non-collagenous proteins [Toledano et al., 2022; Tao et al., 2022]. Therefore, to enhance the preventive potential of fluoride, especially in dentin tissue, there has been a quest for strategies that minimize damage to dentin and are focused on preserving collagen fibrils and promoting structured remineralization.

Among biomimetic and remineralizing agents, sodium trimetaphosphate (NaTMP) is an inorganic cyclic polyphosphate that has been widely tested in fluoride vehicles, mainly in toothpaste, for promoting the precipitation of calcium phosphate onto dentin collagen, leading to the obliteration of dentinal tubules (Favretto et al., 2018). Additionally, NaTMP is capable of creating a protective barrier on the dental surface, preventing acid diffusion and facilitating selective diffusion of calcium and fluoride (Favretto et al., 2018; Amaral et al., 2018; Nunes et al., 2022), and it has significant potential in collagen phosphorylation, mimicking the remineralization induced by dentin matrix phosphoproteins (Nunes et al., 2022).

On the other hand, NaTMP must necessarily be associated with F to present a superior effect compared to conventional toothpaste. Therefore, it is assumed that to increase the effect of NaTMP without using fluoride, it would be interesting to substitute sodium ions with calcium ions in the NaTMP molecule, producing a compound with greater mineralizing activity compared to conventional NaTMP, as this would combine the properties of both (NaTMP and calcium) on remineralization processes, through the deposition of phosphate and calcium, essential for the growth of minerals needed in this process. Considering the above, the objective of this study was to evaluate the ability of toothpastes containing different concentrations of calcium and sodium trimetaphosphate (CaNaTMP) to promote remineralization in dentin caries lesions in an in vitro model. The null hypothesis was that toothpastes containing different concentrations of CaNaTMP would have the same capacity to induce dentin remineralization as one containing 1100 ppm F.

2. Material and Methods

2.1. Synthesis, and Characterization of Calcium and Sodium Trimetaphosphate

Compounds of NaTMP with partial substitution of sodium by calcium (CaNaTMP) were prepared starting with the dilution of 30 g of NaTMP (Na3P3O9, CAS 7785-84-4, Sigma-Aldrich Co., St. Louis, MO, USA) in 200 mL of deionized water (pH 5.6). Next, the solution was treated in a glass column (2 cm × 30 cm) containing strong cationic ion exchange resin in the H^+ form (DOWEX 50WX4, Dow Inc., Midland, MI, USA) for the substitution of sodium ions by calcium ions [Watanabe et al., 1998], with a flow rate of 1.0 mL/min. After passing through the column (pH 1.11), the supernatant of 4.39 g of calcium hydroxide ($Ca(OH)_2$), Code HC09743RA, Exôdo, Sumaré, SP, Brazil) diluted in 800 mL of deionized water was added to the filtrate until reaching the same pH value as the NaTMP solution before ion exchange. The solution was kept in an oven for 24 hours at 37°C for CaNaTMP precipitation [Griffith, 1962], followed by filtration using quantitative filter paper (Quanty JP42, J Prolab, São José dos Pinhais, PR, Brazil) and a Büchner funnel connected to a vacuum system (–600 mmHg) [Amaral et al., 2018]. The precipitate was kept in an oven for 24 hours at 70°C for drying, and the powder was homogenized in a ball mill (Planetary Micro Mill PULVERISETTE 7 classic line, Fritsch GmbH, Idar-Oberstein, Germany) for 5 minutes [Amaral et al., 2018]. Subsequently, the NaTMP and CaNaTMP powders were deposited onto supports coated with carbon tape, subjected to vacuum and cathodic spraying for a thin coverage with gold (Quorum - Q150T E, West Sussex, UK). The morphology of NaTMP and CaNaTMP particles was obtained by scanning electron microscopy (SEM, Carl Zeiss, EVO ILS15, Carl Zeiss NTS LTD, Germany) at 20 kV and 3,000 u magnification. The chemical composition of the samples was determined by energy-dispersive X-ray spectroscopy (EDS, Oxford Instruments, INCAx - act, 133 eV, England) with 20 keV, spatial resolution of 2 µm, and counting time of 150 s. The chemical elements analyzed were phosphorus (P), oxygen (O), sodium (Na), and calcium (Ca).

