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ORIGINAL RESEARCH

THE HYDROXYAPATITE OF QUAIL EGGSHELLS (CORTURNIX COTURNIX) AS REMINERALIZATION MATERIALS IN PRIMARY TEETH (IN VITRO)

Ulfa Yasmin,[1*] Indah Sapitri,[2] Rosada Syntia Dwi,[1] Sulistiawati,[3] Novita Idayani[1]

ABSTRACT

Introduction: *Caries is the most common oral health problem, particularly among children. Calcium deficiency due to demineralization of the enamel could be anticipated with the application of remineralization materials. One of the natural alternatives to support the remineralization process is quail eggshells (Coturnix coturnix) containing high calcium carbonate as a hydroxyapatite precursor.* ***Aim:*** *This study aims to evaluate the effectiveness of quail eggshells as hydroxyapatite materials.* ***Methods:*** *This study was a laboratory experimental study with a pretest-posttest control group design. The sample of this study consisted of 15 mandibular primary incisor teeth that were mounted on self-cure acrylic resin and were divided into 3 groups: the treatment group used 20% quail eggshell gel (Group A), the positive control group used CPP-ACP (Group B), and the negative control group used placebo gel (Group C). The applications were conducted for 30 minutes for 14 days and immersed in artificial saliva. The enamel hardness was measured using a Vickers Hardness Tester. Data were analyzed using Paired T-Test and Post Hoc LSD.* ***Result:*** *The Paired T-Test showed that all the groups were statistically significant (p<0,05), which leads to the remineralization process. Post hoc LSD showed that the significant difference at post-test occurred between the treatment group and both control groups (p<0,05).* ***Conclusion:*** *It can be concluded that 20% quail eggshell gel is effective as a remineralization material in primary teeth.*

Keywords: *hydroxyapatite, remineralization, quail eggshells, vickers hardness tester*

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[*1]Department of Pediatric Dentistry, Faculty of Medicine, Sriwjaya University, Palembang, South Sumatera, Indonesia
[2]Student of dentistry, Faculty of Medicine Sriwjaya University, Palembang, South Sumatera, Indonesia
[3]Department of Periodontia, Faculty of Medicine Sriwjaya University, Palembang, South Sumatera, Indonesia

**corresponding author: ulfayasmin@fk.unsri.ac.id*

INTRODUCTION

Dental caries remains a common oral health problem in Indonesia, especially among children. One of the contributing factors is the low level of parental awareness regarding the importance of maintaining their children's oral hygiene. Based on the Basic Health Research (Riskesdas) 2018, the prevalence of dental caries in primary teeth was reported at 60.7%.[1] The development of dental caries is primarily influenced by the imbalance between demineralization and remineralization processes in the enamel. When demineralization progresses at a faster rate than remineralization, it leads to the gradual loss of essential minerals, particularly calcium and phosphate, resulting in enamel breakdown..[2] Loss of minerals in the teeth could be repaired by the remineralization process.[3] Remineralization occurs when there is adequate calcium and phosphate amounts, these minerals diffuse into the microporosities of demineralized enamel and are absorbed by the hypomineralized enamel. This process leads to the formation of hydroxyapatite (HAp) crystals, which act as fillers to restore mineral content within the enamel microporosities. The increase in enamel hardness reflects the success of the remineralization process.[4]

It can occur naturally in the oral cavity through saliva, or be induced by the application of remineralization materials, either conventional agents such as CPP-ACP (*Casein Phosphopeptide - Amorphous Calcium Phosphate*) or natural substances. Many natural substances, even waste like eggshells, could support the remineralization process on the tooth due to the higher sources of calcium carbonate.[5,6–9]

Numerous studies tried to evaluate the effects of eggshells, like chicken's or duck's, as remineralization materials that showed a reduction in enamel microporosity and an increase in the hardness of enamel surfaces.[5–11] Setyawati and Waladiyah, in their study, concluded that the microporosity of enamel decreased after the application of chicken eggshell paste.[7] Esmaeel et al. showed that there was calcium, phosphorous, and magnesium deposition in enamel after soaking chicken eggshell powder solution.[6] Fahmy and Yunita concluded that the application of duck eggshell gel with a 20% concentration was effective than a 40% concentration. In their study, the 30-minute application duration was most effective in increasing enamel hardness.[9]

No previous study has evaluated quail eggshells as remineralization materials, so this study aimed to evaluate the effectiveness of hydroxyapatite gel derived from quail eggshells as remineralization materials in primary teeth.

