EFFECT OF DUCK EGGSHELL NANO-CALCIUM CARBONATE AND PHOSPHOPEPTIDE-AMORPHOUS CALCIUM PHOSPHATE CASEIN ON RE-MINERALIZATION OF DECIDUOUS ENAMEL SURFACE

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ABSTRACT

Introduction: Dental caries has been the most prevalent disease in humans, particularly in children.

Objectives: This study aimed to determine the difference between duck eggshell's nano-calcium carbonate (NCDe) and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) in increasing enamel re-mineralization.

Material and methods: 5% and 10% NCDe were obtained from 50 g of thick NCDe; then, 2.5 g were taken with 2.5 g of carboxymethyl cellulose (CMC) and 45 ml of distilled water to obtain 5% NCDe gel. Twenty-four primary maxillary first incisor teeth were divided into four groups, i.e., (1) negative control, (2) positive control (10% CPP-ACP), (3) 5% NCDe, and (4) 10% NCDe. Each tooth sample was measured for the enamel surface's micro-hardness using Vickers hardness test. Meanwhile, the enamel surface morphology was captured by means of electron microscopy scanning with magnification of 5,000×.

Results: The average micro-hardness of the enamel in the 10% NCDe and 10% CPP-ACP groups were 343.66 HV and 305.28 HV, respectively. In addition, the 10% NCDe group showed the lowest enamel porosity with strongest positive correlation between treatments' duration, with an increase in enamel re-mineralization. In terms of statistical data, the correlation and linearity parameters for the 10% NCDe group were .800 and 64.1%, respectively.

Conclusions: The 10% NCDe group tended to be more effective than 5% NCDe and 10 CPP-APP groups in increasing re-mineralization of tooth enamel. Therefore, it was confirmed that NCDe 10% group has met the criteria as an excellent agent to re-mineralize primary teeth enamel.

Key words: hardness, calcium carbonate, tooth re-mineralization, dental enamel.

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INTRODUCTION

Dental caries has been the most prevalent disease in humans, particularly in children. According to the World Health Organization (WHO) in 2016, 60-90% of school-aged children in the world experience dental caries [1, 2]. Data from the Ministry of Health of the Republic of Indonesia in 2018 show that 93% of children in early child-

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hood in Indonesia experience caries [3]. One of the high percentage mineral content of tooth enamel is calcium. Dental caries can be inhibited by preventing a de-mineralization process from occurring through a re-mineralization process. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a well-known dental material that can prevent de-mineralization of teeth. It contributes to decrease in progression of carious lesions on the enamel, and has a significant additive effect that slows down de-mineralization process [4]. Amorphous calcium phosphate in CPP-ACP undergoes deposition on the surface and in inter-prismatic cracks of enamel [5, 6].

Other sources of calcium that can be easily found are eggshells. Eggshells are a rich source of calcium, which contains calcium carbonate (94%), calcium phosphate (1%), and organic material (4%) [7]. By stimulating chondrocyte differentiation and cartilage formation, eggshell powder can be utilized in-vitro to improve bone mineral density [8, 9]. Due to its' biocompatibility, lack of risk of disease transmission, convenience of use, boundless accessibility, price, performance, and capabilities to be prepared efficiently and profitably, hydroxyapatite derived from eggshells is a versatile new regenerative substance that can be utilized as a bone graft substitute. The minerals in eggshell powder penetrate into the superficial layer and impede the surface porosities when they come into contact with lesions on the enamel that resemble caries [10-12]. As a re-mineralizing agent, the eggshell powder can be used in preventive dentistry as a supplement or a substitute for fluoride [12, 13].

The highest calcium levels are found in duck eggshell flour, with calcium levels reaching 10.11% [14]. One of the most commonly applied calcium derivatives is calcium carbonate ($CaCO_3$), which shows a significant increase in minerals and re-mineralization of caries lesions [15]. Current scientific and technological developments have evolved and can convert $CaCO_3$ into nano-$CaCO_3$ [16]. When compared to micrometer-scale particles, nano-particles can increase their surface area by up to hundreds of times, which increases the material's capacity to adhere to other substances [17, 18].

