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by Juni Jekti

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THE INCREASE OF TOOTH ENAMEL SURFACE HARDNESS AFTER APPLICATION BLOOD COCKLE SHELLS (*ANADARA GRANOSA*) PASTE AS REMINERALIZATION AGENT

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ABSTRACT

Objective: To determine the increase of tooth enamel surface hardness after application hydroxyapatite paste that was synthesized from blood cockle shells (*Anadara granosa*) as a remineralization agent.

Methods: Laboratory experimental study using twenty-seven maxillary first premolar and randomly divided into 3 groups. All of the samples were immersed in the non-cola carbonated drink (5 min). Thereafter, samples in each group were treated (6 min) with application of blood cockle shells paste that has been synthesized (group 1), casein phosphopeptide-amorphous calcium phosphate paste (GC Tooth Mousse®) (group 2) as a positive control, and stored in saline solution (NaCl) (group 3) as a negative control. Vickers Hardness Number (VHN) measurement was performed at baseline, after immersing in non-cola carbonated drink and after completing of the respective treatment.

Results: Immersion in non-cola carbonated drink reduced the enamel surface hardness significantly. Significant re-hardening after treated occurred in group 1 and 2 also baseline hardness of both groups were achieved. But statistically no significant differences between group 1 and 2 in re-hardening enamel surface hardness (final hardness-hardness after immersion).

Conclusion: Application of blood cockle shells paste as a remineralization agent could increase tooth enamel surface hardness which is nearly the same effective as CPP-ACP paste.

Keywords: Enamel surface hardness, Non-cola carbonated drink, Blood cockle shells paste, CPP-ACP paste

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INTRODUCTION

Demineralization of teeth is caused by an acidic attack through two primary means: dietary acid consumed through food or drink and interaction of bacteria with sugar on tooth enamel surface [1]. The demineralization process is indicated by reduced enamel surface micro hardness [2, 3]. If dietary acid is consumed frequently and not managed through effective interventions, it may result in substantial loss of enamel and subsequent exposure of the underlying dentin, which can, in turn, lead to dentin sensitivity, loss of vertical height, and esthetic problems [4]. Demineralization that happens constantly will cause loss of some enamel prismatic and form a microporosity in the enamel [5, 13]. Hydroxyapatite (HAp) has attracted much interest as a biomaterial due to its similarity in chemical composition to that of human hard tissue and also has outstanding properties like biocompatibility, bioactivity, osteoconductivity, non-toxicity, and non-inflammatory nature [6]. HAp is a bioactive ceramic material that could promote tooth remineralization and can be synthesized from materials that contain calcium and phosphor by some chemically synthetic methods [7, 8]. Calcium and phosphate ions will hinder the decomposition process of hydroxyapatite and cause rebuilding or reconstruction of partially soluble crystalline hydroxyapatite [9]. Over the past years, biologically derived natural materials, such as fishbone, bovine bone, corals, oyster shells, eggshells, and blood cockle shells (*Anadara granosa*) have been converted into useful biomaterials like hydroxyapatite [10-12]. Most all territory of Indonesia consists of waters, where various types of shell reside but this commodity generates not optimally waste utilization. Mostly shells can be consumed because they are rich in protein, however, the cockles generate waste and its utilization is still not optimal [10, 13]. Blood cockle shells (*Anadara granosa*) is one of shell species that contain 98-99% calcium carbonate ($CaCO_3$) which can be used as natural sources of calcium in HAp synthesis process and effective for protection against demineralization of the tooth [7, 8, 10, 14]. In this research, blood cockle shells (*Anadara granosa*), which has a calcium source will be synthesize HAp by a hydrothermal method process. Therefore, the aim of this study was to determine the increase of tooth enamel hardness after application hydroxyapatite paste that synthesized from blood cockle shells (*Anadara granosa*) as a remineralization agent.

MATERIALS AND METHODS

Material

Stone Gips (Moldano, USA), sulfuric acid (used for cleaned the blood cockle shells), reagents, i.e., diamonium phosphate, sodium carboxymethyl cellulose, methylparaben, glycerol and menthol.

