ORIGINAL ARTICLE

Hydroxyapatite Paste of Snakehead (Channa Striata) Fish Bone: The Impact on Primary Teeth Enamel Remineralization

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ABSTRACT

Introduction: Primary teeth have thinner enamel than permanent teeth. Dissolution of hydroxyapatite from the tooth structure at low pH can lead to the irreversible destruction of enamel. Hydroxyapatite particles have been shown to deposit and restore demineralized enamel surfaces. **Purpose:** To analyze the effectivity of hydroxyapatite paste of snakehead fishbone on remineralization of primary teeth enamel. **Method:** A total of twelve primary maxillary incisor teeth were collected, and each tooth was divided into two parts. The left part was applied with control paste, and the right part was applied with 10, 15, and 20% hydroxyapatite or Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) paste as the positive control. Remineralization was analyzed by measuring the enamel microhardness and the enamel microporosity. Kruskal Wallis and Mann-Whitney were used to analyze the differences in the increase of Vickers hardness value between groups. **Results:** Teeth treated with hydroxyapatite paste and CPP-ACP had higher enamel microhardness and smaller enamel microporosity than those treated with control paste. The Vickers hardness value was increased by 89.3, 177.2, 288.7 at 10, 15, 20% hydroxyapatite paste, and 283.1 at CPP-ACP. The increase of Vickers hardness value was significantly different between groups ($p<0.05$), except between 20% hydroxyapatite and CPP-ACP paste. The greater the concentration of hydroxyapatite paste, the smaller the enamel microporosity. **Conclusion:** Hydroxyapatite paste of snakehead fishbones stimulates the remineralization of primary teeth enamel. The highest remineralization occurs at a concentration of 20%, comparable to CPP-ACP paste.

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Keywords: hydroxyapatite, microhardness, microporosity, primary teeth, remineralization

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INTRODUCTION

Primary teeth are more prone to developing caries because their enamel is thinner and less dense than permanent teeth enamel (1). Lactic acid is produced by oral microbes, particularly Lactobacilli and Streptococci. This creates an acidic environment on the tooth surface that causes demineralization and reduces the hardness of dental enamel (2). Demineralization is characterized by depleting calcium, phosphate, and hydroxy ions from hydroxyapatite crystals. The remineralization procedure allows for the reconstruction of tooth minerals (3,4). Restoring minerals to demineralized teeth is known as remineralization. Particulate hydroxyapatite facilitates the deposition of minerals, including calcium and phosphate, onto the tooth surface, which aids in the remineralization and repair of small enamel defects (5).

Hydroxyapatite can be synthesized from natural substances, such as shellfish, cattle bones, fish scales, starfish, and eggshells (6). Snakehead fish (Channa striata) are frequently found in South Kalimantan,

Indonesia. The Snakehead fish meat is widely used in food production, while the bones are simply thrown away and become waste. These fish bones can be used as a source of hydroxyapatite (7–9). Synthesis of hydroxyapatite from different sources or using different techniques can produce different characteristics of hydroxyapatite, including the Ca/P ratio (10). To the author's knowledge, no previous study has been conducted on the effectiveness of hydroxyapatite paste from snakehead fish bone on the microhardness and microporosity of primary tooth enamel. A study by Wahyuni et al. showed that incorporating hydroxyapatite derived from snakehead fish bones into permanent teeth enamel leads to remineralization, increasing enamel microhardness (11).

Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) is another substance that can remineralize teeth to stop caries in primary teeth. Since CPP-ACP is derived from casein, a milk protein, it is not appropriate for individuals who are lactose intolerant or allergic to milk proteins (12). Hydroxyapatite is thought to be less harmful and does not cause allergic reactions (13).

This research investigated the effects of different concentrations (10%, 15%, and 20%) of nano-hydroxyapatite paste derived from snakehead fish bones on remineralization in primary teeth. This was achieved by assessing the microhardness and microporosity of the enamel.