METHODS

This research is an in vitro laboratory experimental study with a pretest-posttest control group design using mandibular primary incisor teeth. The fresh quail eggshells used came from egg farming in Palembang, South Sumatra. The hydroxyapatite extraction from quail eggshells was carried out at the Analytical Chemistry Laboratory, Faculty of Chemistry, Polytechnic of Sriwijaya. The artificial saliva production and gel preparation were conducted at the Biochemistry Laboratory, Faculty of Medicine, Sriwijaya University. The tooth hardness testing was performed in the Materials Laboratory, Faculty of Engineering, Sriwijaya University. This research was conducted from November 2023 to February 2024.

This research uses tools, including stationery, filter paper, microbrush, micromotor set, diamond disk, beaker glass, magnetic stirrer, digital scale, Vickers hardness tester, zoom stereo microscope, furnace, sandpaper, incubator, ball mill, pH meter, and clay jug.

The materials used in this study were quail eggshells, 30 primary mandibular incisor teeth, etching 37%, Phosphoric Acid (H_3PO_4), Aquabides, CPP-ACP paste (GC Tooth Mousse®, USA), Nitric Acid (HNO3), Ammonium hydroxide, Potassium chloride, Magnesium chloride, Propylene glycol, Glycerine, Dipotassium hydrogen phosphate, Calcium chloride, Nipagin, and Sodium carboxymethyl cellulose (Na-CMC).

Production of Hydroxyapatite from Quail Eggshells

The quail eggshells were synthesized using the precipitation method. A total of 400 grams of fresh quail eggshells were cleaned from the inner membrane and washed in distilled water. The eggshells were calcined at 1000°C in a furnace for 6 hours to obtain calcium oxide (CaO).[10,11] The calcined result was refined with a ball mill to get small particles in powder. A chemical solution, such as nitric acid, was added and led to the formation of calcium dioxide ($Ca(OH)_2$). The nitric acid (HNO_3) was stirred until the suspension became homogenous. This process created calcium nitrate ($Ca(NO_3)_2$).[10] Then, phosphoric acid (H_3PO_4) as phosphate precursor was mixed with calcium nitrate ($Ca(NO_3)_2$), and ammonium hydroxide (NH_4OH) was added to maintain a pH of 10 in the pH solution.[10,11,12] It was left to stand for 24 hours to facilitate the removal of by-product ammonium nitrate, resulting in the formation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with white and light-colored powder in the final procedure.[10] The powder was calcined again in a furnace to increase its crystallinity.

The synthesis of quail eggshells is represented by the following reaction:

$$10Ca(NO_3)_2 + 6H_3PO_4 + 20NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 18H_2O$$

Gel Preparation

The gel formulation process began by dispersing 20 g of powder into Aqua Bides. Sodium carboxymethylcellulose (Na-CMC) 5 g, glycerine 10 g, propylene glycol 5 g, and nipagin 0.1 g were added and stirred until the gel became homogeneous.[12,13] The placebo gel was made with the same composition, but without hydroxyapatite powder.

