Chelonian Conservation And Biology

Vol. 19No.1 (2024) | https://www.acgpublishing.com/ | ISSN - 1071-8443

DOI:doi.org/10.18011/2024.01(1). 1257-1271

# THE EFFECT OF CARBOXYMETHYL CHITOSAN AMORPHOUS CALCIUM PHOSPHATE APPLICATION ON PRIMARY TEETH ENAMEL HARDNESS

## (IN VITRO STUDY AFTER EMAIL DEMINERALIZATION)

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**Abstract**

Dental caries is an oral disease that often occurs in children, especially in developing countries. The importance of early caries prevention triggers many studies on new prevention methods, especially remineralizing materials. Tooth remineralization can be observed by testing the hardness of the tooth structure. To analyze the effect of CMC-ACP application on the enamel hardness of primary teeth. Experimental laboratory study. 36 primary tooth enamel samples were used; the samples were demineralized with 37% phosphoric acid etching, then divided into 3 groups, namely the negative control group with no treatment, the positive control group with CPP-ACP application, and the CMC-ACP application group. The enamel hardness test was carried out after applying the material on 7 days and 14 days using Vickers Hardness Tester. The results of the enamel hardness test on day 7 and day 14 of CMC-ACP applications were calculated using the *paired sample t-test* with a significance limit of $p<0.05$. The results of this test $p = 0.001$. The results ($p<0.05$) showed that enamel hardness values on day 7 CMC-ACP application had a statistically significant difference from the group on day 14. There was a significant increase in primary tooth enamel hardness from the application of CMC-ACP on days 7 and 14.

**Keywords:** Carboxymethyl Chitosan Amorphous Calcium Phosphate; hardness test; primary teeth

## INTRODUCTION

Dental and oral disease, especially caries, is a problem that often occurs in children, especially in developing countries.[1-3] According to the Basic Health Research (Riskesdas) of the Ministry of Health of the Republic of Indonesia, 2018, as many as 92.6% of children aged 5-9 years had dental caries.[4] Dental caries that are not treated immediately can affect growth and

development, systemic appearance, language, and self-confidence, which can affect the child's quality of life. Prevention and early dental care are very important, especially in children with a high risk of caries.[1, 5] The importance of prevention and early treatment of dental caries has triggered much recent research on new caries prevention methods.[2, 6]

The etiology of caries that influences lesions' initiation and development is multifactorial, consisting of *host,* substrate, microorganisms, and time.[7] *Host* (host) in this case is tooth enamel, which consists of several main components, namely, hydroxyapatite crystals, water, and also an organic matrix.[8, 9] The caries process begins with enamel demineralization, so alternative remineralization to increase enamel hardness includes administering minerals, which is an effort to prevent caries. Chitosan material has been researched and proven to be used to increase the amount of minerals in permanent tooth enamel.[2, 10, 11]

Chitosan is a polymer obtained by deacetylation of chitin. Chitosan is a biocompatible and environmentally friendly material and is considered non-toxic. [2, 12, 13] Chitosan can be easily extracted from animal exoskeletons, especially shrimp, lobsters, crabs, crabs, clams, and insects.[10, 11] The characteristics of chitosan are environmental friendliness, good biocompatibility, hydrophilic, antibacterial properties. They can be processed into various forms, making chitosan widely used in dentistry as an anti-caries, hemostatic, and pain-reducing agent. Chitosan is also used as an additive to improve the physical properties and increase the mechanical properties of dental materials.[14, 15]

Chitosan has low solubility at physiological pH, thus limiting its use and requiring higher solubility. Another disadvantage of chitosan is its high water absorption, so chemical modification of two hydroxyl groups and one amino in the chitosan chain using some carboxymethyl will change the properties of chitosan, namely its solubility in water in various pH environments regulated by the degree of carboxymethylation. This modification of chitosan creates a material called *Carboxymethyl Chitosan (CMC),* which has various biological properties such as antimicrobial, anticancer, antitumor, antioxidant, and antifungal. CMC is widely used in various fields such as wound healing, tissue engineering, as well as drug or enzyme delivery agents.[15, 16]

