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Original Article

DIFFERENCES IN THE EFFECT OF APPLYING 6% AND 12% HYDROXYAPATITE PASTE FROM DUCK EGGSHELLS (*ANAS PLATYRHYNCHOS*) WITH *CASEIN PHOSPHOPEPTIDE-AMORPHOUS CALCIUM PHOSPHATE* (CPP-ACP) AS REMINERALIZING MATERIALS ON ENAMEL SURFACE HARDNESS (*IN VITRO*)

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ABSTRACT

Objective: Dental caries has been the biggest issue in oral health resulting from the demineralization process. Demineralization can reduce the hardness of the tooth enamel surface, causing caries. The purpose of this research was to assess the difference in the effect of applying 6% and 12% hydroxyapatite paste from duck eggshells (*Anas platyrhynchos)* with casein phosphopeptide amorphous calcium phosphate (CPP-ACP) as remineralizing materials on enamel surface hardness.

Methods: This laboratory experimental research used 30 extracted maxillary first premolars as samples, divided into 5 groups. Samples in group I were demineralized and applied with 6% hydroxyapatite paste; group II were demineralized and applied with 12% hydroxyapatite paste; group III were demineralized and applied with CPP-ACP; group IV were demineralized and not given any remineralizing materials; group V were not demineralized and not given any remineralizing materials. The demineralization procedure was done with hydrochloric acid (HCl) solution for 30 min. The remineralization procedure was done for 30 min. All samples were immersed in artificial saliva. This procedure was repeated every day for 14 consecutive days. The analysis of enamel surface hardness was done twice by Microvickers Hardness Tester, the first was after demineralization and second after remineralization.

Results: T-paired test result showed an increase in enamel surface hardness after application of the test materials in each group ($p<0.05$). The result of the one-way ANOVA test showed the application of 6% and 12% hydroxyapatite paste from duck eggshells showed a more significant increase in enamel surface hardness than CPP-ACP paste. Although the numerical value of 12% was higher than 6%, there was no significant difference between the two based on the results of the Post-hoc Bonferroni test ($p=1$).

Conclusion: The application of 6% hydroxyapatite paste, 12% hydroxyapatite paste, CPP-ACP and control group resulted in an increase of enamel surface hardness. The application of 6% and 12% hydroxyapatite paste from duck eggshells showed a significant increase in enamel surface hardness compared to CPP-ACP.

Keywords: Duck eggshells, Remineralization, Hydroxyapatite, Microvickers hardness tester

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INTRODUCTION

Dental caries has been the biggest issue in the field of oral and dental health. World Health Organization (WHO) in 2022 estimated that around 2 billion people suffer from caries of permanent teeth [1]. According to Indonesia Basic Health Research (RISKESDAS) in 2018, the prevalence of dental caries in Indonesia was 88.8%, among which 45.3% experienced cavities but only 4.2-4.4% were restored [2]. These numbers showed that dental caries was common, but the effort in its treatment was still very low.

The caries balance theory stated that the demineralization process was initiated when pathological factors predominated the protective factors [3]. Biofilms are communities of microorganisms that grew and adhered to the tooth surface. The cariogenic bacteria which are actively involved in the initiation of caries are *Streptococcus mutans*. This bacterium metabolizes salivary proteins and glycoproteins into carbohydrates, peptides and amino acids as its main source of growth [4, 5]. This process will produce acid and, as a result, lower the pH in the oral cavity. The acid will induce the dissolution of mineral ions, including hydroxyapatite crystals, in a process called demineralization. That leads to dissolution of tooth enamel structure and will lower the enamel surface hardness value [4, 5]. However, this can be inhibited using remineralizing material such as CPP-ACP, which contains casein phosphoprotein, calcium and phosphate [6].

CPP-ACP is widely employed as a positive control in remineralization studies due to its proven ability to promote mineral deposition in early carious lesions. CPP-ACP stabilizes calcium and phosphate ions through complexation with casein phosphopeptides, maintaining their bioavailability in saliva and enhancing their deposition into demineralized enamel surfaces [7]. Randomized clinical trials have demonstrated that CPP-ACP exhibits comparable remineralizing efficacy to fluoride and other agents, particularly in the management of post-orthodontic white spot lesions [8, 9].