MATERIAL & METHODS

Ethics Statement

The Ethics Commission of the Faculty of Dentistry, Universitas Lambung Mangkurat approved the protocol with an ethical clearance number 105/KEPKG-FKGULM/EC/X/2023.

Preparation of snakehead fish bones

One kilogram of the snakehead fish bones was cut into 2-5 mm pieces, cleaned, and boiled using 4L distilled water for two hours to eliminate any remaining flesh. The fish bones were then cleaned and allowed to dry in the sunlight for about a day to ensure that all water from the boiling process was completely removed. 100 g dry fish bones were then soaked in 1L 1 M HCl solution for two hours to dissolve calcium carbonate, phosphate salts, and other mineral impurities, then rinsed to a pH of ±7 and dried in an oven for two hours at 105°C. The pH was measured using a pH meter (Hanna HI98107, Hanna Instruments, Indonesia). After oven drying, the fish bones were ground using ball milling machine and sieved through a 60 mesh. The fishbone powder was then calcined for four hours at 1000°C for 5 hours. The calcination procedure aimed to eliminate organic materials and other metals and break down calcium carbonate ($CaCO_3$) into calcium oxide (CaO). The resulting CaO is a precursor for producing hydroxyapatite powder (11,14).

Synthesis of hydroxyapatite

The precipitation method was used to synthesize hydroxyapatite. 1M HNO_3 solution was combined with fish bone-derived CaO powder to dissolve the remaining mineral impurities. Stir the solution at a speed of 300 rpm for 30 minutes. This process produced a calcium nitrate ($Ca(NO_3)_2$) solution. This $Ca(NO_3)_2$ solution was then heated to 40°C for 30 minutes using a stirrer at a speed of 300 rpm. After that, $(NH_4)_2HPO_4$ 1.66 M was added to the mixture. The mixture was stirred at a speed of 300 rpm using a magnetic stirrer for ±2 hours while a 1 M NH_4OH solution was added until the pH reached ±10. After that, the solution was covered and left for 24 hours at room temperature to produce a precipitate. The white precipitate formed was separated by filtration using filter paper. The precipitate was re-sintered using a furnace at 900°C for 5 hours. A particle size analyzer (NanoPlus Particle Size Analyzer, Georgia,) was used to measure the size of the hydroxyapatite particle (11,14).

Preparation of hydroxyapatite paste

Na-CMC was added to heated distilled water (60°C) and stirred at 300 rpm for 30 minutes until homogeneous; then, the solution was left to cool to form a semi-solid base. Transfer the base to another container, add methylparaben, glycerine, and hydroxyapatite, stir at 300 rpm for 30 minutes until smooth, and create a paste. Add a few drops of 10% acetic acid to adjust the pH at ranges of 7 to 11 using a pH meter (Hanna HI98107, Indonesia). The paste formulation can be seen in Table I (8).

Table I: Toothpaste formulation

Ingredients	F0 (control paste)	F1 (10% hydroxyapatite paste)	F2 (15% hydroxyapatite paste)	F3 (20% hydroxyapatite paste)
Hydroxy-apatite	0	10	15	20
Na-CMC	2	2	2	2
Methyl-paraben	0.2	0.2	0.2	0.2
Glycerine	1	1	1	1
10% acetic acid	qs	qs	qs	qs
Distilled water	Ad 100	Ad 100	Ad 100	Ad 100

Preparation of the teeth samples

The samples in this study consisted of twelve primary maxillary incisor teeth that had been extracted from children aged 3-7 years at the dental practice due to pre-shedding mobility or tooth root resorption. The children's parents obtained consent for tooth extraction for research

purposes. These incisors were chosen because incisors produce more consistent results in remineralization experiments due to their flat surfaces. The teeth surfaces were mechanically cleaned from calculus and debris using a low-speed handpiece (W&H, Bьrmoos, Austria), rubber cups, and fluoride-free pumice paste. The teeth were dried using an air syringe and visually inspected. The teeth were inspected for enamel defects, carious lesions, or cracks. Teeth exhibiting noticeable structural defects, cavitated lesions, visible white spot lesions, or discoloration were excluded from the study. Samples were kept in 0.1% thymol solution until the time of use.