Other agents for remineralizing tooth enamel to increase enamel hardness or prevent carious lesions are *Casein Phosphopeptide-Amorphous Calcium Phosphate* (CPP-ACP). The casein used is a cow's milk protein derivative.[17-19] The advantages of CPP-ACP include stabilizing calcium phosphate and maintaining ions in the oral environment, thereby increasing calcium phosphate levels in the biofilm and providing benefits to the tooth enamel structure, namely remineralization. CPP-ACP products are widely available in paste and solution and widely used to treat incipient caries lesions. However, CPP-ACP has a drawback, namely that it can cause allergies in children who are allergic to cow's milk protein because the essential ingredients are made from cow's milk protein derivatives.[17, 18]

The combination of chitosan and ACP has been proven to reduce demineralization of permanent tooth enamel and can remineralize enamel to prevent caries lesions.[6] In another study, the CMC-ACP material could remineralize demineralized dentin and stated that the duration of CMC-ACP application had an effect on the remineralization of dentin.[20, 21] This increase in remineralization is significant to restore the balance between demineralization and remineralization of problematic tooth enamel tissue so that the progression of carious lesions can be prevented.[6, 22] The occurrence of remineralization can be measured through changes in the hardness of tooth enamel.[19]

Until now, research on the application method of CMC-ACP as an enamel remineralization agent for primary teeth is still limited compared with research on permanent tooth enamel and dentin. So, the researchers wanted to know the extent of the effect of applying the CMC-ACP combination by measuring the hardness of primary tooth enamel that had been demineralized in vitro with a frequency of 7 days and 14 days.

## Library Review

Email

Enamel consists of several main components, namely, hydroxyapatite crystals, water, and also an organic matrix.[8, 9] The main mineral component of enamel is calcium hydroxyapatite, $That_{10}(AFTER_4)_6(OH)_2$.[9, 12] These minerals comprise approximately 88-90% of the enamel tissue by volume and approximately 95-96% by weight. The core of the crystal dissolves more quickly than the edges of the crystal.[9] The water component of enamel is essential, because ions such as fluoride will move through the water component.[8, 9] Mature email contains 1-2% organic matrix weight. The most critical components of the organic matrix are enamel proteins consisting of amelogenin and non-amelogenin.[9] The basic structural unit of email is the email prism or *enamel rods*. Each enamel rod consists of millions of hydroxyapatite crystals packed into long, thin rods with a 5-6 μm diameter and up to 2.5 mm long.

Email is the most complex biological tissue in the human body. Enamel will withstand shear and impact forces well when mineralized because it has high abrasion resistance, allowing it to wear out slowly. This is an important property because enamel cannot be repaired or replaced. The elastic modulus, together with the flexible support of the underlying dentin, minimizes the possibility of fracture of the tooth.[8, 9]

Primary tooth enamel differs from permanent teeth in terms of morphology, chemical content, and physiology. [23] The outer layer of primary teeth has a higher mineral density than other structures. Studies conducted on primary and permanent teeth show that primary teeth are more susceptible to erosion.[24] The average depth of the lesion is related to the density *prism-junction* and interprismatic enamel volume, both of which are significantly larger in primary teeth than in permanent teeth.[23, 24] Primary tooth enamel, with a higher organic material content, dissolves more quickly than permanent teeth. The mineral content of primary teeth is smaller than permanent

teeth, namely 81.33–94.2%, while for permanent teeth, it is close to pure synthetic apatite (around 97%); the rest consists of water and organic matrix.[23, 24] According to studies conducted on primary and permanent teeth immersed in acidic media, primary teeth show a significantly higher susceptibility than permanent teeth to demineralization.[24]

Demineralization and Remineralization of Teeth

Demineralization (mineral loss) and remineralization (mineral gain) of teeth is a dynamic physicochemical process.[25, 26] The ongoing demineralization process can cause carious lesions caused by *host,* substrate, microorganisms, and time.[7] The demineralization process includes hydroxyapatite dissolution, mainly caused by acid attack originating from the sugar fermentation of biofilm bacteria. Acid attacks cause the pH to decrease and diffuse into dental plaque so that the plaque pH can reach 4 - 4.5. This mineral loss damages the mechanical structure of the teeth and, over time can form demineralized lesions.[27, 28] Demineralized lesions can develop into subsurface lesions with rapid remineralization of the upper part of the lesion in the presence of *fluoride* in saliva.[19, 27, 29] Demineralized lesions formed without a cavity are called "*white spot*".[6, 27] Enamel demineralization depends on the degree of mineralization and chemical content of the enamel to which it is exposed. In more porous enamel, deeper lesions will develop.[25]