Clinically, CPP-ACP has been shown to effectively manage white spot lesions, reduce dentin hypersensitivity, and aid in the prevention of early caries, particularly in paediatric and orthodontic populations [8]. Nevertheless, CPP-ACP presents several no limitations. Derived from milk protein, it is unsuitable for individuals with dairy allergies or those adhering to vegan lifestyles [7]. Furthermore, it lacks intrinsic antibacterial activity against cariogenic biofilms, and its efficacy may be compromised in highly acidic oral environments [7]. The relatively high cost and limited availability of CPP-ACP based products in certain regions also restrict their accessibility. These limitations underscore the need to explore alternative, natural-based remineralizing agents that are more affordable, sustainable, and possess multifunctional properties, including antimicrobial activity. One of the food wastes by products that can be repurposed in the medical field is duck eggshells. Various studies have reported

that duck eggshells possess potential benefits as a remineralizing agent for dental applications.

Hydroxyapatite compound as remineralizing material can be obtained from natural sources that contain calcium carbonate ($CaCO_3$), among which are the shells of duck eggs. It contains 94% $CaCO_3$, 1% magnesium carbonate ($MgCO_3$), 1% calcium phosphate ($CaPO_4$), and 4% organic matter [10]. Based on the study by Asmeati, *et al.* (2022), X-ray fluorescence (XRF) analysis of duck eggshell powder revealed a calcium oxide (CaO) content of 99.50% and an elemental calcium content of 99.71% [10]. According to Buasri, *et al.* (2013), X-ray diffraction (XRD) analysis showed that calcined duck eggshells contained 98.925 wt.% of CaO, which was higher than the 98.124 wt.% found in chicken eggshells [11]. Similarly, Seesanong, *et al.* (2024) reported that XRF analysis identified a higher CaO content in duck eggshells (97.4 wt.%) compared to chicken eggshells (97.0 wt.%) [12]. These findings indicate that duck eggshells contain a higher amount of calcium (in the form of CaO or elemental Ca) and exhibit greater mineral purity than chicken eggshells. Therefore, duck eggshells represent a superior natural calcium source for applications such as hydroxyapatite synthesis in dental medicine. The high content of calcium carbonate in duck eggshells can be utilized as an alternative for tooth remineralizing material. Haghgoo, *et al.* (2016) stated that 3% hydroxyapatite from duck eggshells can increase the enamel surface hardness [13]. Concentration is a key factor that influences the effectiveness of a material. Based on general principles observed in chemical kinetics, the effect of increasing a substance's concentration can vary. When the concentration of a substance is doubled, its effect may remain unchanged (zero order), increase proportionally (first order), or have more than a proportional effect (second order), depending on the mechanism of interaction. In this study, the concentration of duck eggshell derived hydroxyapatite was increased twofold to observe whether a higher concentration would result in greater improvement in enamel surface hardness. This comparison is expected to provide insight into the optimal hydroxyapatite content for potential biomedical or material science applications. Based on the above description, the researchers are interested in analysing the effect of applying 6% and 12% hydroxyapatite paste from duck eggshells compared to CPP-ACP as remineralizing material on the enamel surface hardness.

MATERIALS AND METHODS

The research design was laboratory experimental research using pre and post control groups to evaluate the significance between the demineralized and after the application of experimental materials. This research had followed the research ethics from The Health Research Ethics Committee, Faculty of Medicine, Universitas Sumatera Utara 1032/KEPK/USU/2023. The samples consisted of 30 extracted human maxillary first premolars that had been extracted for orthodontic purposes and obtained from several public health centers and dental clinics in Medan City. Inclusion and exclusion criteria were applied to ensure consistency and reliability of the samples. Teeth included in the study were non-carious, had intact crowns, no visible fractures, and no previous restorations. Teeth were excluded if they showed signs of attrition, erosion, or abrasion; exhibited white spot lesions; or presented any degree of fluorosis. After extraction, the teeth were thoroughly cleaned of soft tissue residues using a scaler and rinsed with distilled water. They were then stored in phosphate buffered saline (PBS) at pH 7.2-7.4 at 4 °C to prevent dehydration and maintain their physiological condition until further processing. PBS was chosen as the storage medium because it maintains pH stability and mimics physiological conditions without altering the chemical structure of enamel or dentin [14]. Each sample was numbered and divided into five groups. Demineralization was carried out by immersing the specimens in 0.1 M HCl solution at pH 2.3 for 30 min.