OBJECTIVES

The goal of this study was to see how the nano-calcium carbonate (NCDe) in duck eggshell differed from CPP-ACP in terms of promoting enamel re-mineralization. In this study, we hypothesized that nano-$CaCO_3$ duck eggshell and CPP-ACP tend to have different effects on micro-hardness due to de-mineralization of the primary maxillary first incisors.

MATERIAL AND METHODS

This work employed a true laboratory experimental design with a pre-test/post-test control group design. Samples used were primary maxillary incisors extracted due to persistence. Primary incisors are teeth that fall out faster than the other teeth. Therefore, it was assumed that the anatomy and structure of the teeth were not damaged or deformed, and the initial homogeneous surface micro-hardness of the enamel could be obtained. Exclusion criteria were as follows: (1) teeth clinically diagnosed with caries, (2) clinically abraded (non-carious lesions due to mechanical wears of tooth), (3) clinically eroded (non-carious lesions due to chemical factors), (4) fracture, (5) restored teeth, and (6) anomaly. The sample size was determined using Federer formula: $(t - 1)(n - 1) \geq 15$, where n is the sample size for each group, and t is the number of groups, and 6 samples for each group were obtained [19]. There were four sample groups consisting of untreated negative control (C–), positive control (C+) applied 10% CPP-ACP, treatment group 1 with 5% NCDe, and treatment group 2 with 10% NCDe gel. Therefore, there were 24 primary maxillary first incisor teeth in total.

SAMPLE PREPARATION

Teeth were stored and immersed in 0.9% NaCl solution. Before treatment, teeth were rinsed with distilled water and dried with an air blower. The surface of the sample was cleaned with pumice. Preparation of duck eggshell powder was done by washing the duck eggshells and boiling them for 15 minutes, then putting them in the oven for 2 hours at 60°C. Afterward, it was milled in a blender and sieved with a 200-mesh sieve to obtain a fine powder of duck eggshells, which contained 94% $CaCO_3$ [20, 21].

PREPARATION OF DUCK EGGSHELL CALCIUM-CARBONATE NANO-PARTICLES

In generating NCDe, 200 g of $CaCO_3$ powder of duck eggshell (CDe) was required. About 2/3 of the CDe powder was taken and then smeared into the inside of the high energy milling (HEM) tube. The aim was to avoid eroding iron layer (Fe) inside the high energy milling tube during milling, so that Fe would not be mixed with NCDe powder. After CDe was inserted into HEM tube, milling was carried out with a span of 20, 24, 30, and 48 hours. To obtain a particle size below 100 nm, the milling results were carried out ultrasonically. Before the ultrasonic process, the milled powder was dissolved in 99.8% isopropanol as a medium for wave propagation to transmit energy to destroy the powder particles, so that their size was getting smaller. Ultrasound was performed at several different times for 30 minutes, 1 hour, and 2 hours. The powder was dried and then placed on a petri dish covered with a tissue at the bottom; then, the size of particles was measured using particle size analysis (PSA) [22]. The outcome used as a research sample was called NCDe powder.

PREPARATION OF DUCK EGGSHELL CALCIUM CARBONATE NANO-PARTICLES GEL

100 g of milled NCDe powder was dissolved in 1.2 l of 96% ethanol, stored for 48 hours, stirred using a stirrer, and concentrated with a rotary evaporator at 50°C for 1 hour until a thick NCDe was obtained and weighed to obtain 50 g of NCDe. A 2.5 g of thick NCDe (5% of 50 g) and 2.5 g of carboxymethyl cellulose (CMC) (5% of 50 g) as well as 45 ml of distilled water were taken to obtain 5% NCDe gel. To get 10% NCDe gel, 5 g of NCDe was needed (10% of 50 g), with 2.5 g of CMC (5% of 50 g) and 42.5 ml of distilled water. The addition of CMC mixed with distilled water was added to make a thick NCDe turn into NCDe gel.