Remineralization is a process in which calcium and phosphate ions are taken from external sources of the tooth to increase ion deposition into the crystal cavities in demineralized enamel.[19, 29] Remineralization occurs when lost tooth minerals are replaced by the deposition of dissolved ions in saliva or biofilm.[27] The mechanism of remineralization is the opposite of demineralization. When the pH of the oral cavity returns to near neutral, Ca ions $^{2+}$ and $PO^{4}$ in saliva combine into the thinned mineral layer of enamel as new apatite. Demineralization zones in crystals act as nucleation sites for depositing new minerals. As is *fluoride* at high concentrations, the original carbonate hydroxyapatite loses the remaining carbonate and is replaced by hybrid hydroxyapatite and fluorapatite. A sudden drop in pH results in less saturation of essential ions (Ca and PO) in the plaque fluid, which causes the enamel to dissolve. At high pH, ionic supersaturation of plaque shifts the balance in the other direction, causing mineral deposition on the teeth.[28] As long as the demineralization and remineralization processes occur at the same rate and there is a balance, the enamel structure will not be lost.[12]

Minimally invasive approaches, such as remineralization therapy to treat incipient lesions are essential in modern pediatric dentistry.[6, 28] This aims to prevent incipient caries lesions by inhibiting the demineralization process, preventing further mineral loss and increasing the remineralization process.[6, 30] Currently, many calcium phosphate-based systems are being developed as remineralization agents.[6]

*Casein Phosphopeptide-Amorphous Calcium Phosphate* (CPP-ACP) is a remineralization agent often used in dentistry. CPP-ACP contains casein in the form of casein phosphoprotein (CPP), which has high levels of calcium and phosphate to inhibit demineralization. Casein can

stabilize calcium ions ($Ca^{2+}$) and fosfat ($P.O_4^{3-}$), derived from phosphorus, by releasing a group of peptides through enzymatic work at neutral pH until the process *buffer* by saliva is maintained. The remineralization process can occur.[19, 31] *Amorphous calcium phosphate* (ACP) is the initial solid phase precipitating from a highly saturated calcium phosphate solution. ACP can be easily converted into a stable crystal phase such as octacalcium phosphate or apatite products. Therefore, ACP requires a combination of materials to maintain its stability. CPP is one of the materials that can maintain the stability of ACP.[31, 32] Even though CPP-ACP is now widely used, this material has a drawback, namely that it can cause allergies in children who are allergic to milk protein because the basic ingredient is made from milk protein derivatives. Until now, other remineralization agents continue to be researched to accommodate the shortcomings of previous materials.[17, 18]

*Carboxymethyl Chitosan Amorphous Calcium Phosphate* (CMC-ACP)

Chitosan is a straight-chain, cationic polysaccharide that occurs naturally or is obtained from the deacetylation of chitin. Chitin (Figure 5) is a natural polysaccharide from crustacean shells, insect cuticles, and in fungal cell walls. Chitin is the second most abundant polymerized carbon found in nature. Chitosan is obtained from the deacetylation of chitin base.[12, 9, 10] The copolymer obtained has properties *that are biodegradable*, consisting of D-glucosamine units containing free amino groups.[1, 14] Chitosan dissolves in acid solutions, such as acetic acid, formic acid, lactic acid, as well as inorganic acids, after prolonged agitation. However, solubility depends on several parameters, such as the degree of deacetylation, molar mass, acid and biopolymer concentration, and ionic strength.[12, 14] Chitosan can be widely used in various fields because of its biocompatibility, biodegradability, antibacterial, emulsifier and antibacterial properties. *chelating*.[2, 12, 14]

