The Hardness of Tooth Enamel Post-Application of CPP-ACP as Remineralization Material on Demineralized Tooth

Opik Taofik Hidayat[1], Lini Meriani[2], Irmaleny[1]*

1. Department of Conservative Dentistry, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia.
2. Dental Student of Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia.

Abstract

CPP-ACP is a cow milk protein derivate which is a source of calcium and phosphate ions. These calcium and phosphate ions may replace hydroxyapatite calcium structures that are damaged due to the demineralization process, resulting in the process of tooth remineralization.

In vitro true experimental research using 30 maxillary premolar post-extraction teeth with the criteria of no fillings, no caries, no fractures, and completely closed root apex. The teeth were divided into two groups: 15 premolar teeth as a control group and 15 premolar teeth as a test group. Both groups were submerged in Pocari Sweat isotonic drink for 30 minutes to be demineralized. Then the control group was submerged only in artificial saliva. The test group was submerged in artificial saliva and had CPP-ACP applied for 21 days, after which the hardness of each tooth was measured using a Vickers hardness tester.

Data analysis done with the ANOVA statistical test and continued by a post hoc test showed a meaningful difference in the increase of tooth hardness before and after application of the remineralizing material CPP-ACP on the test group. Statistical test results showed no meaningful differences between the test group and the control group which didn't have CPP-ACP applied.

The application of CPP-ACP as a remineralizing material for 21 days can increase the hardness of demineralized tooth enamel.

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Introduction

Demineralization is the process of removal of mineral ions from hydroxyapatite crystals on hard tissues such as enamel, dentin, cementum, and bones.[1] Demineralization of the tooth is caused by acid attacks. These acids may originate as a result of bacterial metabolism, in which carbohydrates are fermented into acid, or directly from consumed foods and drinks that are acidic.[1,2] The consumption of acidic drinks such as carbonated drinks, isotonic drinks, fruit juice, and fruit-flavored tea, has increased for several decades in both developed and developing countries, especially on children and teenagers.[3]

Corresponding author:
Irmaleny
Department of Conservative Dentistry, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia.
E-mail: irmaleny@fkg.unpad.ac.id

Demineralization may happen if the enamel is surrounded by an environment of lower pH levels than 5.5. Low pH levels will increase the concentration of hydrogen ions and these ions will destroy hydroxyapatites of the enamel.[2] Continuous demineralization may cause the formation of porosity on the surface of the enamel which decreases the hardness of the enamel.[4] A demineralizing enamel cannot fully repair itself due to its non-regenerative quality.[5] Demineralization will stop if the acid concentration becomes low and calcium concentration or saliva phosphorus increases which result in the process of remineralization.[6]

Remineralization is the regeneration of minerals that have been lost as a result of demineralization. This process involves calcium mineral ions and phosphate ions which reform hydroxyapatite crystals on the enamel.[7] The ions which were lost during demineralization may be regenerated if pH levels are neutralized and enough calcium and phosphate ions are present in the tooth's environment.[6] Remineralization is an important process which significantly influences the hardness and strength of teeth.[8] Remineralization may be naturally achieved by the saliva which serves as a buffer as well as a carrier of essential ions which may accelerate remineralization.[9] However, the effect of natural remineralization from saliva sets slowly and are not sufficient to protect individuals from the process of demineralization without the help of additional agents to increase the effect of remineralization.[10]

Remineralizing materials such as fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP), functionalized tricalcium phosphate (fTCP), bioactive glass, nanohydroxyapatite, dicalcium phosphate dihydrate (DCPD), xylitol-coated calcium phosphate, and arginine are materials that are used to accelerate the process of remineralization.[7] Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a remineralizing agent which has been studied and extensively accepted to this day.[11] CPP-ACP is a cow milk protein derivate which is a source of calcium and phosphate ions. These calcium and phosphate ions may replace hydroxyapatite calcium structures that are damaged, resulting in the process of tooth remineralization.[12] CPP-ACP can localize calcium and phosphate ions on the tooth's surface, therefore guarding the buffering process by the saliva which helps preserve the neutral pH levels of the enamel by suppressing demineralization and accelerating remineralization.[6] CPP-ACP possesses a topical anti-cariogenic effect due to its ability to stabilize calcium and phosphate under amorphous conditions.[13]