Group I was demineralized and then applied with 6% hydroxyapatite paste from duck eggshells. Group II was demineralized and then applied with 12% hydroxyapatite paste from duck eggshells. Group III was demineralized and applied with CPP-ACP. Group IV was only demineralized and not applied with experimental material. Group V was not demineralized and not applied with experimental material. Demineralization process was using HCl solution for 30 min and remineralization process was done in a 30 min duration. All of the samples were immersed in artificial saliva to resemble the oral cavity. The artificial saliva was renewed every 24 h. Its preparation was carried out based on the Fusayama Meyer's formulation [15]. The composition of the artificial saliva is presented in the following table 1.

Table 1: Component of the fusayama meyer's artificial saliva

Component	Concentration: g/l
Sodium chloride (NaCl)	0.4
Potassium chloride (KCl)	0.4
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.795
Monosodium phosphate dihydrate (NaH_2PO_4)	0.78
Sodium sulfide nonahydrate ($Na_2S.9H_2O$)	0.005
Urea ($CO(NH_2)_2$)	1
Hydrochloric acid (HCl) (1M)	until pH 6.8

Duck eggshells were made into hydroxyapatite paste using the wet sedimentation method. A total of 370 g of duck eggshells were cleaned and dried for 2 h in 100 °C. The shells were ground and calcined for 2 h in 900 °C resulting in CaO powder. A 37 g of strained CaO powder was then dispersed in aquadest up to 500 ml volume and then stirred for 1 h at 90 °C temperature. The 1 M calcium hydroxide ($Ca(OH)_2$) solution was dissolved in 0.6 M phosphoric acid (H_3PO_4) drop by drop and then stirred until it reached pH 8.5 using a magnetic stirrer on a hot plate heated to 40 °C for 30 min. The solution was left for 24 h until white sedimentation formed and then strained and washed three times with distilled water. The strained result was dried at 100 °C for 2 h and calcined for 2 h at 900 °C. The resulting hydroxyapatite powder was analyzed with fourier transform infrared spectroscopy (FTIR) characteristic test to confirm the presence of hydroxyl, carbonate, and phosphate groups that indicate hydroxyapatite.

The 6% hydroxyapatite paste from duck eggshells was made by mixing 6 g of hydroxyapatite powder, 5 g of CMC-Na, 1 ml glycerin, and distilled water until the weight of the paste reached 100 g. The 12% hydroxyapatite paste from duck eggshells was made by mixing 12 g of hydroxyapatite powder, 5 g of CMC-Na, 1 ml glycerin, and distilled water until the weight of the paste reached 100 g. The role of each component in the toothpaste formulation is presented in the following table 2.

The test was done in 14 consecutive days and samples were kept in incubator at 37 C to simulate the oral cavity temperature. The enamel surface hardness test was done twice, once after demineralization and second after 14 days of treatment. The measurement was done on three points which represented the upper, middle and lower thirds of the tooth crown. After collection, the data were statistically analyzed with IBM statistical package for the social sciences (SPSS) version 30 program.

Table 2: Components of the hydroxyapatite-based paste and their respective functions

Components	Role
CMC-Na	• Thickening and gelling agent → Enhances the viscosity of the paste, allowing for easy application and prolonged retention on the tooth surface. • Suspension stabilizer → Prevents sedimentation of active particles such as nano hydroxyapatite. • Ion release controller → The gel structure regulates the diffusion of Ca^{2+} and PO_4^{3-} ions from the paste to the enamel surface [16].
Glycerin	• Humectant → Retains moisture within the paste and prevents drying during storage. • Binding agent → Provides a smooth and homogeneous consistency [17].
Aquadest	• Primary solvent: Aquadest is the foundational medium in toothpaste, enabling uniform dispersion and hydration of all hydrophilic components including CMC-Na (thickener), humectants, surfactants, and active ingredientsthus ensuring batch to batch consistency and stable texture [18].
Hydroxyapatite	• Active remineralizing agent: Fills the micropores of enamel and dentin caused by demineralization [18].