Chitosan has low solubility at physiological pH (6.0), thus limiting its application in systems that require higher solubility. Chitosan has poor solubility in water due to its rigid crystal structure, which limits its practical work in various processes.[15, 34] This problem can be overcome by making derivatives of chitosan that are soluble in water. The presence of hydroxyl and amino functional groups in its structure results in chemical modifications to increase physical solubility and electrical charge. [15] Chemical modification of the two hydroxyls and one amino group in the chitosan chain using a carboxymethyl group will change the properties of chitosan. One derivative of chitosan is *carboxymethyl chitosan* (CMC).[20, 34, 35] The carboxymethylation reaction occurs primarily at the C-6 hydroxyl group or at the NH2 group which produces the compound N, O-carboxymethyl chitosan which is soluble in water and consists of either an amino group as a primary ($-NH_2$) or as a secondary amine ($–NH–CH_2COOH$). [15] CMC is rich in carboxyl groups, so it has a high calcium content in the CMC-ACP combination and plays a role in the remineralization process.[20, 34] ACP is a material that has an important role in biomineralization. ACP and its modifications can reduce demineralization and potentially be a caries prevention method. The stabilization of ACP has been studied, and CMC is one material proven to stabilize ACP and prevent its particles from transforming into crystals.[14, 34, 35]

Chitosan is used to prevent dental caries because it provides bactericidal and bacteriostatic activity.[2, 6, 10] Group amino-$NH_2$ chitosan inhibits the reaction to cariogenic acids and reduces the solubility of the acid in hydroxyapatite. This amino group can capture hydrogen ions so that the hydrogen ions become positively charged. [10] This positive charge makes chitosan an adhesive on negatively charged surfaces such as tooth enamel and cell membranes. Recent studies show that chitosan has an effect on the remineralization process and has anti-cariogenic activity because chitosan acts as a *barrier* against acid penetration, contributes to inhibiting demineralization, and inhibits the release of phosphorus from enamel, thereby interfering with enamel demineralization.[2, 6, 10] Chitosan has been used as a structure-forming agent in the formation of calcium phosphate, and hybrid materials obtained in this way are widely used for restoration of the mineral structure of teeth.[6] The remineralization agent based on chitosan and calcium phosphate is CMC-ACP. Hybrid materials based on chitosan micro/nano gels with in situ calcium phosphate have never been reported or used for tooth remineralization studies.[6]

In the study, a chitosan/calcium phosphate hybrid microgel was developed for the first time. Its potential and effectiveness as a remineralization agent were discovered, increasing the nucleation and growth of calcium phosphate on the surface of demineralized enamel. The formation of hybrid microgels, guided by chitosan macromolecules, which act as templates for calcium phosphate deposition, resulting in the in situ formation of amorphous hydroxyapatite. This material has several advantages as a remineralization agent, including bio-adhesive and antimicrobial properties, and a source of sustainable reserves of calcium and phosphate ions to enhance the remineralization of incipient caries lesions.[6] This study reveals a simple and easy-to-apply remineralization procedure based on a newly developed hybrid material that can be used effectively to stop as well as enhance remineralization in the early stages of caries development.[1, 6]

Another study also revealed that chitosan hydrogel can be used as an enamel remineralization agent and has been proven to be an effective treatment modality. [13] Results of gel enamel hardness research *fluoride* and chitosan hydrogel with the results of these two materials can produce remineralization of enamel tissue. The highest microhardness values of demineralized enamel tissue were found after gel application *fluoride*. Chitosan hydrogel demonstrated biomimetic remineralization of enamel, which provides a breakthrough for enamel tissue regeneration in the modern era of preventive dentistry.[13]

In a study conducted by Niu et al., the release of calcium ions was studied in a combination of chitosan with ACP, which was applied for 7 days and 14 days. The 14-day application was shown to have a significant increase in the number of calcium ions compared to the 7-day application. An increase in the amount of calcium released indicates that remineralization is occurring.[36]

In an in vitro study conducted on premolars, demineralization was carried out using chitosan hydrogel for 5 and 10 days. The results under SEM showed that the samples at day 10

showed superior and uniform re-establishment of surface integrity compared to the 5 day and control groups. The porosity that arises during demineralization decreases, and crystals on the enamel surface regenerate to form a homogeneous and dense layer of mineral tissue.[12]