Synthesis and characterization of hydroxyapatite from blood cockle shell

The uncrushed blood cockle shells were cleaned by H_2SO_4 solution with composition (5% H_2SO_4 and 95% aquades). The blood cockle shells were brush, washed with water until it is clean and dry in oven at 80 °C for 24 h. Crushed blood cockle shells until it becomes powder. Further put it into a crucible and calcined at 1000 °C for 5 h to turn $CaCO_3$ structure to CaO. Dissolved 3.035 g diamonium phosphate $(NH_4)_2HPO_4$ with aquades until its saturated solution (15 ml) formed. Diamonium phosphate solution streamed to 5 g of calcined blood cockle shells powder using titration (rate 2 ml/minute) while stirred with a constant speed to form a white precipitate. pH was adjusted around 11-12. The precipitate was washed with aquades, filtered with filter paper, and dried in 160 °C with 200 mesh particle size for 20 h. The hydroxyapatite (HAp) was characterized with X-Ray Diffraction (XRD) and made into paste.

Blood cockle shells paste process

This blood cockle shells paste form was made by added Nipagin and 0.2 g NaCMC. The HAp from synthesis process was washed with 1 g gliserol. Also, 0.05 g menthol was mixed with alcohol until it dissolve after then put it into washed HAp.

Sample preparation

The inclusion criteria are maxillary first premolar teeth which were extracted with orthodontic reason, rooted in two and the apex tip has closed perfectly. While the exclusion criteria are the presence of email caries, anomalies structure or shapes.

Twenty seven maxillary first premolar and randomly divided into 3 groups. Each group consists of 9 samples. The teeth were sectioned to separate the crown and root at the 2 mm below cemento-enamel junction by using a carborundum disc bur. After preparation, the crowns were embedded in gypsum and the buccal of enamel surface placed upward. The enamel surface hardness of the samples was determined three times: for initial (baseline), after demineralization and after treated (final measurement) for each group. For each measurement, indentation were made three times on the same spot of enamel surface (indentation time 10 s) with the Universal Hardness Tester device (Affri®, Italy) and measured by Vickers indenter. The hardness results were in Vickers Hardness Number (VHN) units and the mean surface hardness was calculated.

Study design

All samples were demineralized with immersion in the non-cola carbonated drink (Sprite®) for 2 min. Samples were rinsed with tap water to stop the demineralization process. The enamel surface hardness was determined again. Application of blood cockle shells paste (group 1), CPP-ACP paste (GC Tooth Mousse®, group 2) as a positive control, and immersion in saline solution (NaCl, group 3) as a negative control. Paste covered the entire buccal surface of the sample and each group was treated for 6 min. Thereafter, the surface hardness was determined again (final measurement).

Statistical analytics

The results were presented as mean±standard deviations (SD). The fit of the data to the normal curve was tested using Shapiro-Wilk's test. The distribution of the analyzed variables were normal; therefore, parametric tests were used for further statistical analysis with Repeated Analysis of Variance (ANOVA), paired t-test and *Least Significant Different* (LSD) test.

RESULTS AND DISCUSSION

The compositions of synthesized blood cockle shells were measured using X-Ray Diffraction. The quantitative analysis results shows the crystalline material formed 96.9% hydroxyapatite compound and the rest of the synthesis product form 3.06% CaO as seen in fig.

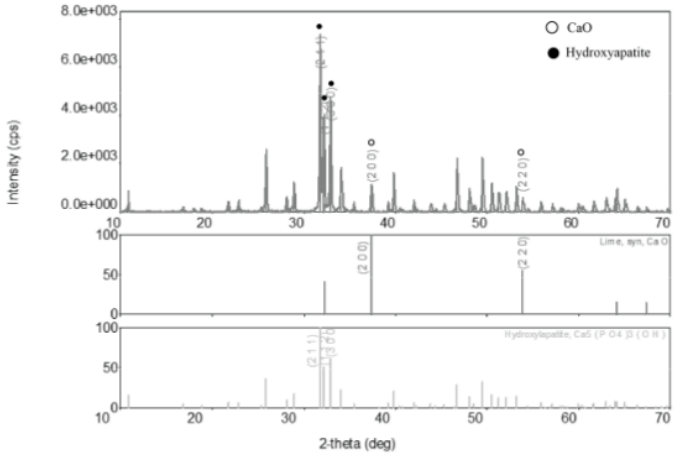

Fig. 1: X-Ray Diffraction patterns of blood cockle shell powder after synthesized