Production of Artificial Saliva

The materials were prepared and weighed, including 0.65 g/L of potassium chloride, 0.058 g/L of magnesium chloride, 0.165 g/L of calcium chloride, 0.804 g/L of dipotassium hydrogen phosphate, 0.365 g/L of potassium dihydrogen phosphate, and 2 g/L of carboxymethyl cellulose. All materials were mixed in 1 litre of Aqua bides and stirred with a magnetic stirrer until homogeneous.[14]

Sample Preparation

The sample size was calculated using a formula for comparing two means based on normal distribution, incorporating Z-values for a 95% confidence level (Z = 1.96) and 90% power (Z = 1.28). This method was chosen to maintain consistency with previous in vitro studies employing similar designs.[15] The result was n = 4.9, which was rounded up to 5 samples per group. Considering three groups (quail eggshell hydroxyapatite gel, commercial remineralization gel, and placebo), a total of 15 samples was required.

The crown of the anterior primary tooth was cut horizontally approximately ± 1 mm below the cemento-enamel junction (CEJ). The root of the anterior primary tooth was cut using a diamond disk bur. The teeth were mounted on self-cure acrylic resin, and the surfaces were smoothed using sandpaper with grit sizes 400, 1000, and 1500.[14] Surface smoothing is performed to facilitate the measurement process using the Vickers Hardness Tester (Figure 1). The specimens were divided into 3 groups, the treatment group used 20% quail eggshell gel (Group I), the positive control group used CPP-ACP (Group II), and the negative control group used placebo gel (Group III).

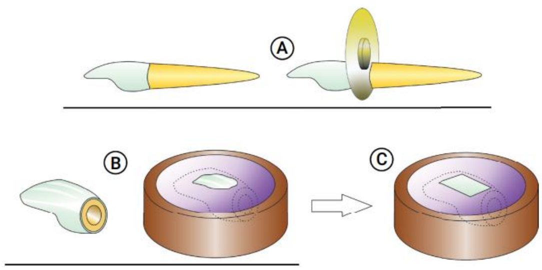

Figure 1. (a) Process of cutting the primary crown, (b) the labial surface mounted in the resin, (c) and the surface were smoothed by the sandpaper

Demineralization and Remineralization Process

Enamel hardness was measured three times: before treatment, after the application of demineralizing agents, and after the application of remineralizing agents. Enamel hardness was measured using a Vickers Hardness Tester with a 5-kilogram load for 15 seconds. Demineralization was carried out by applying a 37% etching solution for 20 minutes to the entire surface of the lower mandibular primary incisor crown until early enamel lesions were formed.[16,17] The specimens were washed with running water and dried. The enamel surface hardness after demineralization was measured using a Vickers Hardness Tester. The remineralization process was carried out using experimental materials, including hydroxyapatite gel from quail eggshells (Group I), CPP-ACP (Group II), and placebo gel (Group III), which were applied to the entire surface of the tooth crown using a microbrush. The samples were left for 30 minutes each day for 14 days, then cleaned with Aqua bides to eliminate any residual gel and soaked in 100 mL of artificial saliva in a container at 37°C in an incubator.[8] Afterward, the enamel surface hardness was measured again.

Surface Hardness Assessment

The measurement was accomplished with a Vickers hardness tester (Torsee VKH 2E) in the Materials Laboratory, Faculty of Engineering, Sriwijaya University. The used load was 5 kg for a period of 15 seconds. Two measurements were taken for each sample, and the average of these readings was calculated. The Vickers hardness test was performed with a Zoom Stereo Microscope, which features a diamond indenter with a square tip. The angle between the opposing faces of the indenter is 136°.

Statistical Analysis

Data analysis in this study was performed using SPSS. Normality testing was conducted using the *Shapiro-Wilk test ($p > 0.05$)*, and homogeneity testing was performed using *Levene's Test ($p > 0.05$)*. If the data are normal and homogeneous, the data analysis employed a

Paired T-Test to compare enamel hardness before and after the application of the remineralizing gel. A *post hoc LSD* test was then conducted to identify the differences between the groups.

RESULTS

The precipitation method used in this study showed the final powder of the quail eggshells with a white and bright color (Figure 2).

(A) **(B)** **(C)**

Figure 2. (a) The quail eggshells after removing the inner membrane, (b) quail eggshells after calcined after 6 hours, (c) the result of hydroxyapatite powder from quail eggshells.