Dental Microhardness Test

Hardness is the ability of a material to resist plastic deformation, scratches or abrasion due to penetration. The hardness of tooth enamel is an indicator of tooth weakness against demineralization.[30, 31] Hardness is measured by indenting the specimen with a standard force or weight. Symmetrical indentation is measured under a microscope for the depth, extent, or width. Indentation dimensions are inversely proportional to penetration resistance. Lighter loads are used for softer materials.[8]

A Microhardness test is a method for determining the hardness or resistance of a material when the test sample cannot be tested with a microhardness test. The microhardness test is ideal for evaluating the hardness of small, thin, or complex samples. Hardness test *Button* and *Vickers* are classified into microhardness tests, whereas *Brinell* and *Rockwell* include macro hardness tests. Test *Button* and *Vickers* using a load of less than 9.8 N. The resulting indentation is very small and limited to less than 19mm depth.[31] Micro hardness test *Vickers* uses form indentation *136-degree pyramid-shaped indenter diamond* which forms an indent shaped like in figure 2.6.[8] Microhardness *Vickers* has a unit of account, namely *Vickers Hardness Number* (VHN). The Vickers microhardness test is suitable for testing the hardness of brittle materials and is also widely used to measure the hardness of tooth structure.[31]

## METHODS

This research uses a laboratory experimental design with samples of human deciduous teeth extracted as indicated, physiological resorption or persistence, and treated by soaking in distilled water until the research time. The application of CMC-ACP material in this study was the application of 5% CMC-ACP to the enamel surface of primary teeth after demineralization with 37% phosphoric acid etching for 30 seconds. CMC-ACP 5% application for 7 and 14 days, on primary tooth enamel after demineralization. The microhardness value of primary tooth enamel after treatment is the material's resistance to indentation, measuring the hardness of primary tooth enamel after demineralization and the application of 5% CMC-ACP. The hardness test in this study used a *Vickers Microhardness Tester* (VHN) with a pressure of 200 g for 15 seconds.

The hardness value was measured as many as (four) times/email of the first tooth, namely, initial hardness after first tooth enamel demineralization, CMC-ACP application day 7, and CMC-ACP application day 14.

This research was carried out in October-November 2022 with the inclusion criteria of intact human deciduous incisors. The exclusion criteria in this study were teeth with restorations, caries, enamel defects, fractures, attrition/erosion/abrasion, and teeth stored without storage media.

The sample size calculation used the Federer formula with three groups, resulting in 12 groups for each group and a total sample of 36 primary teeth. [39, 40]

The research began with approval from the FKG UI Ethics Commission, and then 36 primary teeth were collected according to the criteria. Sample preparation was carried out for 36 primary tooth samples by implanting the teeth in acrylic resin. After complete preparation, an initial hardness test is carried out on all samples. All samples were then demineralized using 37% phosphoric acid etching. For 15 seconds, rinse with distilled water for 30 seconds, and dry with *a chip blower.* After demineralization, a hardness test will be repeated on all specimens.

CMC-ACP material is made by mixing 2.5 grams of CMC with 40 ml of distilled water, then stirring at 1000 rpm until the CMC powder dissolves and forms a gel. 0.498 gr K4 is added to the CMC and stirred at a speed of 500 rpm. A total of 0.555 gr $CaCl_2$ was added in 10 minutes of distilled water, and this solution is dripped into the CMC gel slowly for 5 minutes so that there is a CMC-ACP gel.

36 primary tooth samples were divided into 3 test groups, namely, group I with no treatment (only soaked in distilled water), group II (application of CPP-ACP material), and group III (application of CMC-ACP material). In group I, the specimens were soaked in distilled water, and the distilled water was replaced every day (24 hours). This procedure was carried out 7 times for 7 days; then, a hardness test was carried out. After the hardness test is carried out, it is re-immersed in distilled water, which is replaced every day (24 hours). This procedure was repeated 7 times for 7 days (14 days of treatment), and the hardness test was carried out again. In groups II and III, the material for each group was applied by applying a thin layer of material to the specimen once a day using an applicator. After application, leave for 5 minutes, then soak in distilled water for 12 hours in an incubator. After 12 hours, the specimens were rinsed with distilled water and dried with a *chip blower*. Next, the specimen was soaked again in distilled water for 12 hours. This procedure was carried out 7 times for 7 days; then a hardness test was carried out. After carrying out the hardness test, soak the specimen again in distilled water. Next, repeat the application with the same procedure, namely repeat this procedure 7 times for 7 days (14 days), then carry out a hardness test.