RESULTS AND DISCUSSION

FTIR analysis was done on duck eggshell powders to identify the hydroxyapatite groups. The result of the FTIR spectrum characterization in fig. 1 showed several functional groups, namely hydroxyl (OH^-), carbonate (CO_3^{2-}), and phosphate (PO_4^{3-}). The results of the test are presented in the following table 3. Hence, FTIR test result showed that the peak wavelengths were inside the specified range, confirming that the powder contained hydroxyapatite compound. Due to instrument availability, XRD and energy-dispersive X-ray spectroscopy (EDX) were not performed. This limitation is acknowledged and will be addressed in future studies.

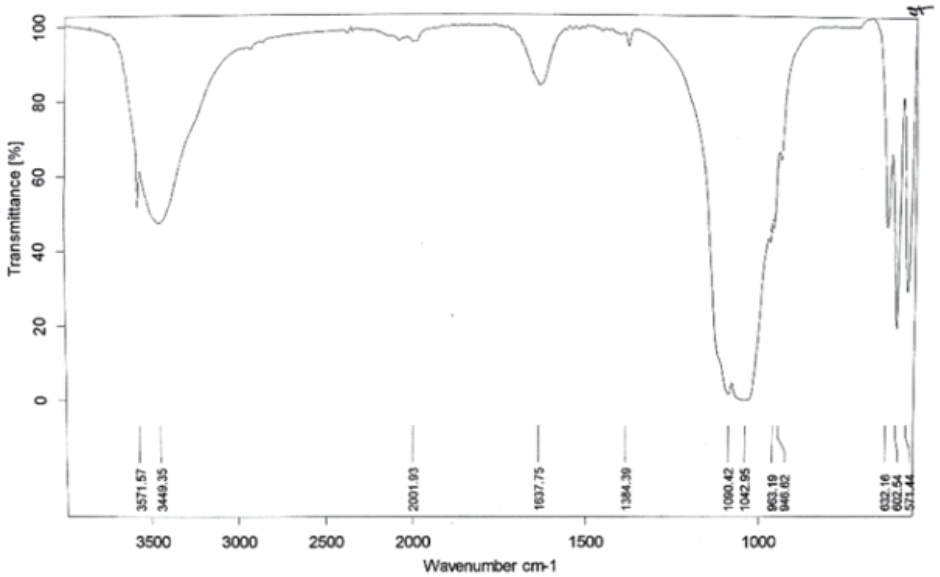

Fig. 1: FTIR characterization result of hydroxiapatite powder from duck eggshells

Table 3: FTIR peak assignments of functional groups

Functional group	Observed peak wavelength (cm^{-1})	Reference range (cm^{-1})	Source
Hydroxyl (OH^-)	3449.35, 3571.57	3300-3600	Batubara *et al.* [19]
Phosphate (PO_4^{3-})	963.19, 1042.95, 1090.42	950-1100	Batubara *et al.* [19]
Carbonate (CO_3^{2-})	1384.39, 1637.75	1384-1637	Rajabiyan *et al.* [20]

The result of enamel surface hardness test after demineralization and after remineralization is shown in table 4, where all the groups experienced an increase of enamel surface hardness. The highest increase in enamel surface hardness was found in Group II (12% duck eggshell paste), while the lowest increase was found in Group V (control). The mean increase of the enamel surface hardness in Group II (12% duck eggshell paste) was higher than Group I (6% duck eggshell paste) and Group III (CPP-ACP). The mean increase of the enamel surface hardness in Group I (6% duck eggshell paste) was higher than Group III (CPP-ACP).