ENAMEL HARDNESS TEST

Twenty-four labial surfaces of primary maxillary first incisors were implanted in acrylic resin. The labial part was planted into a 1 cm diameter polyvinyl chloride (PVC) pipe using acrylic resin. This procedure stabilized the position of samples when measured. The surface of the sample was cleaned with a brush. Before being treated, each tooth sample was evaluated for enamel surface's micro-hardness using Vickers hardness tester. The tip of the diamond indenter was pressed against the sample's surface with a load of 300 g for 10 seconds. Test loads of 300 g was chosen in this study, because it has been used in a number of previous studies. A higher load may be impractical for a softer surface in pre-post experiment because, after the treatment, it produces a larger impression than the optical microscope can measure [23]. Hardness was measured in the middle area. Penetration results were observed through a microscope lens with a magnification of 400×, revealing a pyramidal shape. Then, two lines were placed at the end of pyramid formation on Vickers micro-hardness tester tool to measure the diagonal length produced by the indenter. Then, the read button was pressed, and the results of surface hardness with units of Vickers hardness (HV) were revealed. The initial enamel hardness range in this study was 247.8-432.3 HV. The samples were immersed in a de-mineralization solution of 2.2 mM/l $CaCl_2$, 2.2 mM/l KH_2PO_4, and 50 mM acetate buffer for 1 hour, with a pH of 4.06 [24, 25]. The samples of incisors were divided into 4 groups using the sample random sampling method, including a negative control group C– (without treatment), positive control C+ (with 10% CPP-ACP0 application), treatment 1 (with 5% NCDe application), and treatment 2 (with 10% NCDe application). The materials were applied to the surface of the enamel using a tip applicator. The four groups' samples were placed in different petri dishes openly, and left to stand for 5 minutes and 10 minutes. Their hardness was measured again at the 5th and 10th minutes, like the previous method of measuring enamel hardness. Provision of de-mineralized material was made prior to administration of re-mineralization material C+ and NCDe to determine the increase in micro-hardness of the first primary maxillary teeth enamel surface, which was decreased due to de-mineralization [26].

A scanning electron microscope (SEM) with a magnification of 5,000× was applied to observe each sample group's enamel surface morphology, and to analyze mineral de-mineralization and re-mineralization on the enamel surface. The sample to be tested for SEM musted to have a size that follows the capacity of standard SEM/calibration tool. Each sample was coated with liquid gold (5-20 nm). Then, the coated sample was placed in a vacuum chamber right in the center. The appliance was powered at 20 kV. The sample image would be seen on a monitor, and the sample was controlled from the outside to be shifted to obtain the area photographed [27].

In this study, the researchers employed a paired *t*-test and one-way analysis of variance (ANOVA) for data analysis. A paired *t*-test was applied to analyze enamel micro-hardness differences of samples in each group, before and after the treatment (at 5 and 10 minutes). While one-way ANOVA was performed to analyze the enamel micro-hardness differences of the samples between groups at 5 and 10 minutes of treatment.

RESULTS

Based on the nano-particle size analysis (Nano-PSA) test (Figure 1), it was observed that the average size of the NCDe powder was 41.83 nanometer (nm), with the highest peak at 27.49 nm. In Figure 2, the results of SEM showed that the 10% of NCDe group had the lowest porosity after treatment compared with the 5% NCDe, 10% CPP-ACP, and negative control (C–) groups at the 5th and 10th minute measurements.

According to the results of a paired t-test, there was a significant difference in enamel micro-hardness between the treatments C+ 10 min group and the de-mineralized group (p = 0.016) and the C+ 5 min group (p = 0.039). In the 5% of NCDe group measurements, there was no significant difference (p = 0.089). However, it was significantly different from the de-mineralization group at 5th minute (p = 0.014) and 10th minute (p = 0.011). The 10% NCDe group treatments at 5th and 10th minutes had no significant difference (p = 0.065), but significantly different from the de-mineralization group at 5th minute (p = 0.011) and 10th minute (p = 0.008). Meanwhile, in the group C–, there was no significant difference at the 5th and 10th minutes (Figure 3).