2.2. Formulation and pH Determination, Fluoride Concentration in Experimental Toothpastes

The toothpastes were prepared in the pediatric dentistry laboratory at FOA/UNESP with the following components: titanium dioxide, carboxymethylcellulose, sodium methyl p-hydroxybenzoate, saccharin, peppermint oil, glycerin, abrasive silica, sodium lauryl sulfate, and water. For this study, toothpastes containing CaNaTMP at different concentrations were prepared, namely: 0.25%; 0.5%; 1.0%. Additionally, a toothpaste containing 1100 ppm F in the form of NaF (Merck, CAS 7681-49-4, Germany) was manufactured as a positive control, and a toothpaste without the addition of CaNaTMP or NaF (placebo/negative control) was also prepared.

To determine the pH, each toothpaste was suspended with deionized water at a ratio of 1:3 (weight:weight), and the pH was measured using a pH electrode (Orion 290A, Orion Research Inc., Boston, USA) with constant stirring. The fluoride (F) content of the toothpastes was determined according to the method described by Delbem et al. [2002]. Approximately 100-110 mg of each toothpaste were weighed in triplicate into polypropylene tubes, to which deionized water was added to a final volume of 10 mL. After homogenization, for the determination of total fluoride (TF), 0.25 mL of this suspension was kept under agitation for 1 hour at 45°C with 0.25 mL of 2 mol L^{-1} HCl. It was then neutralized with 0.5 mL of 1 mol L^{-1} NaOH and 1.0 mL of TISAB II (total ionic strength adjustment buffer). An ion-specific electrode combined for fluoride ions was used for fluoride determination, with a reference microelectrode (9609 BN – Orion) coupled to an ion analyzer (Orion 720 A), previously calibrated with five standards (1, 2, 4, 8, and 16 µg F/mL). For the determination of ionic fluoride (IF), the remaining toothpaste suspension was centrifuged at 906 ×g for 20 minutes. Then, 0.25 mL of the supernatant was pipetted and added with 0.25 mL of 2 mol L^{-1} HCl, 0.5 mL of 1 mol L^{-1} NaOH, and 1.0 mL of TISAB II, and the fluoride was determined in the same way as for TF.

2.3. Preparation of Dentin Specimens

For the study, initially, 200 freshly extracted bovine incisors without apparent damage were selected. The teeth were cleaned and stored in a 0.1% thymol solution. Subsequently, the selected incisors had their dental crowns separated from their roots using a diamond disc attached to a metallographic cutter Isomet 1000 (Buehler Ltd, Lake Bluff, IL, USA). Dentin blocks with dimensions of 4.0 mm x 4.0 mm x 2.0 mm (Figure 2A) were obtained from the root portion closest to the cervical (± 1 mm) and verified using a high-precision digital caliper (Mitutoyo América do Sul Ltda, Suzano, SP, Brazil). These blocks were then planed and polished using a polishing machine (BETA – grinder polisher, Buehler, Lake Bluff, Illinois, USA) with silicon carbide abrasive papers at grits #600, #800 at low rotation, and #1200 (Extec Corp, Enfield, CT, USA) at high rotation, with constant water irrigation during all abrasion stages. After the use of each grit size, the blocks were cleaned in deionized water using an ultrasonic bath (Unique USC 1400, Indaiatuba, SP, Brazil), operated at 40 Hz and 135 W for 20 minutes at room temperature [Favretto et al., 2018; Nunes et al., 2022]. The final polishing was performed with felt discs moistened with a 1µm diamond solution (Extec Corp. Enfield, CT, USA) and again washed in an ultrasonic bath as previously described. The blocks were then stored in a humid environment with deionized water at 4°C during the preparation process.

2.4. Initial Specimen Selection and Sample Size Calculation

The samples were selected based on the initial surface hardness (SHi), which was determined using the Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and Buehler Omni Met software (Buehler, Lake Bluff, USA), with a Knoop indenter, a static load of 10 g, and 10-second dwell time. Five impressions, separated by a distance of 100 µm, were made in the central region of each block for SH analysis [Gonçalves et al., 2018]. Blocks with SHi between 60 and 90 KHN were selected for induction of artificial caries lesions. The mean initial surface hardness

(SHi) for all blocks was 84.5 (7.7), ranging between 84.3 (8.1) and 84.9 (8.3) KHN in the experimental groups ($p > 0.05$). There were no significant differences between the groups after allocation ($p > 0.05$). Sample size calculation (n = 12 blocks/group) was based on a previous study [Nunes et al., 2022], considering the mean values of mineral concentration ($g_{Hap} \times cm^{-3}$) and artificial caries lesion depth (μm) as primary outcomes, a minimum detectable difference between means (3 and 12, respectively), an expected standard deviation (2 and 8, respectively), an α error of 5%, and a β error of 20%.