Table 4: The mean value of enamel surface hardness after demineralization and after application of the test material

Group	Enamel surface hardness (HV)		
	After demineralization (Mean±SD)	After remineralization (Mean±SD)	Difference (Mean±SD)
I (Duck Eggshell Paste 6%)	262.56±6.40	337.11±5.30	74.55±8.93
II (Duck Eggshell Paste 12%)	264.60±10.73	345.08±4.44	80.48±7.69
III (CPP-ACP)	268.05±4.87	330.16±3.35	62.11±7.61
IV (Demineralization+artificial saliva)	269.62±12.63	314.9±3.60	45.36±14.08
V(Nondemineralization+artificialsaliva)	316.09±1.37	322.86±2.70	6.77±2.48

HV: Vickers hardness

Data obtained from Microvickers Hardness test were subsequently analyzed using t-paired, Cohen's d, one-way ANOVA followed by Post-hoc Bonferroni test because the data were found to be normally and homogenally distributed. T-paired test to see the difference of the effect before and after treatment on every group. The t-paired test showed a statistically significant increase of enamel surface hardness in all groups with $p<0.05$.

A subsequent analysis, specifically Cohen's d was carried out to improve our recognition of the differences in all groups. The statistic result in table 5, it can be observed that there was an increase in the mean values from the pretest to the post test across all groups. In addition, significance value of 0.000 (<0.05) was obtained for all groups, indicating that the differences between the pretest and post test were statistically significant. Although the p-value indicates statistical significance, it does not reflect the magnitude of the observed effect. Therefore, the effect size is also reported. The Cohen's d values are as follows: group I is 8.930, group II is 7.697, group III is 7.617, group IV is 14.087, and group V is 2.487. The analysis demonstrated a significant increase in enamel surface hardness following hydroxyapatite application. The Cohen's d value exceeding 0.8 indicates a large effect size, suggesting that remineralization with hydroxyapatite has a strong and clinically meaningful impact on enamel hardness.

The difference between before and after application of test material was calculated using one-way ANOVA to see the effect of the treatment in every group. The result was shown in table 6, in which p-value of 0.0001 ($p<0.05$) was found, suggesting a significant difference in the effect of the treatment in all groups. Afterwards, the data was analyzed using Post-hoc Bonferroni test to see which group had the greatest increase in enamel surface hardness. The statistic result in table 6 showed no significant difference between applying 6% and 12% hydroxyapatite paste in the enamel surface hardness test. However, there were significant differences between other groups ($p<0.05$). The most significant difference was found between the 12% hydroxyapatite paste group and control group (non-demineralization) with p-value of 0.0001 and mean difference of 73.70 HV, followed by the 6% hydroxyapatite group and control group (non-demineralization) with p-value of 0.0001 and mean difference of 67.78 HV. The application of 6% and 12% hydroxyapatite paste from duck eggshells increased the enamel surface hardness more significantly than CPP-ACP ($p<0.05$).

Table 5: Cohen's d values for comparing the effects of remineralization treatments on enamel surfaces

Pretest (HV)	Posttest (HV)	Sig.	Cohen's d
262.563	337.118	0.000	8.930
264.603	345.083	0.000	7.697
268.056	330.165	0.000	7.617
269.625	314.990	0.000	14.087
316.096	322.868	0.000	2.487

HV: Vickers hardness

Table 6: The result of one-way ANOVA on the effect of the treatment in each group

Group	n	mean±SD (HV)	p-value
I (6% duck eggshell paste)	6	74.55±8.93	0.0001
II (12% duck eggshell paste)	6	80.48±7.69	
III (CPP-ACP)	6	62.10±7.61	
IV (demineralization+artificial saliva)	6	45.36±14.08	
V (nondemineralization+artificial saliva)	6	6.77±2.48	

*$p<0.05$ = significant with the *one-way* ANOVA test. N=6, HV: Vickers hardness

Table 7: The result of post-hoc bonferroni test on the difference in the increase of enamel surface hardness between groups after treatment