The average micro-hardness value of the enamel surface after 5 minutes in the C+, 5% NCDe, and 10% NCDe treatment groups were 290.10 HV, 308.78 HV, and 324.66 HV, respectively. On the other hand, the negative control C– group showed micro-hardness of 278.64 HV. At 10 minutes, the treatment group C+, 5% NCDe, and 10% NCDe presented micro-hardness of 305.28 HV, 315.78 HV, and 343.66 HV, respectively. In the control group C–, micro-hardness was 278.84 HV. One-way ANOVA showed that at 5 minutes, there was no significant difference between the three treatment groups, except between the 10% NCDe treatment and C–, which differed significantly ($p = 0.021$). At 10th minute measurement, there was a significant difference between the 5% and 10% NCDe treatments groups with the C– group ($p = 0.045$; $p = 0.018$). The 10% NCDe treatment group differed significantly with the C+ group ($p = 0.036$). Meanwhile, in the treatment group C+ with 5% NCDe and 5% NCDe with 10% NCDe, there were no significant differences seen (Figure 4).

Pearson's correlation test results (Table 1) showed a significant positive correlation between the testing material and the level of re-mineralization. The positive correlation showed that the longer the time for the 10% CPP-ACP, 5% NCDe, and 10% NCDe on the tooth enamel surface, the better the re-mineralization effect detected. The highest correlation coefficient value occurred in the 10% NCDe treatment group ($r = 0.800$, $p = 0.000$).

In the positive control C+ 10% group, the regression coefficient was positive, which was 3.480. It implied that each additional time of 1 minute would increase re-mineralization by 3.480 ($p = 0.040$). The R^2 value was 28.6% for the C+ 10% group, indicating that the length of time for the C+ 10% application contributed 28.6% to the increase in re-mineralization. For the 5% NCDe group, the regression coefficient was positive, i.e., 3.666. Each additional 1 minute on 5% NCDe can increase re-mineralization by 3.666 ($p = 0.011$). The R^2 value was 40.2%, indicating that the 5% NCDe group contributed 40.2% to the increase in re-mineralization. For the 10% NCDe group, the regression coefficient was 6.670 ($p = 0.000$). From its' R^2 value, it was revealed that the 10% NCDe group contributed 64.1% to the re-mineralization increase (Figure 5).

DISCUSSION

The mineral content of enamel can be calculated indirectly by measuring micro-hardness of the enamel surface [28]. In addition to the measurement of micro-hardness of the enamel surface, inhibition of de-mineralization in tooth enamel can be evaluated by analyzing the surface morphology of tooth enamel, with scanning electron microscope (SEM) testing [29]. SEM results depicted that the 10% NCDe treatment group had the lowest porosity. The reduced enamel porosity indicates a potential for re-mineralization on the enamel surface [30]. Enamel re-mineralization is essential for teeth to reduce dental caries because teeth cannot re-mineralize, hence re-mineralization materials are needed. In this case, calcium carbonate nano-particles can reduce the caries score by 31.1% [16, 31]. This study proved that 10% NCDe could be a re-mineralizing agent after de-mineralization of tooth enamel. In an acidic environment, CPP-ACP would buffer plaque pH to

Results

			Size (d.n...)	% Number	SD (d.n...)
Z-average (d.nm):	**41.83**	**Peak 1:**	27.49	100.0	7.032
PdI:	**0.269**	**Peak 2:**	0.000	0.0	0.000
Intercept:	**0.883**	**Peak 3:**	0.000	0.0	0.000
Result quality:	**Refer to quality report**				