2.5. Formation of Artificial Caries Lesion in Dentin

The dentin specimens were covered with two consecutive layers of acid-resistant varnish (nail polish - Colorama Ltd., SP, Brazil), leaving only the polished dentin surface free for contact with the treatments proposed in the study. The specimens were divided into 3 areas [Gonçalves et al., 2018], as follows: I- Control Area: 1/3 of the surface was covered with acid-resistant varnish, which will not come into contact with the demineralizing solution and the proposed treatments; II- Demineralized Area: this area was placed in a demineralizing solution, after which it was covered with acid-resistant varnish, avoiding contact with the treatment solutions; III- Treated Area: this area was subjected to demineralization and one of the experimental toothpastes.

To induce artificial dentin caries lesions, these specimens were placed in a demineralizing solution (50 mmol L-1 acetate buffer containing 2.2 mmol L^{-1} CaCl2; 2,2 mmol L^{-1} KH_2PO_4; 0.04 μg F/mL, pH 5.0, 37°C). After 30 minutes, they were immersed in 30 mL of remineralizing solution (1,5 mmol L^{-1} Ca $(NO_3)_2 \times H_2O$; 0,9 mmol L^{-1} $NaH_2PO_4 \times H_2O$; 150 mmol L^{-1} KCl in 0.02 mol L^{-1} sodium cacodylate buffer; 0.04 μg F/mL, pH 7.0, 37°C) for 2 hours. This cycle was repeated 3 times per day for 2 days, and the blocks remained in the remineralizing solution for 18 hours and 30 minutes between days [Nunes et al., 2022].

Subsequently, the blocks were individually immersed in 30 mL of demineralizing solution for an additional five days at 37°C, totaling seven days of protocol. Afterward, half of the surface with artificial caries lesion was covered with acid-resistant varnish (Artificial Caries area) and randomly allocated into five experimental groups (n=12), as described previously, for subsequent pH cycling and treatment.

2.6. pH Cycling and Treatment

The dentin blocks underwent pH cycling [ten Cate & Arends, 1978; Lagerweij &

ten Cate, 2006] for 7 days and were treated with dentifrice suspension in deionized water (1:3 - weight:weight) for 1 minute twice a day (before and after immersion in demineralizing solution), with agitation. The blocks were thoroughly rinsed with deionized water between treatments and pH cycling solutions. After the 7 day of cycling, the blocks remained in remineralizing solution for 24 hours [Nunes et al., 2022].

2.7. Analysis of Dentin Mineral Concentration using X-ray Micro-computed Tomography

After pH cycling and treatment, the nail polish was removed, and the mineral concentration was determined using X-ray micro-computed tomography (Sky-Scan1272 High-resolution Micro-CT, Bruker-Micro-CT, Kontich, Belgium). The Micro-CT was operated at 55 kV and 180 µA, with an aluminum filter of 0.25 mm, spatial resolution at 5 µm, rotation at 0.400°, and random movement at 10. Image projections were reconstructed using NRecon software (version 1.6.10.2, Skyscan1272, Bruker Micro-CT) with smoothing at 3, ring artifact correction at 9, and beam hardening correction at 60% [Favretto et al., 2018; Nunes et al., 2022].

After image reconstruction, virtual two-dimensional (2D) slices in the sagittal and coronal planes were obtained using Data Viewer software (Skyscan1272). The sequences of 2D images were imported into ImageJ software (version 1.47v, National Institutes of Health, Bethesda, MD, USA) to produce an overall profile of mineral concentration (g_{Hap} x cm^{-3}) as a function of depth (µm). The mineral concentration profile was obtained from areas 1, 2, and 3 based on a calibration curve calculated with reference mineral standards (0, 0.25, and 0.75 g_{Hap} x cm^{-3}). The surface layer thickness (µm) and lesion depth (µm) were also determined [Nunes et al., 2022].

2.8. Statistical analysis

The results were analyzed taking into account the different experimental treatments and the areas of analysis (Artificial Lesion and Treated). The variables considered were the values of ΔZ ($g_{Hap} \times cm^{-3} \times$ µm), surface layer thickness (µm), and lesion depth (µm). After verifying normal distribution (Kolmogorov-Smirnov test) and homogeneity (Cochran's test) of the data, they were subjected to two-way repeated measures analysis of variance (ANOVA), followed by the Student-Newman-Keuls multiple comparisons test. The analyses were performed using SigmaPlot software (version 12.0) with a significance level of 5%.