The samples in this study consisted of twelve primary maxillary incisor teeth that had been extracted. These incisors were chosen because incisors produce more consistent results in remineralization experiments due to their flat surfaces. The samples were randomly categorized into four groups, as shown in Table II. Each group consists of three teeth divided into two parts, namely the left and right sections. The teeth surfaces were mechanically cleaned from calculus and debris using a low-speed handpiece (W&H, Bьrmoos, Austria), rubber cups, and fluoride-free pumice paste. The teeth were inspected for enamel defects, carious lesions, or cracks. The teeth were sectioned at the cementoenamel junction (CEJ). CEJ is the boundary between enamel and cementum. Before cutting, the CEJ area was first indicated with a pencil. The cutting procedure was performed using a high-speed diamond rotary bur with water-air spray to remove the root from the crown. Subsequently, the crowns were indicated using a pencil on the median part of the tooth crown, then vertically divided into two equal sections with a separating disk. Each tooth section was implanted in self-cure acrylic resin, with the labial portion facing the surface. The samples were then immersed in an 80% lactic acid pH 2 for 48 hours, then rinsed with distilled water and left to dry.

Table II: Mean and standard deviation values for enamel microhardness for different groups

Treatment groups		Vickers microhardness value (Mean±SD) (VHN)	Difference of Vickers microhardness value (Mean±SD) (VHN)	Sig. (2-tailed)
Group 1	Left section (F0)	46.2 ± 6.9	89.3 ± 2.8	0
	Right section (F1)	135.5 ± 10.4		
Group 2	Left section (F0)	46.7 ± 7.2	177.2 ± 8.3	0
	Right section (F2)	224.0 ± 34.0		
Group 3	Left section (F0)	47.2 ± 4.2	288.7 ± 6.4	0
	Right section (F3)	335.9 ± 28.8		
Group 4	Left section (F0)	43.4 ± 3.8	283.1 ± 9.1	0
	Right section (CPP-ACP)	326.5 ± 39.3		

Sample treatment

Each experimental group's left section of the teeth was treated with a control paste (F0). In contrast, the right section was treated with hydroxyapatite paste [F1 (10%) for Group 1; F2 (15%) for Group 2; and F3 (20%) for Group 3] or CPP-ACP paste (GC America INC, America) for Group 4. The left section of the teeth in each experimental group was treated with a control paste (F0). In contrast, the right section was treated with hydroxyapatite paste (F1, F2, and F3) or CPP-ACP paste (GC America INC, America). The paste was applied for 30 minutes, once daily, for seven days. After application of the paste, the samples were then rinsed with distilled water and placed in artificial saliva. After each application of the paste, the teeth were then rinsed with distilled water and put into artificial saliva for 24 hours. The treatment was repeated every day until day 7 with daily replacement of artificial saliva.

Microhardness testing

The microhardness tests were carried out using a Micro Vickers Hardness Tester (HMV-G31DT Shimadzu, Japan) with a load of 100 gf for 10 seconds. The microhardness was determined using three indentations on each sample and expressed as Vickers hardness number (VHN). The VHN was calculated using the formula:

$$VHN = 1854.4 \times \frac{F}{d^2}$$

Where VHN = Vickers Hardness Number (gf/μm^2), P = Applied load (gf), and d = Mean diagonal length of the indentation (μm). The average of the three results of VHN was computed to represent the Vickers hardness value of the sample (8,11).

Enamel microporosity analysis

Enamel microporosity was analyzed using scanning electron microscopy (SEM) (JEOL JSM IT-200, Japan). One sample of each treatment group was randomly chosen. The samples were fixed on an SEM stub and then coated with a thin layer of gold under vacuum. Sample stub kept in SEM chamber, operate at 10 kV. Run the sample and capture the images at magnifications of x2000. Each field of view's microporosity diameter was measured and computed. The mean of enamel microporosity was measured by selecting ten representative porous points per field of view. The enamel microporosity diameter was then totaled, and the mean was calculated. The difference in the diameter of the enamel microporosity of the right section of the teeth was subtracted from the left to determine whether there was a decrease or increase in the diameter of the enamel microporosity of primary teeth (15).