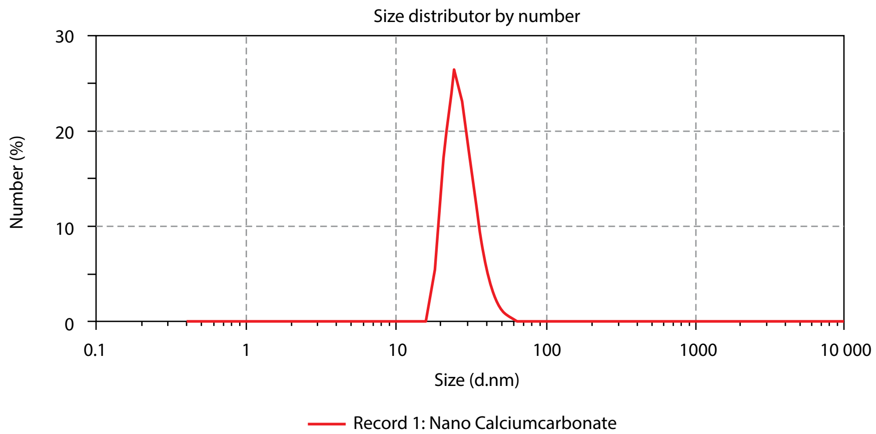


FIGURE 1. Nano-PSA result of NCDe powder (41.83 nm)

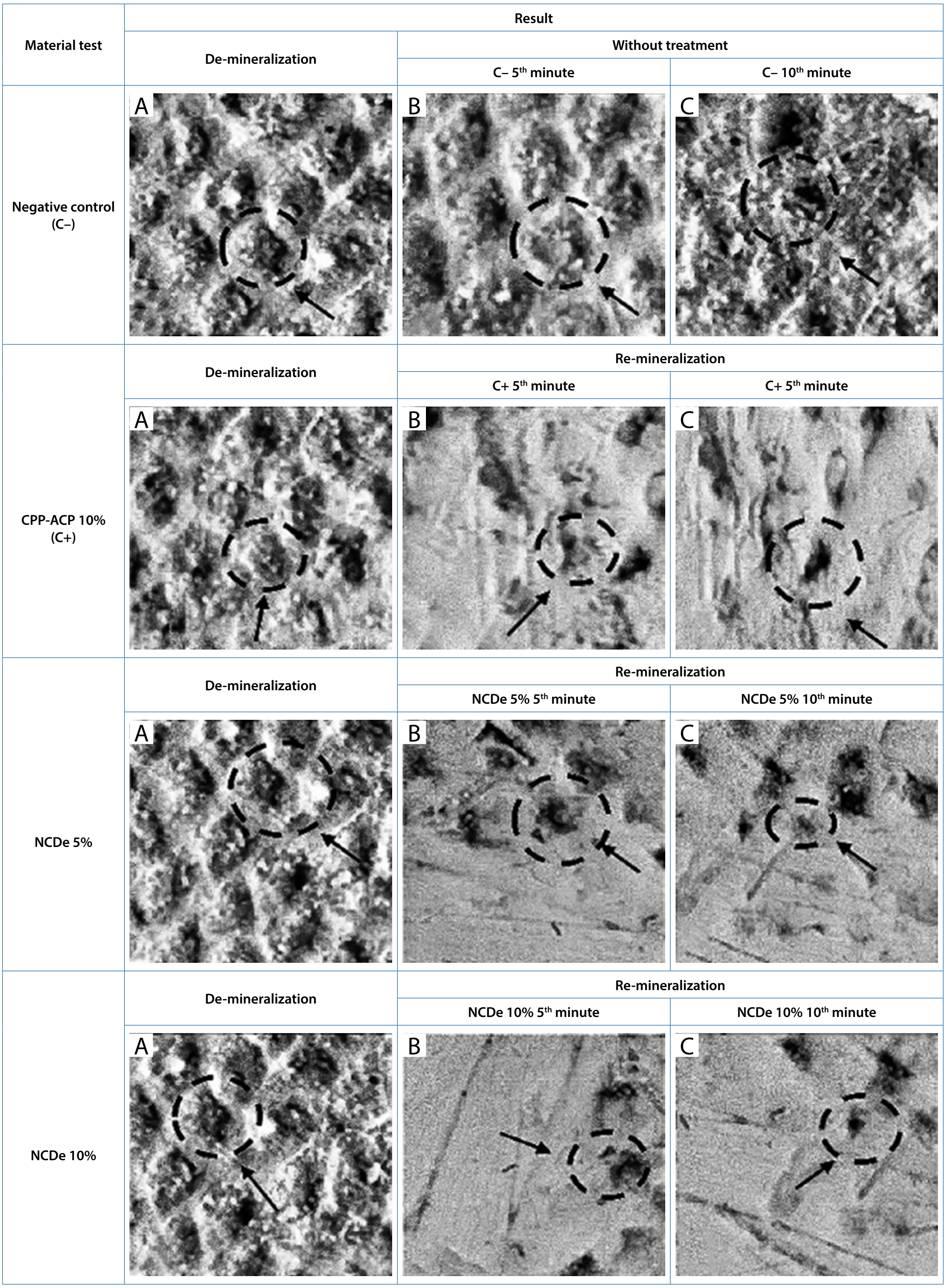
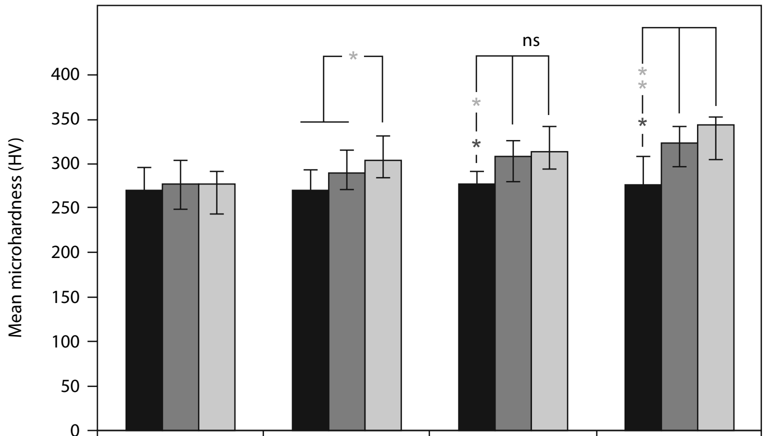