Data and statistical analysis begins with a data normality test; because the data is less than 50, a test is used, *Shapiro-Wilk*. Analysis of paired comparative statistical tests to determine changes in enamel hardness after CMC-ACP application on day 7 and day 14, tested using *Paired sample t-test* when the data distribution is normal, test *Willcoxon* done when the data distribution is not normal.

## RESULT

In this study, the sample used was 36 primary incisors by the research sample criteria. The samples taken are processed according to established procedures, and then four hardness tests are carried out, namely, initial violence, subsequent violence demineralization, hardness after

application on the 7th day, and hardness after application on the 14th day. Each hardness test on each sample is carried out three times using *Vicker's Microhardness Tester*, and then the average value is taken. Samples were tested at the Metallurgical Engineering Laboratory, Tarumanegara University, Jakarta. The test result values obtained are as follows: *Vickers Hardness Number*.

The first step is to test the normality of the data using a test *Saphiro-Wilk.* In the normality test results, all data groups (after CMC-ACP application after 7 days and 14 days) showed p>0,05 (normal distribution), so a parametric test is carried out, namely *paired sample t-test* with a significance limit of p<0.05 between the enamel hardness values of primary teeth after application of CMC-ACP on day 7 and after application of CMC-ACP on day 14. This statistical test uses the numerical sample of two paired groups. The results of the statistical analysis can be seen in Table 5.1. The statistical analysis results showed p<0.05, meaning there is a significant difference between email hardness primary teeth after CMC-ACP application *on the* 7th day and after CMC-ACP application on day 14.

Table 1. Analysis of mean enamel hardness values for primary teeth after CMC-ACP application on the 7th day and after CMC-ACP application on the 14th day

| **Email Severity Rate** | **Mean (SD)** | **P** |
|---|---|---|
| After the 7th day of the CMC-ACP application | 332,67 (7.58) | 0,001* |
| After the CMC-ACP application on the 14th day | 353 (9.67) | |

Based on the results *of the paired sample t-test,* *significant p < 0.05

## DISCUSSION

This study aims to determine the effect of CMC-ACP application after application on the 7th day and 14th day on the hardness of primary tooth enamel. The enamel hardness of primary teeth was measured to see the remineralization process after applying the CMC-ACP material.[19] Increasing remineralization is very important to restore the balance between demineralization and remineralization of tooth enamel hard tissue so that the progression of caries lesions can be prevented. New methods of preventing caries in children's teeth are often carried out using remineralization therapy and remineralization agents.[27] It is hoped that early caries prevention can improve a child's quality of life.[1, 5]

The hardness test carried out in this study took one of the tooth structure samples, namely enamel. Enamel is the most muscular outer biological tissue of teeth in the human body. Enamel will be more resistant to forces and abrasion that occur during chewing when it is well mineralized.[8, 9] During the mineralization process if demineralization and remineralization occur at the same and balanced rate, there will be no loss of enamel structure. as a trigger for initial caries lesions.[12] The enamel samples used in this study were primary tooth enamel because the researchers wanted to see the remineralization effect of CMC-ACP on primary teeth, which are

more susceptible to demineralization than permanent ones. This is caused by the lower mineral content of primary teeth than permanent ones, so they dissolve more efficiently, especially in acidic media. Research on the hardness of primary teeth after CMC-ACP application has never been done before.[23, 24] In this study, 36 primary tooth samples were obtained, and enamel samples were taken from human primary incisor teeth. The enamel hardness test is carried out on the middle third of the labial because the surface contour of this part tends to be flatter than other surfaces, making the test easier.[32]