The levels of biologically available calcium correlates significantly with the increase of remineralization of lesions under the enamel's surface. The presence of saliva and biofilm is necessary for the working mechanism of CPP-ACP. In an acidic environment, ACP is separated from CPP so that calcium and saliva phosphorus levels increase. CPP may stabilize levels of ACP in the saliva by preventing the sedimentation of calcium and phosphate, while also stabilizing levels of calcium.[13] The combination of CPP-ACP with fluoride results in the localization of calcium and phosphate ions with fluoride ions on the surface of the enamel which is known as casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP). The advantage of CPP-ACFP is the availability of calcium, phosphate, and fluoride all in one product. Calcium phosphate in this complex is biologically available for lesion remineralization under the surface of the enamel.[14] CPP is also a carrier of good fluoride ions and can carry fluoride ions to deeper lesion areas while also increasing its permeability. The increase in fluoride ions results in more fluorapatite and hydroxyapatite, promoting more of the effects of remineralization.[15] However, the amount of fluoride in drinking water, foods, and toothpaste must be taken into account, because excessive intake of fluoride may cause fluorosis.[16] The advantage of CPP-ACP, when compared to CPP-ACFP is that the former does not contain fluoride which removes the risk of fluorosis from its usage.[12,17]

The *in vitro* model of enamel remineralization is often used to predict the anticaries effectiveness of CPP-ACP, but several questions regarding CPP-ACP's remineralizing effect are yet to be answered.[13] Based on the previous description, the authors will study the hardness of tooth enamel post-application of the remineralizing material casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on demineralizing teeth.

Materials and methods

This research has earned the ethical approval from the Research Ethics Commission of Padjadjaran University in Bandung, no. 217/UN6.KEP/EC/2021. This research has also earned the approval of the Dean of the Faculty of Dentistry of Padjadjaran University with the Research Approval Number of 3407/UN6.F.1/PT.01.04/2021. This research is done in the Laboratory of Tooth Conservation and Laboratory of Oral Biology of the Faculty of Dentistry of Padjadjaran University and the Laboratory of Solid Oxide Systems, Metallurgical Engineering of the Faculty of Mining and Petroleum Engineering of the Bandung Institute of Technology from 5 April to 5 May 2021.

This research was an *in vitro* true experimental research which used 30 maxillary premolar post-extraction teeth with the criteria of no fillings, no caries, no fractures, and completely closed root apex. The following tools were used: a diamond disc bur, a micromotor, an incubator, a Vickers hardness tester, a pH meter, trays, basic instrument (explorer, cotton pliers), medicine pots, cotton rolls, microbrushes, masks, and gloves. The following materials were used: 0.9% NaCl solution, sterile aquadest, artificial saliva, Pocari Sweat isotonic drink, GC Tooth Mousse (Recaldent GC Corporation Tokyo, Japan) which contains 10% CPP-ACP, and self-cured acrylic resin.

Remineralizing material CPP-ACP was the independent variable while enamel hardness post-application of the remineralizing material CPP-ACP was the dependent variable. The samples were prepared by first cleaning the teeth, then washing them using aquadest and storing them in medicine pots filled with 0.9% NaCl solution at room temperature until given treatment. The teeth were then cut horizontally to separate the crown from the root more or less 1 mm under the cementoenamel junction using a diamond disc bur.[12] The cut crowns were then planted in self-cured acrylic resin with their buccal surface facing upwards and given a number.[12] If the sample was to be used, it was first washed using sterile aquadest.

The samples were given initial treatment in the form of submersion for 30 minutes in an isotonic drink.[18] The samples were then randomly divided into 2 groups: the first group consisted of 15 maxillary premolar teeth which were submerged in artificial saliva as the control group and were not given CPP-ACP. The second group consisted of 15 maxillary premolar teeth which were submerged in artificial saliva and had CPP-ACP applied to them routinely for 3 minutes twice a day for 21 days.

Measurements of hardness on each group were done 3 times. On the control group, measurements were done before submersion in isotonic drink, after submersion in isotonic drink, and after submersion in artificial saliva for 21 days. Measurements on the test group were also done 3 times which are before submersion in isotonic drink, after submersion in isotonic drink, and after submersion in artificial saliva whilst being applied CPP-ACP for 21 days to observe the enamel surface's hardness. Measurement of hardness on the samples was done through the use of a Vickers hardness tester with the unit of Vickers Hardness Number (VHN) or kg/mm^2.

The acrylic was then clamped with the surface of the tooth facing upwards using the clamps on the Vickers hardness tester (Figure 1). The sample was then positioned to be exactly in the middle of the objective lens which was then focused by rotating the handle on the right of the tester clockwise.

Figure 1. The sample was positioned to be in the middle of the objective lens.