Group	Mean difference (HV)		p
Group I	Group II	-5.92	1.000
	Group III	12.44	0.239
	Group IV	29.19	0.0001
	Group V	67.78	0.0001
Group II	Group I	5.92	1.000
	Group III	18.37	0.016
	Group IV	35.11	0.0001
	Group V	73.70	0.0001
Group III	Group I	-12.44	0.239
	Group II	-18.37	0.016
	Group IV	16.74	0.034
	Group V	55.33	0.0001
Group IV	Group I	-29.19	0.0001
	Group II	-35.11	0.0001
	Group III	-16.74	0.034
	Group V	38.59	0.0001
Group V	Group I	-67.78	0.0001
	Group II	-73.70	0.0001
	Group III	-55.33	0.0001
	Group IV	-38.59	0.0001

*$p<0.05$ = significant with the Post-Hoc Bonferroni test. N=6, HV: Vickers hardness

Demineralization was carried out by immersing the specimens in 0.1 M HCl solution at pH 2.3 for 30 min. This protocol was selected to simulate early enamel erosion under acidic oral conditions. HCl is a strong acid that has been shown to effectively induce surface demineralization by creating interprismatic porosities on enamel surfaces, similar to what occurs in the initial stages of erosive tooth wear. After food or acidic beverage consumption, salivary pH in the oral cavity can drop below the critical pH of enamel (5.5) and may remain acidic (pH 5.5–6.0) for 30 to 60 min before returning to the normal physiological range (pH 6.5–7.5), depending on the individual's salivary buffering capacity. Thus, a 30 min exposure to HCl was chosen to mimic the temporary acidic challenge commonly experienced in the oral environment and to create a standardized demineralized surface for further remineralization testing [21]. As an alternative, lactic acid is a weak organic acid naturally produced by cariogenic bacteria during carbohydrate metabolism that induces demineralization more gradually at a higher pH, closely mimicking the natural progression of dental caries and subsurface lesion formation. Recent studies have demonstrated that lactic acid effectively produces superficial enamel demineralization resembling early caries lesions when applied over extended periods (7 days) with pH levels around 5.4. Therefore, lactic acid offers a physiologically relevant alternative for *in vitro* demineralization protocols, especially in studies focused on early lesion development and remineralization dynamics [22].

This research was carried out with a duration of 30 min duration each day for 14 consecutive days. Nugroho (2021) in his research stated that the application of CPP-ACP and jackfruit seeds (*Artocarpus heterophyllus Lamk*) as remineralization materials for 30 min could increase the enamel surface hardness [23]. It was in accordance with the manufacturer's instructions that by keeping CPP-ACP and tooth enamel in contact for as long as possible in the oral cavity, the diffusion of calcium and phosphate ions to the enamel structure would occur optimally. After application, the patient was recommended not to eat and drink for 30 min. The 14 days duration was based on Mona *et al.* (2023) research which stated that the application of duck eggshell paste for 14 days can increase the enamel surface hardness [24]. This was also in accordance with the Ostwald Ripening Theory that remineralization process which occured in longer duration will produce a larger enamel crystal. In time, the smaller crystals will merge and form a larger crystal until reaching maximal size and more stable, resulting a better remineralization process [25]. Therefore, the enamel surface hardness value will also increase along with the increase of the duration of remineralization material application [26].

Demineralization is the process of dissolution of minerals in hydroxyapatite crystals without breaking the structural integrity, a process which was caused by acid. The acid will diffuse to the hard tissues of the tooth through water in between crystals, reaching the bottom of the apatite crystal surface. When reaching the bottom surface, the calcium and phospate ions will dissolve into the water phase in oral cavity. This demineralization process can be hampered by remineralization materials such as CPP-ACP. CPP-ACP consists of calcium and phosphate ions stabilized in an amorphous, non-crystalline complex by casein phosphopeptides, which prevents premature crystallization and enables efficient ion delivery into subsurface enamel lesions. In contrast, hydroxyapatite is crystalline, providing a direct mineral source at the surface. This crystalline structure offers scaffold-like deposition, but limits ion release and penetration depth compared to amorphous systems. CPP-ACP will keep the calcium and phospate ions in amorph situation which was needed in tooth enamel. CPP-ACP reacted with enamel hydroxyapatite crystal, binding the hydroxyl group and forming hydroxyapatite calcium phosphate which stand the acidic demineralization and increase the remineralization process. CPP-ACP can also stabilize calcium and phosphate ions, which helps to keep the neutral situation on enamel [27, 28].