Different techniques the preparation routes of HAp have been reported, including the hydrothermal method. This method has important advantages over other methods for the synthesis. In figure, X-Ray Diffraction analysis shows the formation of HAp (96.9%) and CaO (3.06%) as the product of synthesized of blood cockle shell by hydrothermal reaction. Based on the results of a study conducted by Elizondo-Villareal *et al.*, using the hydrothermal method to synthesized eggshell successfully form hydroxyapatite and $CaHPO_4$ in a 3:1 ratio [8]. The main advantages of the hydrothermal method are high crystallinity and excellent homogeneity of hydroxyapatite [8, 15]

Table 1: Enamel surface hardness at baseline, after demineralization, and final measurement, reduce of hardness value (ΔVHN_a), an increase of hardness value (ΔVHN_b)

Group	N	Baseline mean±SD (VHN)	After demineralization mean±SD (VHN)	Final measurement mean±SD (VHN)	ΔVHN_a mean±SD (VHN)	ΔVHN_b mean±SD (VHN)	P-value
1	9	106.96±20.84	85.88±25.56	128.17±21.42	21.08±11.46	42.29±15.82	0.000*
2	9	115.66±25.25	95.89±21.41	125.70±14.48	19.77±12.51	29.81±17.28	0.001*
3	9	112.14±33.93	96.10±36.14	95.82±35.55	16.04±10.48	-0.28±0.91	0.000*

*Repeated ANOVA Test<0.05, Significant, Group 1. Blood cockle shells, Group 2. CPP-ACP, Group 3. Saline Solution

Mean surface hardness (VHN) of the different group (1-3) were determined at baseline, after demineralization and at final measurement. Table showed the mean of enamel surface hardness were increase after application of blood cockle shells paste (group 1) (128.17±21.42 VHN) and the CPP-ACP paste (group 2) (125.70±14.48 VHN) and both groups resulted in higher enamel surface hardness than their baseline. While immersing in saline solution could (group 3) reduce enamel surface hardness. Based on the result of repeated ANOVA test, there were significant changes of enamel surface hardness at baseline, after demineralization and at final measurement in all groups.

ΔVHN_a were show reduce of enamel surface hardness from baseline to after demineralization. Based on statistical analysis (paired t-test)

there were significant reduce of enamel surface hardness in all groups (1-3). The demineralization process in this study performed by immersion samples in an acidic non-cola carbonated drink (pH 3.26, 20 °C) for 2 min. The type of carbonated drink that used in this study contains citric acid. Citric acid has complex interactions and strong ability to bind calcium from the enamel surface [16]. The acidity can affect the physical and chemical structure of the enamel, so that it reducing surface hardness rapidly [17]. As expected, the results in ΔVHN_a showed that demineralization for 2 min in a non-carbonated drink significantly reduced enamel surface hardness. There were some others studies have to prove the significant reduce in surface enamel hardness after immersion in a carbonated or non-carbonated drink [18-21]. Particularly those with low pH because the acids can easily dissolve the enamel surfaces [22].

ΔVHN_b were show increase of enamel surface hardness from (after demineralization) to (final measurement). Application of blood cockle shells paste and CPP-ACP paste were increase enamel surface hardness significantly. While immersing in saline solution (group 3) reduce enamel surface hardness but statistically not significant. Based on the results of statistical analysis (paired t-test), application of blood cockle shells paste and CPP-ACP paste were increase enamel surface hardness significantly. Immersing in saline solution (group 3) reduce enamel surface hardness but statistically not significant. The increase of enamel surface hardness (ΔVHN_b) after application of blood cockle shells paste (group 1) (ΔVHN_b = 42.29±15.82 VHN) is higher than CPP-ACP paste (group 2) (ΔVHN_b = 29.81±17.28 VHN) but based on *Least Significant Different* test between group 1 and group 2 obtained p-value = 0.062 ($p<0.05$ significant), which means that the increase of enamel surface hardness in both of groups statistically were not significantly different.