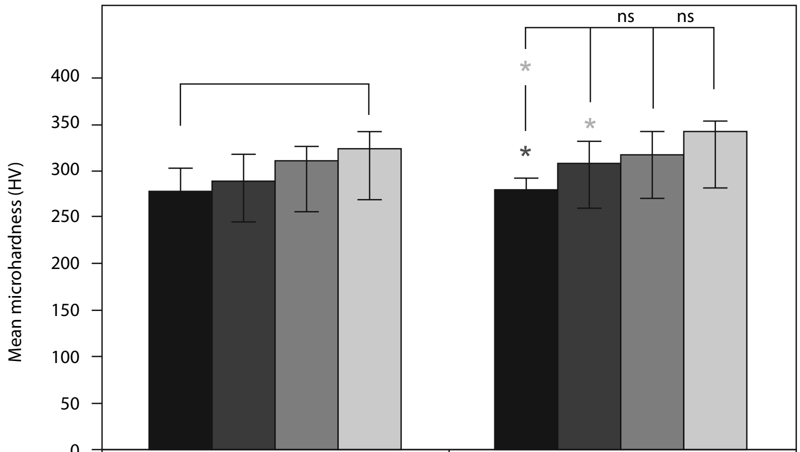
FIGURE 2. Scanning electron microscopy images of the enamel surface morphology before (de-mineralization) and after (re-mineralization) treatment in (1) negative control (C–), (2) 10% CPP-ACP, (3) 5% NCDe, and (4) 10% NCDe 10% groups