3. Results

The SEM images revealed larger and irregularly shaped crystals for NaTMP (Figure 1A) and smaller, more regular grains for CaNaTMP (Figure 1B). EDS analysis identified peaks of the chemical elements Na at 1.04 keV, O at 0.52 keV, and P at 2.02 keV, common to both NaTMP and CaNaTMP samples (Figure 1C and 1D), along with the presence of peaks at 0.26 and 3.69 keV characteristic of the Ca element only in CaNaTMP (Figure 1D). The percentage (%) of P and Na mass in the CaNaTMP samples is lower compared to NaTMP (Figure 1C and 1D), while the % of O was higher for CaNaTMP.

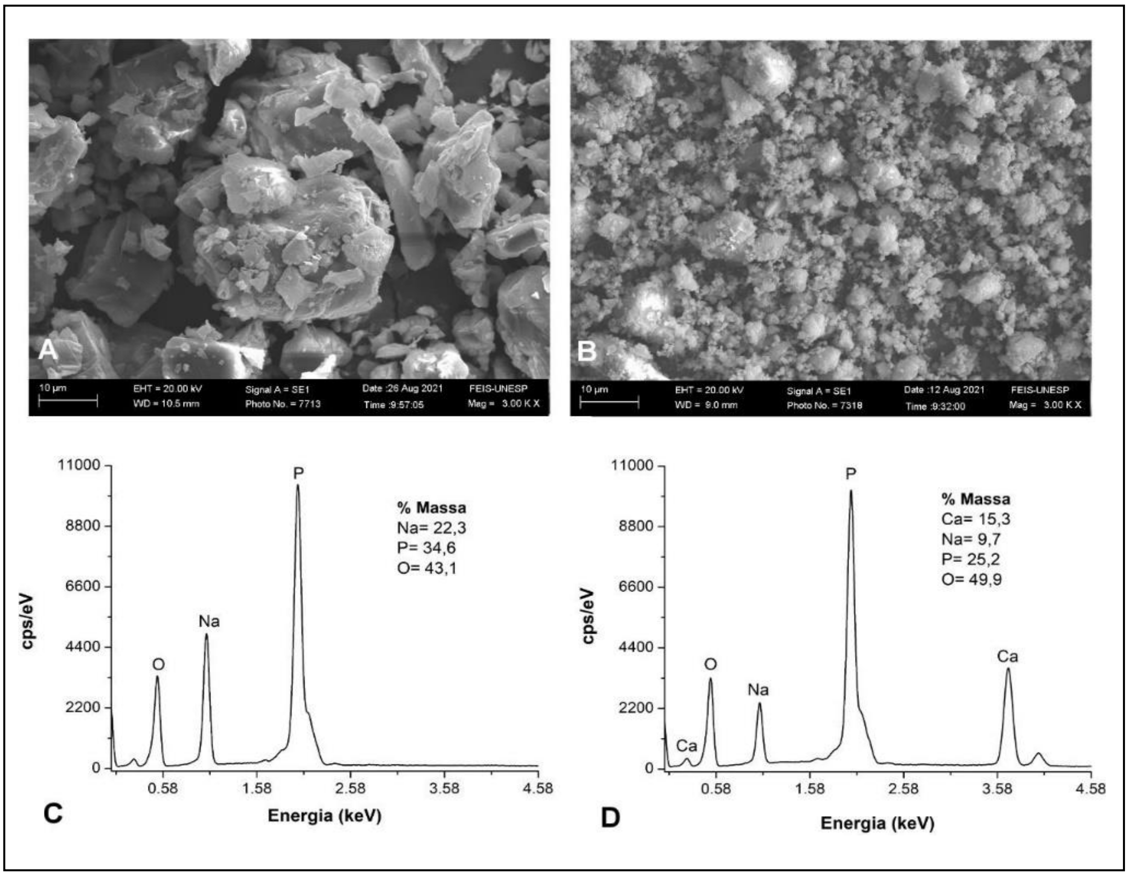

Figure 1. Scanning electron microscopy photomicrographs of NaTMP (A) and CaNaTMP (B) samples, and their respective EDS histograms (C and D).

The pH values of the dentifrices ranged from 6.9 to 7.4 (as shown in Table 1). The concentrations of ionic and total fluoride remained within established limits for each dentifrice, with a variation of less than 10%. The pH cycling model used led to the induction of artificial dentin caries with similar mineral loss in all blocks ($p > 0.05$) (Table 1). Regarding the treated area, during pH cycling, the use of the placebo dentifrice resulted in greater mineral loss in dentin affected by caries (ΔZ) ($p < 0.05$; Table 1). The dentifrice containing 1% CaNaTMP demonstrated the highest mineral gain ($p < 0.001$), with a remineralization rate ≅ 50% higher than 1100 ppm F. Meanwhile, the dentifrice with 0.25% CaNaTMP showed a remineralizing effect similar to 1100 ppm F ($p > 0.05$). Additionally, a dose-response curve for the concentration of CaNaTMP in the dentifrice was observed (Table 1).