In a 12 w randomized controlled clinical trial, self-applied 10% CPP-ACP paste resulted in significant regression of enamel demineralization. Both visual assessments and laser fluorescence measurements confirmed that the extent of remineralization in the CPP-ACP group was notably greater than in the control and fluoride varnish groups, and comparable to the CPP-ACP plus fluoride varnish group. The study by our previous study demonstrated that CPP-ACP significantly increased enamel surface hardness compared to the demineralized control group. This indicates that CPP-ACP is comparably effective in enhancing enamel microhardness [6].

Batubara (2024) and Haggho (2016) in their research stated that natural ingredients such as fish scales and eggshells can be used as calcium source in manufacturing tooth remineralization material [13, 19]. Duck eggshells which have high calcium carbonate can be used in hydroxyapatite synthesis to be formed into paste as remineralization material. Hydroxyapatite molecules can reduce the formation of biofilms, making it a suitable preventive agent in caries treatment [29].

Saliva has an especially significant role in oral cavity, especially in keeping the balance between demineralization and remineralization process. Saliva acted as the source of calcium and phosphate ions to help neutralize acid and therefore assist the occurrence of remineralization [30]. Artificial saliva was used in this study to simulate the oral environment and maintain physiological relevance during the remineralization phase. While artificial saliva itself contains calcium and phosphate ions that may contribute to baseline remineralization, it was uniformly applied across all groups, including the control. Therefore, any potential remineralizing effect from the artificial saliva is considered consistent and non differential. Nevertheless, its presence may still act as a confounding factor by partially masking the net effects of the test materials. Future research may consider evaluating the ion release profile separately and including a dry storage control group to better isolate treatment-specific outcomes.

T-paired test result showed a significant increase in enamel hardness surface in Groups I, II, III, IV and V ($p<0.05$), meaning there was remineralization process occurred on the enamel after the application of 6% and 12% hydroxyapatite paste, CPP-ACP and immersion in artificial saliva. Calcium and phosphate ions contained in the hydroxyapatite paste of duck eggshells enter the enamel through porous enamel subsurface via the diffusion process. The calcium and phosphate ions will settle inside the enamel rod and afterwards will be absorbed by the hypomineralized enamel. The ions would later react with the remaining enamel hydroxyapatite crystals, binding with hydroxyl groups and forming hydroxyapatite structure which stands against acid demineralization and increases the occurrence of remineralization process. With the rearrangement of mineral ions of the enamel, which are hydroxyapatite crystals, the tooth structure will be denser, hence increasing the tooth enamel hardness [24].

One-way ANOVA statistic test showed that there was a significant difference of the effect between groups which were applied with 6% duck eggshells hydroxyapatite paste, 12% duck eggshells hydroxyapatite paste, CPP-ACP and immersion in artificial saliva, according to the p-value of 0.000 ($p<0.05$). Post-hoc Bonferroni results showed that Group I (6% duck eggshells hydroxyapatite paste) and Group II (12% duck eggshells hydroxyapatite paste) did not have a significant difference, but 12% duck eggshells hydroxyapatite paste had a greater increase in the enamel surface hardness than the 6% group. This was because the higher calcium and phosphate concentration in enamel environment, the faster the enamel microporosity becomes sealed [23].

Although the 12% hydroxyapatite formulation showed a numerically greater increase in enamel surface hardness compared to the 6% concentration, the difference was not statistically significant. This outcome may be attributed to several physicochemical factors. First, increasing the concentration can lead to higher viscosity, which may impede ion mobility and limit the diffusion of calcium and phosphate into demineralized enamel surfaces. Additionally, ion supersaturation at higher concentrations could reduce the driving force for further mineral exchange with the enamel [31].

Another plausible explanation is particle agglomeration. As hydroxyapatite concentration increases, particles tend to aggregate, thereby decreasing the effective surface area and reducing ion bioavailability. These factors may collectively contribute to

remineralization efficacy observed between the 6% and 12% formulations [32].