	C–	C+	NCDe5%	NCDe10%
De-mineralization	270.9600	270.4800	279.1200	276.9600
Re-mineralization 5th minute	278.6	290.1000	308.7800	324.6600
Re-mineralization 10th minute	278.8400	305.2800	315.7800	343.6600

**p < 0.05, **p < 0.01, ns – not significant*

FIGURE 3. Mean differences in enamel micro-hardness before (de-mineralization) and after treatment (re-mineralization) with 10% CPP-ACP (C+), 5% NCDe, and 10% NCDe



	Re-mineralization 5th minute	Re-mineralization 10th minute
C–	278.6400	278.8400
C+	290.1000	305.2800
NCDe5%	308.7800	315.7800
NCDe10%	324.6600	343.6600

FIGURE 4. Mean differences in enamel micro-hardness after treatment (re-mineralization) with 10% CPP-ACP (C+), 5% NCDe, and 10% NCDe at 5th and 10th minutes

inhibit de-mineralization, but has a short-term re-mineralization effect [32,33]. In this study, 10% CPP-ACP, 5% NCDe, and 10% NCDe presented an increasingly long-term effects. The re-mineralization increased for a long time (Table 1). However, de-mineralization still occurred in all groups marked by porosity (Figure 2). The 10% NCDe application duration contributed 64.1% to the increase in re-mineralization compared to C+ 10% and 5% NCDe (Figure 5). The 10% NCDe had the potential to be a re-mineralizing agent after de-mineralization of primary tooth enamel. The mean occlusal micro-hard-

TABLE 1. Correlation between material test and re-mineralization

Material test	Re-mineralization at 5th and 10th minutes	
	r-value	*p*-value
CPP-ACP 10%	0.535 (strong enough)	0.040
NCDe 5%	0.634 (strong)	0.011
NCDe 10%	0.800 (very strong)	0.000