Table 1 – Means values (SD) of ionic fluoride concentration (FI), total fluoride concentration (FT), pH toothpaste, mineral concentration (ΔZ) in dentin with artificial caries and after treatment and pH cycling, and percentage of mineral recovery (%ΔZ) according to the treatments (n = 12)

Treatments	Toothpaste analysis			ΔZ ($g_{HAp} \times cm^{-3} \times \mu m$)		%ΔZ
	FI (ppm F)	FT (ppm F)	pH	Artificial Caries	Treated	
Placebo	10.2 (0.2)	10.6 (1.1)	7.2 (0.6)	-54.3^{A} (11.6)	-2.0^{B} (4.3)	-4.8^{a} (9.9)
1100 ppm F	1148.9 (22.3)	1146.7 (22.5)	7.3 (0.4)	-54.0^{A} (10.9)	9.9^{C} (4.9)	17.8^{b} (7.6)
CaNaTMP 0.25%	10.7 (0.9)	11.1 (0.5)	6.9 (0.4)	-54.8^{A} (11.9)	7.9^{C} (3.3)	14.9^{b} (6.7)
CaNaTMP 0.5%	9.8 (1.3)	10.4 (0.3)	7.1 (0.3)	-55.8^{A} (6.7)	13.9^{C} (5.4)	25.3^{c} (10.3)
CaNaTMP 1%	10.3 (0.9)	10.8 (0.3)	7.4 (0.7)	-54.2^{A} (6.9)	19.0^{D} (4.4)	35.0^{d} (5.9)

Equal superscript capital letters indicate no statistical difference for the variable ΔZ. Distinct superscript lowercase letters indicate statistical difference among the treatments at %ΔZ (Two-way ANOVA; Student-Newman-Keuls; $p < 0.05$). SD stands for the standard deviation of means.

After the formation of artificial caries lesions, the values for surface layer thickness and lesion depth did not show significant differences among the experimental groups ($p > 0.05$; Table 2). The group with 1100 ppm F exhibited the greatest surface layer thickness (µm) (p < 0.001). Conversely, dentifrices containing CaNaTMP showed the lowest surface layer thickness, regardless of the concentration used (Table 2; Figure 2). Although the depth of the lesion body was reduced with all treatments except the placebo, the dentifrice containing 1% CaNaTMP promoted the lowest lesion depth (µm) (p < 0.001), showing superior performance by ≅ 43%, 38%, and 26% compared to the 0.25% CaNaTMP, 1100 ppm F, and 0.5% CaNaTMP groups, respectively (Table 2). Treatment with 1100 ppm F led to the formation of a laminar subsurface lesion (Figure 2), while the toothpaste with 1 % NaCaTMP led to a greater reduction in the depth of the lesion (Table 1).

Table 2 – Mean values (SD) thickness of surface layer and depth of lesion on artificial caries and treated area according to treatments (n=10)

Treatments	**Thickness of surface layer** (µm)		**Depth of lesion** (µm)	
	Artificial Caries	**Treated**	**Artificial Caries**	**Treated**
Placebo	17.8^{A} (1.6)	20.1^{B} (2.8)	223.9^{A} (59.3)	$203.2^{A,B}$ (57.2)
1100 ppm F	$19.9^{A,B}$ (1.6)	31.0^{C} (2.2)	225.7^{A} (36.7)	$169.2^{B,C}$ (31.7)
CaNaTMP 0.25%	20.3^{B} (3.2)	19.5^{B} (2.2)	232.3^{A} (44.2)	$174.9^{B,C}$ (40.2)
CaNaTMP 0.5%	20.6^{B} (2.8)	19.0^{B} (2.8)	220.8^{A} (47.7)	154.6^{C} (39.4)
CaNaTMP 1%	$18.8^{A,B}$ (1.4)	$18.5^{A,B}$ (2.5)	223.5^{A} (37.1)	122.3^{D} (20.9)

Different superscript capital letters indicate the difference between artificial caries and Treated values, and treatments for each analysis (Student-Newman-Keuls; p<0.05). SD stands for the standard deviation of means.