After a focused image was formed on the ocular lens, the sample was then moved by sliding it to the right so that it is positioned under the diamond penetrator. The penetrator was activated and lowered by pressing a button. A green light turned on once this happens and a red light then turned on once the penetrator had made contact with the sample. After 30 seconds, the penetrator rose and both green and red lights must be turned off before the sample can be slid back under the objective lens and refocused. Afterward, an image of a rhombic cut could be seen from the ocular lens. Its diagonal length was directly measured with a micrometer on the ocular lens. From these measurements, the mean value was taken.

The following formula was used to calculate the hardness of the sample:[12]

$$VHN = 1{,}854 \times P/d^2$$

VHN = hardness of sample (kg/mm^2)
P = weight of load (100 grams)
d = diagonal length (1/1000 mm)

Measurement of the initial surface hardness of each sample was done before submersion in the isotonic drink. But before the demineralization procedure could be done, the isotonic drink was first measured for its pH using a pH meter. The samples were then demineralized by submersion in 5 ml Pocari Sweat isotonic drink inside medicine pots for 30 minutes and then rinsed using aquadest before being dried using cotton rolls. After submersion, the surface hardness of the samples was measured again using the Vickers hardness tester to obtain the value of enamel surface hardness post-treatment. In the control group, the samples were submerged in artificial saliva. On the test group, 0.02 mL *GC Tooth Mousse* which contains CPP-ACP was applied to the samples using a microbrush for 3 minutes routinely twice a day for 21 days to achieve an effective result of remineralization.[12,19] During the period of *Tooth Mousse* application, the samples were submerged in medicine pots filled with artificial saliva which was replaced once every 24 hours. The artificial saliva used was made by an integrated laboratory department of the faculty using the AFNOR method. Composition of the artificial saliva included 0.7g NaCl, 0.33g KSCN, 1.5g $NaHCO_3$, 1.2g KCl, 1.3g urea, 0.26g Na_2HPO_4, 0.2g KH_2PO_4, 1000mL aquadest, and 37% HCl. Storage was done via an incubator with the temperature set to 37℃.

The samples were washed clean using sterile aquadest and then dried using cotton rolls. The Vickers hardness tester was then prepared, and the specimens were remeasured for its enamel surface hardness post-treatment of application of remineralizing material CPP-ACP. Tooth hardness post-application of remineralizing material was measured on day 21.[12]

The method of data analysis used in this research was the paired t-test which was used to determine the difference in enamel hardness between the two groups before and after the application of CPP-ACP with the criterion of a fulfilled normality test which was the chi-square test. Then a one-factor ANOVA test was done to discover the significance of the difference in enamel hardness between the two groups and followed by a post hoc test. Testing of the data was done using the Excel MegaStat software.

Results

The mean measurement results of the enamel surface's hardness on each group after 3 instances of measurement can be seen in Table 1. The first measurement is the measurement of initial enamel hardness before being submerged in Pocari Sweat isotonic drink for both groups. The second measurement was done on the same day after the isotonic drink submersion for 30 minutes for both groups. Next, the third measurement on the control group was done after submersion in artificial saliva for 21 days. On the test group, measurement was done after the application of remineralizing material CPP-ACP and submerged in artificial saliva for 21 days. Research results showed that there were differences between the mean values of tooth hardness before and after treatment. (Figure 2)

	Group	Mean	Std. Dev
1st measurement	Control	414.75	52.513
	CPP-ACP	440.04	33.811
2nd measurement	Control	285.95	64.990
	CPP-ACP	260.11	32.974
3rd measurement	Control	372.59	93.635
	CPP-ACP	348.45	51.727

Table 1. Mean results of tooth enamel hardness measurement (in units of VHN).

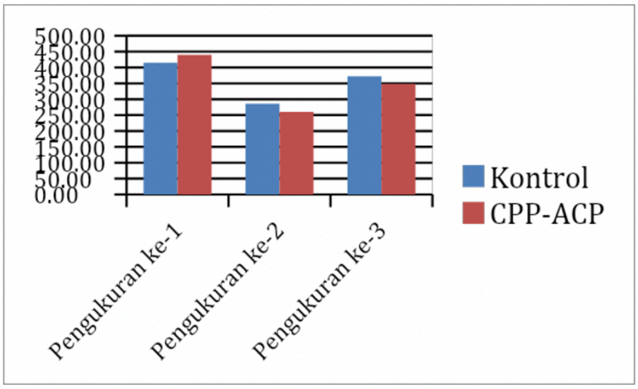

Figure 2. Mean results of tooth enamel hardness measurement (in units of VHN).