Calcination at 900 °C may induce the formation of β-tricalcium phosphate (β-TCP) as a secondary phase alongside hydroxyapatite, which can significantly alter material properties. Recent studies have shown that the proportion of β-TCP increases with higher calcination temperatures, reaching up to 40% at 1000 °C. The presence of β-TCP can enhance ion release, which may benefit bioresorbability. However, excessive β-TCP can compromise mechanical integrity and reduce the stability of the remineralized enamel layer. Therefore, controlling the proportion of β-TCP in the hydroxyapatite formulation is crucial for optimizing remineralization outcomes [33].

In the present study, HCl was selected to achieve a standardized demineralization protocol under controlled conditions. However, for future investigations that incorporate microbial models or aim to simulate cariogenic biofilm activity, lactic acid would be a more appropriate choice due to its biological relevance as a metabolic byproduct of oral bacteria. We acknowledge that this study did not include XRD or EDX analyses to confirm the crystallinity and elemental composition of the synthesized hydroxyapatite. Future research should incorporate these characterization methods to ensure material purity and to better understand the relationship between structural properties and remineralization efficacy.

In this study, enamel remineralization was evidenced by an increasing trend in surface microhardness values and favourable morphological changes in the groups treated with hydroxyapatite and CPP-ACP paste compared to the control. The sample size (n=6 per group) was determined based on feasibility and resource constraints, consistent with preliminary or exploratory *in vitro* studies in similar fields. While no formal power analysis was performed due to the absence of reliable effect size estimates from previous studies, this limitation is acknowledged. However, due to the limited sample size the statistical power of this study did not reach an optimal level. This limitation may have contributed to the inability to detect statistically significant differences, even when clinically or visually relevant effects were observed. Therefore, further studies with larger sample sizes and more robust experimental designs are warranted to confirm these preliminary findings and ensure their statistical and clinical validity.

This study did not include a negative control group (vehicle paste only). Instead, the control group consisted of enamel samples that were not treated with any remineralizing agents and were immersed solely in artificial saliva. While this design allowed for the evaluation of baseline remineralization potential under simulated oral conditions, the absence of a true negative control group formulation may limit the ability to fully isolate the vehicle effect. Future studies are recommended to incorporate a negative control group to strengthen the validity of comparative assessments and eliminate potential confounding from the delivery medium. Ion release data were not collected on this study, and no measurements via inductively coupled plasma optical emission spectrometry (ICP-OES) or comparable methods were performed. Future research is warranted to employ more sensitive and accurate analytical methods, such as ICP-OES, to confirm and more precisely quantify the availability of calcium and phosphate ions released from the paste, thereby providing a deeper understanding of the material's potential bioactivity.

CONCLUSION

Based on our analysis, there were significant effects of the application of 6% and 12% duck eggshell hydroxyapatite paste in remineralizing enamel, but no significant difference between them. The application of 6% and 12% duck eggshells hydroxyapatite paste showed more significant results than the application of CPP-ACP.

This study demonstrates that hydroxyapatite synthesized from duck eggshells possesses key functional groups essential for dental remineralization. Given its high calcium content and biocompatibility, duck eggshell derived hydroxyapatite presents a promising, cost effective, and locally available alternative to conventional agents such as CPP-ACP. Its potential use is particularly valuable in resource-limited settings, where accessibility and affordability are critical for clinical application.

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AUTHORS CONTRIBUTIONS

Fitri Yunita Batubara: conceptualized the study and supervised the overall research design; Widi Prasetia: developed the methodology and contributed to the preparation of materials; Dennis Dennis: performed the laboratory experiments and data collection; Wandania Farahanny: assisted with sample preparation and quality control; Nevi Yanti: conducted the statistical analysis and data interpretation; Cut Nurliza: supported the experimental validation and documentation; Trimurni Abidin: contributed to literature review and theoretical background; Mayvira Annisa: writing reviews, editing and revising of the work, resources and visualization; Dina Masrura: drafted the initial manuscript and coordinated with co-authors; William Sahala Markus Sitompul: critically revised the manuscript and handled correspondence with the journal. All the authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTERESTS

Declared none

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