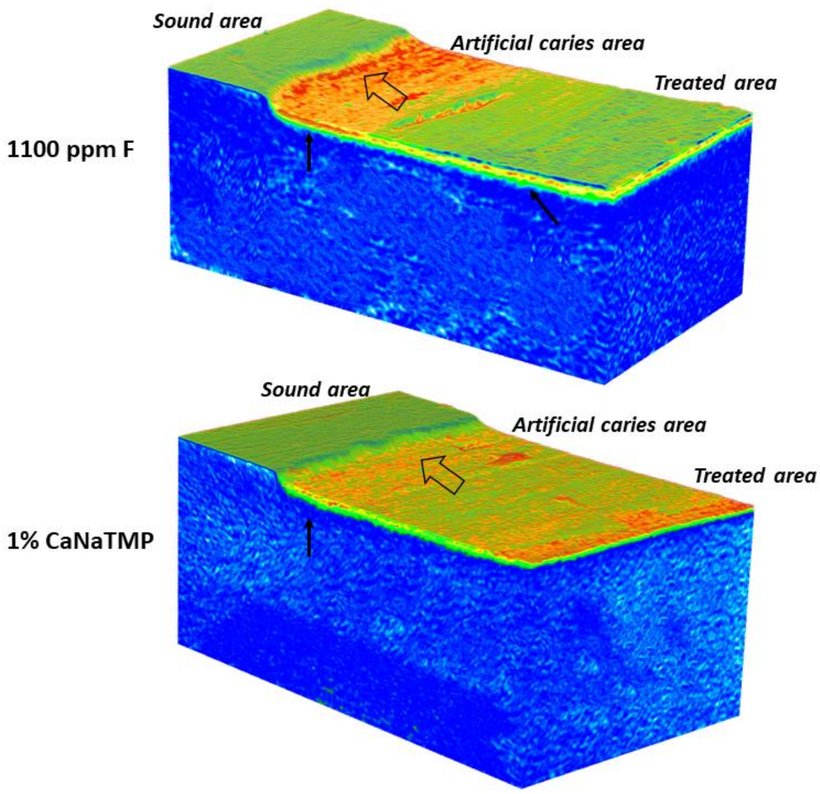

Figure 2. Photomicrographs of the 3D volume rendering by the CTVox software (version 3.00, Brucker Micro-CT) of the projections of the dentin blocks (central region) of the and 1100 ppm F, and 1 % CaNaTMP groups. Open arrow indicates the beginning of the loss of dentin structure about the sound area; black arrow in the vertical direction, from bottom to top, indicates subsurface lesion; slanted black arrow indicates laminar lesion.

4. *Discussion*

Literature confirms that the addition of sodium trimetaphosphate-based agents enhances the effect of fluoride dentifrices on the remineralization/demineralization processes of dental substrates [Favretto et al., 2021; Emerenciano et al., 2018; Favretto et al., 2018; Danelon et al., 2017; Danelon et al., 2015; Takeshita et al., 2015]. However, the impact of dentifrices formulated with calcium and sodium trimetaphosphate, obtained by partial substitution of sodium ions with calcium in dentin tissue, remains unclear. The results of this study showed that the synthesis of calcium trimetaphosphate and its application in dentifrices provided a higher level of remineralization of dentin caries lesions compared to conventional dentifrice (1100 ppm F). Thus, the null hypothesis postulated for this study, which speculated that dentifrices containing CaNaTMP would not produce a superior effect on the *in vitro* remineralization process of dentin compared to dentifrice containing 1100 ppm of F, was rejected.

It is worth noting that dental caries progresses rapidly, and the exclusive use of fluoride therapy as a preventive measure does not ensure a comprehensive solution, as the formation of fluoride reservoirs and the remineralization potential of saliva are limited by the availability of calcium and phosphate ions. Therefore, it is essential to explore new strategies to stabilize and strengthen the dentin matrix, stimulating remineralization and promoting mineral nucleation. The model used [Gonçalves et al., 2018] allowed for verifying the dose-response relationship and its impact among the Placebo, 1100 ppm F, and CaNaTMP (0.25, 0.5, and 1%) groups, validating the adopted pH cycling model.