Before the analysis was done, a normality test was done beforehand. Results of the test displayed p>0.05. Based on these results, the data used was data with normal distribution which meant the next step was analysis with the ANOVA test.

From the ANOVA table above (Table 2) a p-value of 2.34E-12< α=0.05 was earned which showed a meaningful difference in the hardness of tooth enamel surface before and after treatment. It may then be concluded that the results on each group differed from one another. Therefore, a follow-up post hoc test in pairs was necessary.

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	336,272.398	5	67,254.4796	19.79	**2.34E-12**
Error	244,657.551	72	3,398.0215		
Total	580,929.949	77			

Table 2. Group ANOVA test.

	ACP-2	K-2	ACP-3	K-3	K-1	ACP-1
ACP-2						
K-2	0.25					
ACP-3	0.00	0.01				
K-3	0.00	0.00	0.30			
K-1	0.00	0.00	0.01	0.08		
ACP-1	0.00	0.00	0.00	0.00	0.27	

Table 3. Follow-up post hoc test.
*Note:
K-1: Control group, 1st testing (initial enamel hardness before submersion in isotonic drink)
ACP-1: CPP-ACP group, 1st testing (initial enamel hardness before submersion in isotonic drink)
K-2: Control group, 2nd testing (enamel hardness after submerged in the isotonic drink after 30 minutes)
ACP-2: CPP-ACP group, 2nd testing (enamel hardness after submerged in the isotonic drink after 30 minutes)
K-3: Control group, 3rd testing (enamel hardness after submerged in artificial saliva for 21 days)
ACP-3: CPP-ACP group, 3rd testing (enamel hardness after application of remineralizing material CPP-ACP and submerged in artificial saliva for 21 days)

The values on the table above display the *p-value*. If the *p-value* < 0.05, it means that a significant or meaningful difference exists. The follow-up post hoc test (Table 3) was done to determine the location of the difference in means between the groups. From the table, it may be derived that significant differences in the enamel hardness existed between ACP-3 and ACP-2; ACP-3 and K-2; K-3 and ACP-2; K-3 and K-2; K-1 and ACP-2; K-1 and K-2; K-1 and ACP-3; ACP-1 and ACP-2; ACP-1 and K-2; ACP-1 and ACP-3; and ACP-1 and K-3. From the same table, it may also be derived that no significant differences in the enamel hardness existed between K-2 and ACP-2; K-3 and ACP-3; K-1 and K-3; and ACP-1 and K-1.

Discussion

Data collection has been conducted to determine the effect of topical application of CPP-ACP on the demineralized tooth. The results showed that there was a change in the average hardness of tooth enamel before and after the application of CPP-ACP remineralization material.

The results of statistical analysis using the one-factor ANOVA test followed by a post hoc follow-up test state that the hardness of tooth enamel decrease after the demineralization and show a significant difference when compared before and after the demineralization. These happen after soaking the tooth in the isotonic drink, i.e. formulated drink that was useful to rehydrate the body.[20]

Isotonic drinks can prevent dehydration, supply enough carbohydrates to increase energy, and provide electrolytes to replenish electrolytes lost during sweating.[21] Most soft drinks including isotonic drinks contain sugar, minerals, and several types of acids, such as; phosphoric acid, citric acid, malic acid, and tartaric acid.[2] The consumption of isotonic drinks increases the potential for erosion as a non-bacteriogenic, the loss of tooth structure caused by acids, and the risk of caries by bacteria that occupy the tooth surface.[21] This is because isotonic drinks have a low pH ranging between 2.4 - 4.5, which is below the critical pH limit of enamel causing the demineralization.[2]

The demineralization process related to acid attack results in the chemical dissolution of the organic and inorganic matrix components. This is due to the water content in the enamel and dentin which facilitates the diffusion of the acid.[1] The demineralization is characterized by the breakdown of hydroxyapatite crystals, which begins with the reaction between phosphate ions (PO_4^{3-}) of hydroxyapatite crystals with H^+ ions resulting in the dissolution of apatite crystals, H^+ ions react with OH^- ions resulting in H_2O and converts PO_4^{3-} ions into HPO_4^{2-} ions. When in contact with the acid, the HPO_4^{2-} ion will turn into H_2PO_4.[12] In this condition the enamel surface becomes rough and uneven.[9]

In this study, the results of the one-factor ANOVA test followed by the post hoc test show an increase in tooth enamel hardness after the 21 days of CPP-ACP application. This is due to a remineralization process that can maintain the hardness or even increase the hardness of the tooth enamel surface.[4]