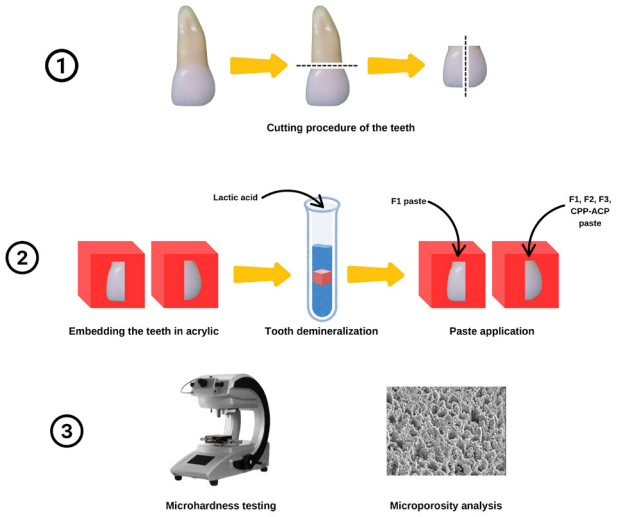

Figure 1: Schematic representation of treatment procedures

Statistical analysis

A T-test was performed to determine the difference in the Vickers hardness value between the left and the right tooth sections. The differences in the Vickers hardness value between the left and right tooth sections were measured and then compared between treatment groups using Kruskal-Wallis, followed by post hoc Mann-Whitney. Statistical difference was set at probability value (p) <0.05.

Statistical difference was set at p<0.05.

RESULTS

Hydroxyapatite has been successfully synthesized from snakehead fish bones using the precipitation method with an average size of 458.9±72.0 nm. The enamel microhardness results revealed variations in enamel microhardness between the left and the right samples. The right samples that were treated with hydroxyapatite (F1, F2, F3) and CPP-ACP paste had a higher enamel microhardness than those treated with control paste (F0). The mean of Vickers microhardness value can be seen in Table II. The T-test results revealed a significant difference in the Vickers microhardness value between the left and right sections of the teeth after hydroxyapatite paste application. A significant difference in the Vickers microhardness value was also observed after the CPP-ACP application.

As the hydroxyapatite concentration rises, so does the enamel's microhardness. The highest increase of enamel microhardness occurred in Group 3. The increase of

Table III: Mann-Whitney test results of enamel microhardness

Treatment groups		Sig. (2-tailed)
Group 1	Group 2	0.049*
	Group 3	0.049*
	Group 4	0.049*
Group 2	Group 3	0.049*
	Group 4	0.049*
Group 3	Group 4	0.051

*significantly difference

Table IV: Mean and standard deviation values of enamel microporosity for different groups

Treatment groups		Diameter of enamel microporosity (Mean±SD) (mm)	Difference of Microporosity Diameter (Mean±SD) (mm)
Group 1	Left section (F0)	3.6 ± 1.0	0.8 ± 0.9
	Right section (F1)	2,8 ± 0.6	
Group 2	Left section (F0)	4.3 ± 1.2	2.3 ± 1.4
	Right section (F2)	2.0 ± 0.6	
Group 3	Left section (F0)	4.2 ± 1.4	3.1 ± 1.5
	Right section (F3)	1.1 ± 0.5	
Group 4	Left section (F0)	4.4 ± 0.7	2.6 ± 0.9
	Right section (CPP-ACP)	1.8 ± 0.7	

the enamel microhardness in Group 3 was also higher than in Group 4. Mann-Whitney test results showed the increase in Vickers microhardness values of Groups 1 and 2, which were applied with 10 and 15% hydroxyapatite paste, was significantly lower than in Groups 3 and 4, which were applied with 20% hydroxyapatite and CPP-ACP paste. (p=0.049). The increase in Vickers microhardness value in Group 3 showed no significant difference with Group 4 (p=0.051) (Table III).