As seen in ΔVHN_b, the application of blood cockle shells paste and CPP-ACP paste for 6 min showed a significant effect in increasing surface enamel hardness. Based on the results of a study conducted by Wegehaupt *et al.*, application of CPP-ACP paste for 3 min after immersion in the non-cola carbonated drink for 2 min could increase enamel surface hardness but not achieving initial measurement hardness (baseline) [18]. So that, we propose 6 min for application time to estimate enamel surface hardness achieve the baseline. As can be seen in table, the final measurement of enamel surface hardness in group 1 and 2 were exceeded the baseline. The increased of enamel surface hardness occurs due to the presence of hydroxyapatite in blood cockle paste as well as calcium and phosphate ion in CPP-ACP paste thus could replace calcium and phosphate ions that have dissolved while demineralization process. CPP-ACP paste is a derivative of cow's milk with high-calcium and phosphate, which can potentially act as remineralizing agents on the enamel [23, 24]. Based on a study conducted by Ceci *et al.*, CPP-ACP paste could stimulate the occurrence of remineralization after demineralization by carbonated cola drink for 2 min [25]. The study by Musa *et al.* revealed the hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ synthesized from the blood cockle shells (*Anadara granosa*) proved effective for protection against dental demineralization [10]. Hydroxyapatite or calcium carbonate nanostructures can act as calcium and phosphate sources to retain these ions in supersaturation state in the enamel minerals [26].

As shown in table, the re-hardening (ΔVHN_b) in group 1 was higher than group 2. Because basically the blood cockle shells paste and CPP-ACP paste consists of the almost the same content in increasing the enamel surface, which is calcium and phosphate ions [10]. CPP-ACP paste contains about 18% calcium and 30% phosphate which due to the remineralization process, calcium and phosphate ions in CPP-ACP react through several processes starting from the movement of ions out of CPP and entering the enamel rods then forming apatite crystals [27]. While blood cockle shells paste contain 96.9% hydroxyapatite based on X-Ray Diffraction (XRD) test. HAp is known to have a similar chemical structure to the mineral content of the tooth, thus allowing for covering the pores on the demineralized surface directly [28]. In a study conducted by Porcelli *et al.* there was a significant increase in surface enamel hardness after the application of the HAp paste [30]. According to a study by Sharma *et al.* the use of the nano hydroxyapatite paste is more effective when compared with the CPP-ACP paste in increasing the levels of calcium and phosphate on the enamel surface. Based on the enamel surface hardness test, the increase of enamel surface hardness values is more significant on the HAp pastes than CPP-ACP paste [31]. Irrespective of these findings concerning the re-hardening, statistically no significant difference in re-hardening (ΔVHN_b) enamel surface hardness between group 1 and group 2. In table group 3 (negative control) immersing in saline solution (0.9% NaCl) was reduce enamel surface hardness but statistically not significant.

CONCLUSION

The application of blood cockle shells (*Anadara granosa*) paste containing 96.9% hydroxyapatite as a remineralization agent could increase tooth enamel surface hardness which is nearly the same effective as CPP-ACP paste.

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AUTHORS CONTRIBUTIONS

All authors have made substantial contributions to the work reported in the manuscript. Juni Jekti Nugroho: Conception and designing of the study, drafting the article, critical revision of the article, final approval of the study to be published. Nurhayaty Natsir: critical revision of the article. Aries C Trilaksana: critical revision of the article. Christine A. Rovani: critical revision of the article. Maya M. Atlanta: Data collection, data analysis and interpretation, drafting the article.

CONFLICT OF INTERESTS

All the authors hereby declare that there is no conflict of interest

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