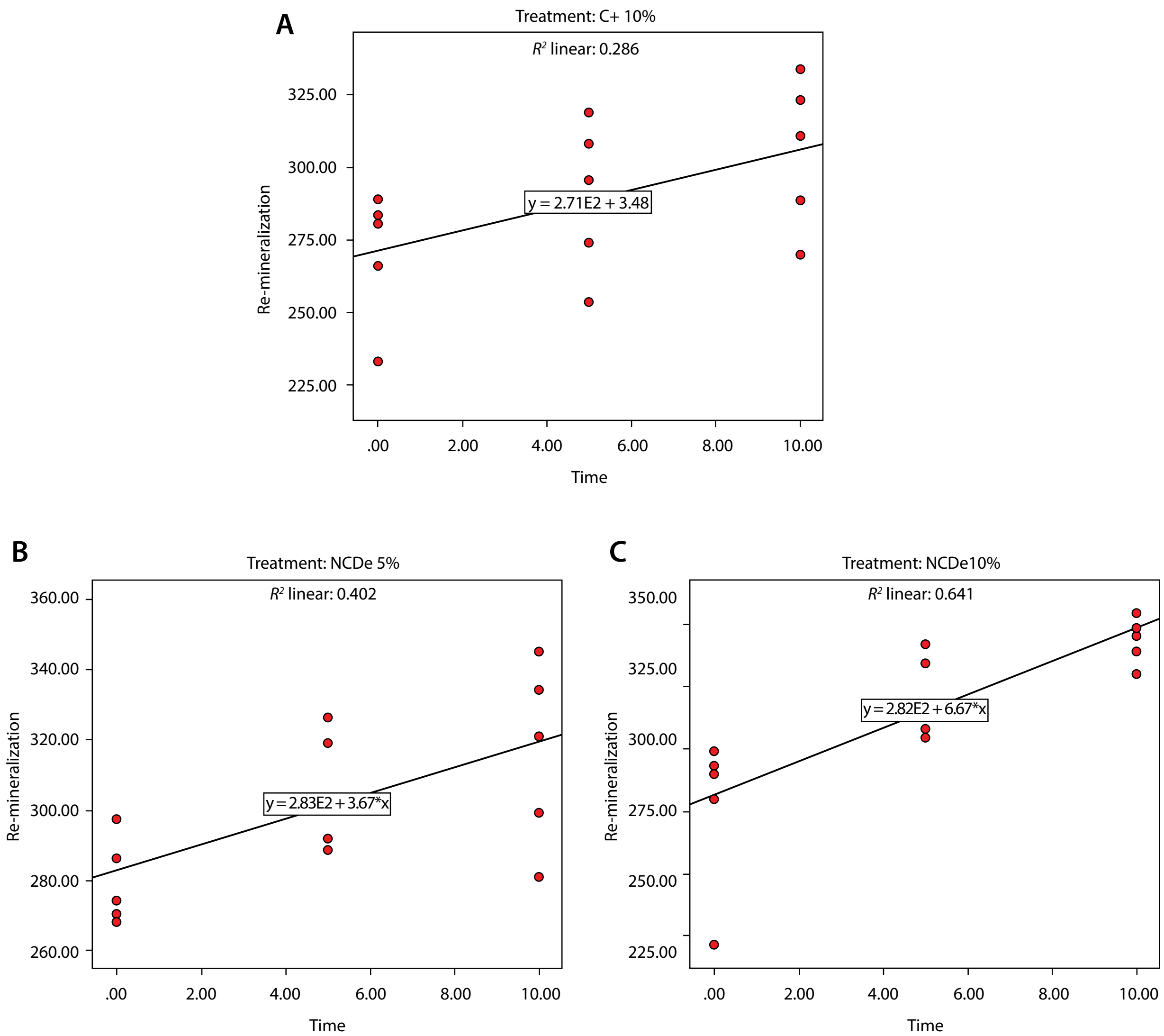


FIGURE 5. Linearity trends for (**A**) C+ 10%; (**B**) 5% NCDe; (**C**) 10% NCDe

ness of enamel was between 359 and 424 VHN (HV), and in the cervical region ranging from 227 to 342 VHN (HV). This variation is caused by several factors, including specimen preparation prior to hardness measurements, reading errors, histological features, chemical composition, and indentation length (IL) [34]. The recommended range of enamel micro-hardness is 227-424 HV [35]. Furthermore, the average micro-hardness values of enamel surface at minute 10 for 10% NCDe treatment showed the highest value as compared with 10% CPP-ACP and 5% NCDe treatment groups.

The main factor that causes treatments with NCDe to performed low porosity and high mean micro-hardness of the tooth enamel surface, is its' particle's size. Studies have confirmed that nano-scale particles can easily penetrate cell membranes, and therefore they can interact with more biological systems [36, 37]. In addition, the NCDe can be used at a sub-cellular scale, with high accuracy in reaching cellular targets and obtaining maximum therapeutic effects.

As a recommendation for future research, the sample's initial hardness range should be reduced, so that it becomes more homogeneous, providing more accurate results. Post-test (re-mineralization) and pre-test (demineralization) micro-hardness measurements need to be considered to minimize bias and improve results' accuracy [18].

CONCLUSIONS

Re-mineralization at the 5th and 10th minutes and the mean micro-hardness of the tooth enamel surface after treatment with 10% NCDe were higher than in groups with 10% CPP-ACP and 5% NCDe. The 10% NCDe treatment group was much more effective than the 10% CPP-ACP and 5% NCDe groups in enhancing the re-mineralization of primary maxillary first incisor enamel. It resulted in the lowest enamel degree porosity. The 10% NCDe group had the strongest positive correlation between the treatments' duration and the increase in enamel re-mineralization ($r = 0.800$, very strong), having the best linearity concerning time. Therefore, the 10% NCDe is proven effective in increasing tooth enamel re-mineralization, and it has the potential as an agent to re-mineralize primary teeth enamel.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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