Characterization of HAp used X-Ray Diffraction (XRD) (Figure 3) to determine the crystalline phases. The phases obtained are compared by the Joint Committee on Powder Diffraction Standards (JCPDS).

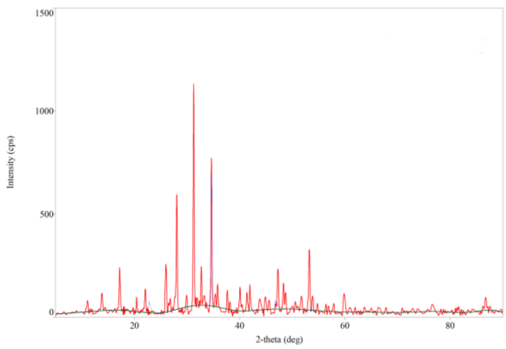

Figure 3. The XRD result of quail eggshell powder

It shows that the sample of quail eggshell powder in Figure 3 has the three highest peaks of the 2θ spectrum at 28.04°, 31.92°, 32.28°, and 34.04°. The analysis results are similar to the standard XRD spectrum pattern of hydroxyapatite based on JCPDS data (01-086-0740) at ranges 31.9°, 32.2°, and 33.9622°. It can be concluded that the powder is purely hydroxyapatite. Therefore, the graph shows sharp and prominent peaks, indicating the powder is crystalline in shape.[10]

The mean of enamel hardness in primary teeth before, after etching, and after application of quail eggshell gel in group I, CPP-ACP in group II, and gel placebo in group III is illustrated in Figure 4.

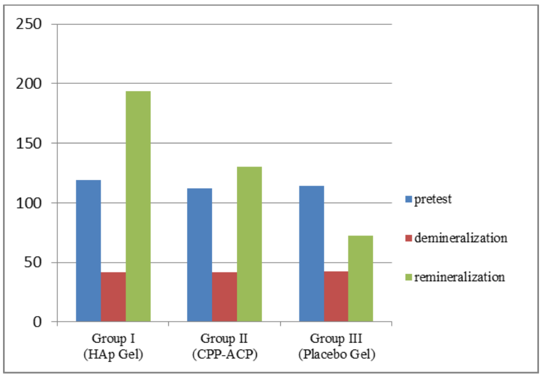

Figure 4. The graph of each group before treatment, after demineralization, and after remineralization.

The highest score of enamel hardness was shown in group I (quail eggshell gel), which was 193,52 VHN, followed by group II (CPP-ACP), which was 130,30 VHN, and group III (placebo gel) was 114,37 VHN. The Shapiro-Wilk test proved that the data were normally distributed ($p > 0.05$) while the Levene test showed that the data were homogeneous ($p > 0.05$).

The result of the Paired T-test statistic to compare enamel hardness before and after treatment in each group of specimens is summarized in Table 1. Paired T-test analysis indicated that the enamel hardness in group I, group II, and group III was significantly different from that of enamel hardness between before and after treatment, with a value of $p<0.05$.

Table 1. Paired T-Test

Specimens		*Sig.*
Group I	before-after	0.002*
Group II	before-after	0.004*
Group III	before-after	0.017*

*significant difference ($p<0.05$)

Based on one-way analysis of variance (ANOVA), it is conclusive that there are significant differences in enamel hardness between group I, group II, and group III ($p<0.05$). The summary of the LSD test result can be seen in Table 2. The LSD test results present that there was a significant difference between group I and group II and group III ($p>0.05$).