Demineralization is a reversible process, therefore partially demineralized hydroxyapatite crystals on teeth can return to their original shape when exposed to an oral environment that supports remineralization.[1] The remineralization process begins with the formation of inhomogeneous deposits of hydroxyapatite crystals that have a small size and irregular arrangement of the ions. After that, it will slowly improve itself in terms of size, structure, and chemical properties. The hydroxyapatite crystals formed will be larger and denser if the remineralization process lasts longer so that the hardness of tooth enamel will increase higher.[22,23] Remineralization is the result of minerals entering the microporosity of enamel.[24]

Although spontaneous remineralization occurs in all cases, the rate of remineralization varies depending on the state of the mouth cavity. A remineralization agent is required to ensure that the remineralization process goes smoothly. Of course, the optimal remineralization agent must be able to transfer calcium and phosphate to the teeth's surface in order to replace dissolved calcium and phosphate.[25] Enamel remineralization can be achieved with the products containing Casein Phosphopeptides - Amorphous Calcium Phosphate (CPP-ACP) derived from milk casein and absorbed through the enamel surface.[26] Casein Phosphopeptide (CPP) can stabilize calcium, phosphate, and fluoride ions in a non-crystalline amorphous state needed in the tooth enamel. Topical application of CPP-ACP causes a chemical reaction, where CPP-ACP reacts with salivary glycoproteins that cover the tooth surface, which is known as a salivary pellicle. Calcium and phosphate in the form of ACP that are not strongly bound to the salivary pellicle will dissolve into the environment around the tooth (saliva and plaque). CPP-ACP also reacts chemically with hydroxyapatite crystals of enamel and dentin, binding the hydroxyl groups and forming calcium phosphate hydroxyapatite which is resistant to acid demineralization. When the CPP-ACP is applied on the tooth surface, it interacts with the hydrogen ions and forms calcium hydrogen phosphate which releases calcium and phosphate ions, which supports the remineralization.[27,28]

The anti-cariogenic activity of CPP-ACP depends on the integration of the nanocomplex into dental plaque and tooth surfaces, thereby serving as a reservoir of calcium and phosphate.[28] Furthermore, the nanocomplex particle size can penetrate the porosity of the enamel surface and release calcium ions and inorganic phosphate at a consistent rate. By producing a crystalline layer, filling interprismatic enamel, and blocking enamel prisms, the CPP-ACP may also inhibit demineralization.[29] CPP-ACP binds to bacterial walls and tooth surfaces. In the event of an intraoral acid attack, calcium and phosphate ions are released, reaching an ion-saturated state in the saliva and subsequently depositing calcium-phosphate compounds on exposed tooth surfaces. The solution of CPP can prevent bacterial adhesion to the tooth surface and suspend the biofilm formation.[26]

The results of the study in the control group showed that there was an increase in the hardness of tooth enamel after the immersion in artificial saliva as a remineralizing agent.[30] Artificial saliva contains minerals such as potassium chloride, magnesium chloride, sodium chloride, metal p-hydroxylbenzoate, dipotassium hydrogen orthophosphate, and calcium chloride. These inorganic components are similar to normal saliva content, such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO^{3-}, and phosphate.[4] The buffering capacity of saliva has an important role in maintaining the pH of saliva and plaque, i.e. neutralizing the effects of acid exposure.[9] Artificial saliva creates an artificial oral environment and prevents bacterial contamination of enamel samples, in addition to matching the makeup of natural saliva.The presence of an organic buffer and supersaturated calcium and phosphate concentrations in artificial saliva aided in remineralization over time, demonstrating saliva's remineralizing action in early carious lesions.[31] The elucidations above show that the application of CPP-ACP to demineralized teeth has a p-value that is smaller than α (α = 0.05) (Table 3). If the p-value < α, then H_0 is rejected. Because H_0 is rejected, the result of this study is that there is a significant difference in the hardness of tooth enamel after the application of CPP-ACP remineralization material compared to before the application of CPP-ACP in the test group. This study shows that the use of CPP-ACP supports remineralization. If the test group was compared with the control group, there was no significant difference between the dental group which was applied CPP-ACP twice a day for 21 days and the control group which was only soaked in artificial saliva. The increase in the hardness of tooth enamel after the application of remineralization material for 21 days is still lower than the initial hardness of tooth enamel before the demineralization. Further research regarding the application of CPP-ACP as a remineralizing material towards more samples and for a longer duration is necessary.

Conclusions

The application of CPP-ACP as a remineralizing material for 21 days can increase the hardness of demineralized tooth enamel.

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Declaration of Interest

The authors report no conflict of interest.

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