Although the fluoridated toothpaste provided greater remineralization of caries lesions than the placebo toothpaste ($p < 0.001$; Table 1), this treatment also increased the thickness of the superficial dentin layer (Table 2; Figure 2). This data is supported by a previous study also conducted on dentin substrate [Nunes et al., 2022]. It is known that fluoride leads to greater remineralization in the outer region of the lesion, at the expense of the deeper part [Nunes et al., 2022; Wierichs et al., 2020; Vieira et al., 2017]. Despite this effect being related to the higher deposition of fluorapatite and calcium fluoride, this superficial layer of fluoride-rich dentin does not prevent acid diffusion into its interior, reaching the deeper layers [Nunes et al., 2022; Oliveira et al., 2022; Favretto et al., 2018; Vieira et al., 2017], as mineral loss occurred in the deepest part of the lesion during pH cycling with fluoride treatment, especially when compared to the experimental toothpastes with 0.5% and 1% CaNaTMP (Table 2, Figure 2). As observed in the present study, related literature has shown that higher fluoride concentrations can lead to the formation of secondary lesions, or laminated lesions [Nunes et al., 2022; Vieira et al., 2017; Walther et al., 2019; Wierichs et al., 2020]. The laminated lesion presents a primary lesion body and a deeper secondary lesion, interspersed by areas of higher mineral density, seen in the presence of high fluoride concentrations [Lippert, 2016; Walther et al., 2019; Wierichs et al., 2018] or calcium phosphate [Vieira et al., 2017], and in lesions that have dilated pores (enamel) or dentinal tubules (dentin) that facilitate mineral and acid diffusion [Nunes et al., 2022]. In contrast, in the Placebo and CaNaTMP groups, the outer part of the lesion is more susceptible to acid attack, with increased demineralization and mineral gain in the deeper part of the lesion (Table 2). The presence of organic matrix and dentinal tubules likely facilitated the flow of mineral deposits into the dentin, and a small portion ends up accumulating in the depth of the lesion, unlike what would occur in enamel. It is important to note that although the fluoridated toothpaste reduced the depth of the lesion (Table 2), it was not as effective as the toothpastes with 0.5% and 1% CaNaTMP.

Despite caries affecting both enamel and dentin, the differences in their structures influence both demineralization and remineralization. Dentin remineralization is a more challenging and less efficient process compared to enamel remineralization [Niu et al., 2014]. This is because the residual mineral seed crystals present in enamel are absent in dentin lesions. This disparity can be attributed to the lower presence of residual mineral crystals and the exposure of the organic matrix, mainly type I collagen, on the surface of acid-conditioned dentin. Additionally, the process of enamel remineralization also differs from dentin remineralization. In the case of enamel, the demineralized tissue is strengthened again, becoming more resistant to acid attacks than the original enamel [He et al., 2019]. On the other hand, in dentin remineralization, there is the regeneration of a new mineralized collagen matrix and the formation of hydroxyapatite crystals, which obstruct the dentinal tubules and protect the dentin-pulp complex [Ning et al., 2012].

The findings of this study reinforce previous hypotheses that NaTMP acts by reducing the diffusion of acid into the inner dentin substrate, minimizing its softening at depth [Emerenciano et al., 2018; Danelon et al., 2018]. In these studies, the combined use of F/NaTMP demonstrated a superior effect compared to its counterpart without NaTMP. In enamel, previous studies have found that the adsorption of NaTMP forms a protective layer [Amaral et al., 2016; Amaral et al., 2018; Nalin et al., 2021] with negative polarity that facilitates the deposition of calcium phosphate [Favretto et al., 2018; Nalin et al., 2021]. During acid attacks, the NaTMP layer has the ability to retain calcium and phosphate from enamel dissolution [Nalin et al., 2021], and when adsorbed to hydroxyapatite, it reduces its dissolution [Amaral et al., 2018]. On the other hand, in dentin, the combination of NaTMP (3%) with fluoridated toothpaste (1,100 ppm F) has been observed to result in greater precipitation of calcium phosphate and increased obliteration of dentinal tubules [Favretto et al., 2018]. Additionally, NaTMP treatment shows a dose-response for the adsorption of these cyclotriphosphates by dentin, reducing surface energy and making the surface more hydrophilic and susceptible to calcium adsorption [Oliveira et al., 2022], which possibly justifies the dose-response remineralizing effect for CaNaTMP in dentin in the present study.

Furthermore, the surface free energy characteristics of NaTMP are plausible to elucidate the present findings. It has been previously observed that NaTMP induces polarity on the dentin surface, making it more anionic (basic or electron-donating: γs^{-}) and with a greater affinity for cations [van Oss, 1993; van Oss, 1995]. An anionic surface has greater wetting and adhesion properties [Oliveira et al., 2022]. Since the dentin surface showed a large number of electron donor sites due to the adsorption of NaTMP, it is capable of adsorbing more Ca^{2+}. The anionic sites of NaTMP bind to the collagen and amino hydroxyl groups present in dentin, dispersed on the dentin surface and altering its polarity [Oliveira et al., 2022].