Table 2. Post Hoc LSD

Specimens	Group I	Group II	Group III
Group I	-	0,016*	0,001*
Group II	0,016*	-	0,024*
Group III	0,001*	0,024*	-

*significant difference ($p<0.05$)

DISCUSSION

The increase of enamel hardness in groups I, II, and III (Table 1) shows a significant difference in pretest and posttest that there has been a process of remineralization on the teeth. Figure 4 shows that all groups increased the mean of enamel hardness. This result is in accordance with calcium and phosphate minerals in quail eggshells, CPP-ACP, and artificial saliva.[9,18] Calcium and phosphate are the main minerals of hydroxyapatite crystals. Both minerals cause the process of remineralization that decreases the microporosity of enamel, thereby increasing the hardness of the enamel.[5,14]

Quail eggshells play an important role in the process of remineralization. It begins with the penetration of nano-hydroxyapatite into the surface of the microporosity of demineralized enamel and increasing surface hardness of enamel.[9] The formed hydroxyapatite fills the interprismatic gap of enamel, thus increasing the enamel hardness. On the other hand, 30-minutes application works optimally in the remineralization process. It is in line with Wiryani et al. that application of duck eggshells on the enamel surface for 30 minutes showed increasing enamel hardness significantly than 60 minutes.[14]

The specimens of group II experienced an increase in enamel hardness due to the application of CPP-ACP. It contains calcium and phosphate ions from milk protein derivatives, allowing it to replace the damaged calcium hydroxyapatite structure. The mechanism of CPP-ACP in increasing tooth hardness involves maintaining calcium and phosphate ions in an amorphous form, enabling them to penetrate the enamel.[19] On the other hand, saturation with calcium and phosphorus ions in CPP-ACP converts the acidic environment to an alkaline environment. It can serve as a calcium source designed to stabilize calcium and phosphate ions on the teeth surface, thus leading to the remineralization capability.[18]

The lowest increase in enamel hardness of primary teeth was observed in group III (negative control). The artificial saliva, which contains minerals similar to those found in normal saliva, plays an important role in increasing enamel hardness. Hidayat et al. in their research reported that the inorganic components of artificial saliva are comparable to those of normal saliva, such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, HCO_3-, and phosphate.[20] Additionally, the pH of the artificial saliva in this study was 7, which involved a buffering process. When apatite. Minerals in saliva can rebuild dissolved apatite crystals, thereby contributing to the natural remineralization process in the oral cavity.[21]

One-way ANOVA statistical analysis was used to compare the presence or absence of significant differences between group I, group II and group III. The results of one-way ANOVA statistical analysis in this study revealed that there were significant differences in enamel hardness between group I, group II, and group III. The result of the LSD test analysis showed that there was a significant difference in enamel hardness between group I, group II, and group III.

Quail eggshells contain calcium carbonate as the precursor of hydroxyapatite, which plays an important role in the process of tooth remineralization.[22,23] The precipitation method used for this research resulted in nanoparticles of hydroxyapatite derived from quail eggshells, which exhibited a crystallization pattern similar to tooth apatite.[10] Additionally, in this study, a 20% concentration works more effectively as a remineralization material. Agusmawati et al. in their study concluded that a 20% concentration of duck eggshells works more optimally than 40% due to its low viscosity.[8]

Based on this study, 20% hydroxyapatite gel from quail eggshells has effectiveness as a remineralization material in primary teeth. Thus, it is a potential alternative remineralization material to prevent caries on children's teeth. On the other hand, the application of this material creates teeth that are opaque in color, which is not aesthetically acceptable. Therefore, it needs to be developed into a lower concentration for the next study, and clinical research should be done to find out more about the effectiveness of quail eggshells as an alternative remineralization material.

CONCLUSION

Based on the results of this study, it can be concluded that hydroxyapatite gel made from quail eggshells is effective in increasing the enamel surface hardness of primary teeth, showing its potential as a natural remineralization material. However, this study has some limitations, such as a small sample size and the use of an in vitro method, which may not fully represent real conditions in the oral cavity. Therefore, further clinical studies are needed to confirm its long-term effectiveness, safety, and possible use in pediatric dental treatment. If proven effective in clinical settings, quail eggshell-based hydroxyapatite gel could be developed into an affordable and accessible preventive product, such as toothpaste or topical gel, especially for children at high risk of dental caries.

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