This research uses a microhardness test with *Vickers* to see the hardness of tooth enamel and indicators of tooth weakness against demineralization. The Vickers hardness test has been used in previous research on primary teeth.[33] The Vickers microhardness test is used on small, thin, and complex samples such as the tooth structure samples used in this study.[31] In this test, Vickers used a load of 200 g, in accordance with the study of Chuenarrom et al., to test enamel; a load of 100, 200, or 300 grams was selected. The greater the test load, the more visible the indentation is on an optical microscope, and the easier it is to test. However, large loads are not recommended for experimental tests that have several test times (*pre-post test*) because after-treatment can produce an indentation impression greater than that which an optical microscope can measure.[34]

In this study, the CMP-ACP material was chosen as a remineralization material because this material has the advantage of having bio-adhesive, antimicrobial properties and is a source of sustainable reserves of calcium and phosphate ions to increase the remineralization of demineralized lesions.[6] CMC is a chitosan derivative in this study, which was processed from prawns. CMC can stabilize calcium ions ($Ca^{2+}$) and fosfat ($P.O._4{}^3$) from ACP.[14, 35, 36] ACP itself is a mineral source with good bioactivity and biocompatibility in the remineralization process.[37] ACP has a crystalline structure at neutral pH. However, a stabilizer is needed to keep the calcium and phosphate ions in an amorphous (shapeless) state so that the calcium and phosphate ions can enter the enamel of primary teeth. CMC is one material proven to stabilize ACP and prevent its particles from transforming into crystals.[14, 35, 36] To date, CMC-ACP, as a remineralization agent, continues to be studied to overcome the lack of previous materials such as fluoride-based materials (if Excessive amounts can cause fluorosis) and CPP-ACP ingredients (can cause allergies in children with cow's milk allergies).[17, 18]

This research uses CMC-ACP in gel form to imitate the initial formation of enamel hydroxyapatite crystals, and gel is the optimal medium for crystal growth.[13] In this study, CMC-ACP with a concentration of 5% was used because in a study conducted by Setiati, it was concluded that the application of CMC-ACP with a concentration of 5% was practical in remineralizing the permanent tooth structure with a dense new layer of mineral.[36, 38] The protocol for preparing 5% CMC-ACP gel was, according to Chen et al., namely by mixing 2.5 grams of CMC with 40 ml of distilled water, then stirring and adding 0.498 grams of $K_2HPO_4$, then stirring. In the gel, 0.555 gr of CaCl was added put it in 10 ml of distilled water, and this solution was dripped into the CMC gel slowly for 5 minutes so that there was a CMC-ACP gel.[21, 36]

The material in this study was applied by applying a thin layer of CMC-ACP 5% all over the work area, leaving it for 5 minutes, then soaking it in distilled water. This is done in an incubator for 12 hours to imitate the length of time a child sleeps, after which the tooth samples are rinsed with distilled water until clean. This procedure is carried out once per day and repeated 7 times (7 days).[6, 39] After 7 days, a hardness test was carried out on the primary tooth enamel. After the hardness test, the CMC-ACP application was repeated 7 times (7 days) for 14 applications (14 days of application). Then the hardness test was carried out again. This research was applied with a frequency of 7 and 14 days because calcium and phosphate ions need time to diffuse into the enamel and become denser crystals, thereby replacing the calcium and phosphate ions from the enamel structure.[12, 40, 41] In another study, the calcium ions released by chitosan combined with ACP experienced a significant increase on days 7 and 14. After 14 days, the release of calcium ions became constant and did not increase significantly.[37] Another study conducted, after remineralization on day 7 of application of chitosan combined with ACP, space *inter rods*, the enamel structure is reduced by the formation of new material such as crystal grains, and at the end of the remineralization procedure, all demineralization lesions are covered by the crystallization structure of the remineralization material.[6]

All primary tooth samples were demineralized using 37% phosphoric acid etching for 15 seconds. According to studies that have been conducted, etching applications can create demineralized lesions. SEM shows that the size of the porosity has increased due to the dissolution of the rim of the enamel rods by acid etching. Decreased hardness values and changes in tooth enamel structure are related to loss of minerals or demineralization of tooth enamel.[42, 43]