SEM analysis revealed that the surface morphology of the left teeth section, which was treated with control paste, shows a wider porous appearance than the right section, which was treated with 10, 15, and 20% hydroxyapatite or CPP-ACP paste. SEM analysis revealed that the surface morphology of the left teeth section shows a wider porous appearance than the right section. This is per the results of the microporosity diameter calculations, as seen in Table IV. A comparison between treatment groups also showed that increasing concentrations of hydroxyapatite paste led to a greater decrease in microporosity diameter. Using 20% hydroxyapatite paste (Group 3) resulted in a greater reduction in microporosity than CPP-ACP (Group 4). However, the enamel area subjected to CPP-ACP treatment in Group 4 visually represents a smoother texture and reduced porosity in certain regions.

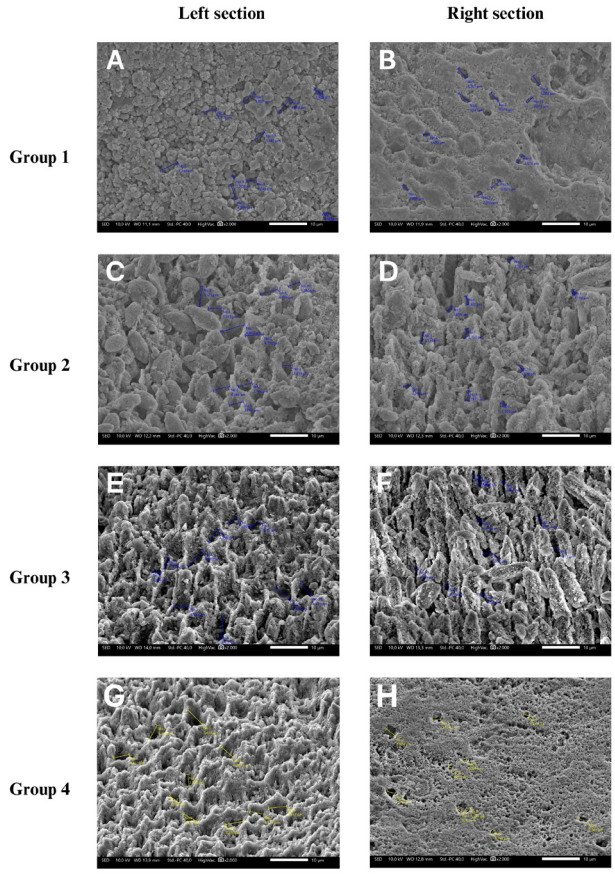

Figure 2: Microporosity of tooth enamel observed using SEM. A. Group 1 left section - after application of F0 paste; B. Group 1 right section - after application of F1 paste; C. Group 2 left section - after application of F0 paste; D. Group 2 right section - after application of F2 paste; E. Group 3 left section - after application of F0 paste; F. Group 3 right section - after application of F2 paste; G. Group 4 left section - after application of F0 paste; H. Group 5 right section - after application of CPP-ACP paste

DISCUSSION

Although fish bones are considered trash in the fish processing business, they are a good source of calcium since they are mostly composed of calcium, phosphorus, and carbonate. Due to its high calcium content, snakehead fish bone (Channa striata) has great potential as a substrate for tooth remineralization pastes. The calcium found in snakehead fish bones can be used to form hydroxyapatite (8,14). The precipitation method is an alkaline acid reaction that yields both water and crystalline particles. This procedure is more straightforward, employing homogeneity and inexpensive basic resources (6). The use of fish bones not only reduces production costs but can also reduce environmental problems caused by fish bone waste from food factories (16).