The dentin remineralizing effect was dose-dependent on the concentration of CaNaTMP, and this finding may also be related to inherent events of intrafibrillar remineralization of dentin, also known as guided tissue remineralization. This form of remineralization, essential for strengthening the mechanical properties of dentin, relies on the presence of the non-collagenous protein dentin matrix protein 1 (DMP1), which acts as a binding agent between Ca^{2+} ions and hydroxyapatite crystals in teeth. Moreover, this justification is corroborated by the intrinsic characteristics of NaTMP, which can absorb type I collagen through an electrostatic mechanism and a phosphorylation chemical process [Zhang et al., 2012; Gu et al., 2010; Li et al., 2008]. When the dentin collagen matrix is phosphorylated, it acts as a template molecule, attracting transient amorphous calcium phosphate nanoprecursors and nucleating apatite within the collagen fibrils. This results in both intrafibrillar and interfibrillar dentin remineralization [Ubolsa-Ard et al., 2024; Liu et al., 2011]. A comparative study investigated the effects of phosphorylated and non-phosphorylated dentin collagen matrix on intrafibrillar dentin remineralization (Cao et al., 2013). It was found that in the absence of transient amorphous calcium phosphate nanoprecursors, the non-phosphorylated dentin collagen matrix failed to induce intrafibrillar dentin remineralization. In the present study, the partial substitution of Na^{+} ions by Ca^{2+} was performed with $Ca(OH)_2$, potentially enhancing the remineralizing effect through the deposition of mineral crystals in dentin.

Additionally, it is presumed that the partial substitution of Na^{+} ions by Ca^{2+} ions in the NaTMP structure may have positively contributed to dentin biomineralization; as many forms of biomineralization have been identified with calcium phosphate precursors [He et al., 2019; Di Foggia et al., 2019]. Furthermore, it is widely recognized that the collagen matrix serves as a framework for crystal deposition but does not provide a mechanism for hydroxyapatite nucleation [Saghiri et al., 2022]. On the other hand, non-collagenous proteins of the extracellular matrix, such as DMP1 and dentin phosphoprotein (DPP, DMP2), are believed to play a crucial role in controlling mineral/apatite nucleation and growth during the dentin biomineralization process [Cao et al., 2013; Boukpessi et al., 2008]. The groups treated with calcium trimetaphosphate, especially the more concentrated ones, likely facilitated greater expression of these proteins, as these proteins have a high affinity for calcium ions and interact with collagen fibrils, presenting highly phosphorylated serine and threonine residues [He & George, 2004].

It is known that the dentin remineralization process also involves the growth of residual inorganic crystals within the lesions [Saghiri et al., 2022]. A previous study demonstrated that collagen phosphorylation using sodium trimetaphosphate, combined with treatment with saturated $Ca(OH)_2$, can promote remineralization of type I collagen found in eggshell membrane [Zhang et al., 2010]. Collagen phosphorylation in dentin lesions aims to mimic the role of non-collagenous proteins in nucleation during the dentin biomineralization process. Additionally, as previously reported, pretreatment with $Ca(OH)_2$ promotes conditions conducive to mineral crystal growth [Di Foggia et al., 2019]. Thus, it is assumed that changes in the bonds between NaTMP molecules increased the ability of CaNaTMP to reduce acid penetration into dentin and mineral loss (Tables 1 and 2), and favored a greater presence of amorphous material in dentin tissue after pH cycles. Overall, the current results show that conventional fluoride toothpaste alone is less effective in repairing dentin caries lesions compared to toothpastes containing CaNaTMP. Thus, the benefits of conventional fluoride toothpaste [Leal et al., 2020] may be insufficient for patients at high risk of developing or with active dentin caries. It is also relevant to emphasize the anticaries potential of CaNaTMP-based toothpastes for reversing active dentin lesions. This is significant information, as its use may also represent a cost-effective strategy compared to the continuous use of high fluoride content toothpastes.

Although the present *in vitro* study was conducted in a controlled and standardized environment, and biomimetic remineralization methods offer the potential for treating carious dentin, it is important to note that laboratory conditions differ significantly from the complexity of the in *vivo* environment. Furthermore, it does not address the impact of saliva on the interaction of CaNaTMP with dentin. This data would be interesting, given that the adsorption of proline and tyrosine-rich proteins (acquired pellicle) by dentin has the ability to bind to calcium and phosphate from saliva [Buzalaf et al., 2012]. Additionally, the presence of the pellicle and dental biofilm should be considered, as they may represent obstacles to the effectiveness of dentin remineralization. Therefore, the results should be evaluated with caution, advocating for *in situ* caries study models and clinical studies to elucidate their effect on the remineralization-des-remineralization process.

5. *Conclusion*

The study concluded that toothpaste containing 0.5% and 1% CaNaTMP resulted in a greater remineralizing capacity of artificial dentin caries lesions, suggesting that they could be an effective therapeutic alternative for improving oral health in individuals, especially those with high caries risk and/or activity.

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