In this study, apart from applying the CMC-ACP material, the CPP-ACP material was also tested as a positive control, and without treatment, the application of the material was a negative control. The positive control results, namely CPP-ACP, are expected to always be in accordance with the results of previous research. CPP-ACP is used as *a gold standard* test because there have been many studies testing this material, and it has been proven that this material can be used as a remineralization agent and can increase the hardness of enamel. Negative control was carried out to prove that there was no increase in enamel hardness in the absence of a material application, and the increase in hardness that occurred in the CMC-ACP group was purely due to the application of this material. Hardness testing on positive and negative controls is carried out to ensure that all procedures are carried out correctly and the results are accurate.[10, 40, 41]

The statistical test results in Table 5.1 show significant results between the 2 groups from the mean hardness value of primary teeth after applying CMC-ACP on day 7 and after applying CMC-ACP on day 14, where the p-value <0.05. The mean hardness value of primary teeth from CMC-ACP application on day 7 increased significantly when compared with CMC-ACP application on day 14.[44, 45]

There is a significant difference in the hardness value of primary teeth between the frequency of application of CMC-ACP material on the 7th and 14th days. This is because the time

factor or frequency of application influences the amount of remineralization that occurs. The remineralization event in this study was associated with an increase in hardness values between the 7th and 14th days of CMC-ACP application. The remineralization in enamel with CMC-ACP application results from calcium and phosphate ions intake. A layer *reservoir* is formed in enamel due to high ionic concentrations, creating strong and stable enamel crystals after the crystal structure dissolves in demineralization.[40] The complete remineralization process to replace the calcium and phosphate ions released from hydroxyapatite requires a period; according to Ibrahim et al., the remineralization process is not complete in the 5-day application time because, in the SEM, it is still visible that there are surface irregularities, there is still reduced porosity in the space. *Inter rods* and the demineralized enamel profile are still visible even though growing crystals almost cover it. [12] This is also by the study conducted by Niu et al., namely, the combination of chitosan with ACP, which was applied for 7 days, experienced a significant increase in calcium ions with an application duration of 14 days, but after 14 days, the increase in calcium ions was constant and the addition was not significant. This study concluded that application frequency influences increased remineralization, and increased remineralization can be characterized by increased hardness of the enamel structure.[37, 41]

In the remineralization process, chitosan acts as a structured assembly template in the biomineralization process. It controls mineral crystallites through molecular interactions between polymers and minerals in the demineralized enamel layer.[12] According to a study from Miftah et al., the duration of application of remineralizing agents can increase enamel hardness. The process of forming apatite minerals occurs immediately after calcium or phosphate ions enter and come into contact with the enamel. The formation of apatite crystals begins with the deposition of small-sized crystals, and these small-sized crystals will melt and form larger crystals up to the maximum size, thus forming dense enamel hydroxyapatite crystals. The longer the application duration, the more perfect the remineralization process will be because the crystals formed become denser until they reach the peak, namely all the crystals have formed and replaced all the ions lost in the demineralization process.[6, 37, 41]

Based on the results of this study, the hardness value of primary teeth after application of CMC-ACP on day 7 experienced a significant increase to day 14. This shows that the frequency of application affects the hardness of primary teeth; that is, the more frequency of application, the higher the hardness value of primary teeth. .[12, 37, 40, 41] The hardness of primary teeth indicates remineralization of primary teeth after demineralization.[6, 37] It is hoped that this research can become the basis for making CMC-ACP as a remineralization material for primary teeth after carrying out other tests such as SEM, EDX (*Energy Dispersive X-ray*), or TEM (*Transmission Electron Microscopy)*.

## CONCLUSION

There is an influence on the hardness of primary tooth enamel that has been demineralized after application of CMC-ACP on the 7th day and the 14th day. The hardness of primary tooth enamel increased significantly after CMC-ACP application from the 7th and 14th days.

### Suggestion

Further research needs to be carried out to support the CMC-ACP material in looking at its remineralization ability to be used as a remineralization material for primary teeth. Tests *in vitro* and other things that can be done include qualitative tests with SEM to see remineralization on the surface microscopically or with EDX to measure calcium and phosphate levels.

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