The application of hydroxyapatite pastes induced remineralization by enhanced enamel microhardness and reduced microporosity of primary teeth. The microhardness of enamel increases and microporosity decreases as the concentration of hydroxyapatite paste increases. This indicates that the remineralization is occurring. This is consistent with the study by Wahyuni et al. and Dewi et al., which demonstrates that hydroxyapatite from snakehead fishbones increases the remineralization process of permanent and primary teeth (8,17). The results of this study are also in accordance with the study by Bajaj et al. and Hadidi et al., which showed that the remineralization capabilities of hydroxyapatite for primary teeth were comparable to CPP-ACP (18,19). According to a study by Davari et al., hydroxyapatite has a superior capacity for remineralization of primary teeth than CPP-ACP (20).

Hydroxyapatite, a calcium apatite mineral, has a molar ratio of 1.67 between calcium and phosphorus with the chemical formula Ca10(PO4)6(OH)2 (21,22). Hydroxyapatite can be used in micro- or nanocrystalline form. The crystal lattice of hydroxyapatite exhibits a remarkable similarity to the molecular composition of apatite crystals found in human enamel (23). Hydroxyapatite also exhibits favorable biocompatibility and bioactivity (24).

It is unclear how hydroxyapatite particles interact with the enamel surface to induce remineralization. One such way is that nano-HAP particles serve as crystal nuclei that draw calcium and phosphate from saliva to cause remineralization due to their strong affinity and adsorption on the tooth surface (25). Hydroxyapatite binds to the damaged enamel surface and fills the micropores on the demineralized tooth's surface (26). It has been demonstrated that hydroxyapatite increases atomicity, elevates surface energy, and demonstrates a strong connection to enamel surfaces (27). The deposition of a new homogenous apatite surface layer on the demineralized surface shields the underlying surface from further demineralization and promotes remineralization. Additionally, hydroxyapatite encourages the accumulation of additional minerals in the outside layer of carious lesions, resulting in a highly mineralized outer layer and preventing mineral ions from penetrating deeper areas of the demineralized lesion (28,29). Hydroxyapatite paste concentration will accelerate hydroxyapatite crystals in the enamel, enhancing its microhardness and decreasing microporosity (15,30).

CPP-ACP—produced from bovine milk protein, casein, calcium, and phosphate—has shown anticariogenic potential in laboratory, animal, and human in situ experiments (31). Hydroxyapatite and CPP-ACP function through slightly different mechanisms but share the goal of restoring lost minerals to the tooth surface. It has been suggested that the anticariogenic mechanism of CPP-ACP results from ACP's localization at the tooth surface, which buffers the activities of free calcium and phosphate ions and helps to keep enamel supersaturated, thereby reducing demineralization and fostering remineralization (32). Based on the study of Sharma et al., compared to hydroxyapatite, CPP-

ACP is less successful at raising enamel's calcium and phosphorus content, and this impact becomes more noticeable over a longer treatment period (33).

However, the present experimental study has some limitations. Since the teeth were extracted from different patients, there may be differences in enamel composition, thickness, and structural integrity due to factors such as age, diet, oral hygiene habits, and individual health conditions. These variations could potentially influence the results and introduce confounding factors that are difficult to standardize. To improve the interpretation of the remineralization process, employing more advanced and reliable methods, such as measuring changes in surface texture using an atomic force microscope (AFM) and measuring the depth of lesion using polarized light microscopy (PLM), can provide a deeper understanding and more accurate results. A study with a larger sample size is also recommended. In addition, the enamel microhardness and microporosity were evaluated under in vitro conditions. In order for the research findings to be more comparable to biological settings, in vivo research is also required.

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CONCLUSION

Hydroxyapatite at concentrations 10, 15, and 20% synthesized from snakehead fishbone can potentially remineralize primary teeth enamel under in vitro conditions by increasing enamel microhardness and reducing enamel microporosity. At a 20% concentration, enamel microhardness increased the most, reaching 288.7±6.4 VHN, and the enamel microporosity diameter decreased the most, measuring 3.1±1.5 µm. This value is higher than CPP-ACP paste with enamel microhardness of 283.1±9.1 VHN and enamel microporosity diameter of 2.6±0.9 µm, but these findings did not indicate a statistically significant difference. Hydroxyapatite derived from snakehead fishbone promotes remineralization to prevent primary dental caries.

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