

## Research Report

## The increasing of enamel calcium level after casein phosphopeptide-amorphous calcium phosphate covering

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### ABSTRACT

**Background:** Caries process is characterized by the presence of demineralization. Demineralization is caused by organic acids as a result of carbohydrate substrate fermentation. Remineralization is a natural repair process for non-cavitated lesions. Remineralization occurs if there are  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions in sufficient quantities. Casein-amorphous calcium phosphate phosphopeptide (CPP-ACP) is a paste material containing milk protein (casein), that actually contains minerals, such as calcium and phosphate. The casein ability to stabilize calcium phosphate and enhance mineral solubility and bioavailability confers upon CPP potential to be biological delivery vehicles for calcium and phosphate. **Purpose:** The aim of this study was to determine the calcium levels in tooth enamel after being covered with CPP-ACP 2 times a day for 3, 14 and 28 days. **Methods:** Sample were bovine incisors of 3 year old cows divided into 4 groups, namely group I as control group, group II, III and IV as treatment groups covered with CPP-ACP 2 times a day. All of those teeth were then immersed in artificial saliva. Group II was immersed for 3 days, while group III was immersed for 14 days, and group IV was immersed for 28 days. One drop of CPP-ACP was used to cover the entire labial surface of teeth. The measurement of the calcium levels was then conducted by using titration method. All data were analyzed by One-Way ANOVA test with 5% degree of confidence. **Results:** The results showed significant difference of the calcium levels in tooth enamel of those groups after covered with CPP-ACP 2 times a day for 3, 14 and 28 days ( $p = 0.001$ ). There is also significant difference of the calcium levels in tooth enamel of those treatment groups and the control group ( $p = 0.001$ ). **Conclusion:** The calcium levels of tooth enamel are increased after covered with CPP-ACP 2 times a day for 3, 14 and 28 days.

**Key words:** Calcium, enamel, casein phosphopeptide-amorphous calcium phosphate

### ABSTRAK

**Latar belakang:** Proses terjadinya karies gigi ditandai oleh adanya demineralisasi. Demineralisasi terjadi oleh asam organik sebagai hasil fermentasi substrat karbohidrat oleh bakteri. Remineralisasi adalah proses perbaikan alami untuk lesi non cavitated. Remineralisasi terjadi jika terdapat ion  $\text{Ca}^{2+}$  dan  $\text{PO}_4^{3-}$  dalam jumlah cukup. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) adalah bahan berbentuk pasta berisi suatu protein susu (kasein). Pada kasein terkandung mineral kalsium dan fosfat. Kemampuan kasein untuk menstabilkan kalsium fosfat dan meningkatkan kelarutan dan bioavailabilitas sehingga CPP memiliki potensi menghantarkan kalsium dan fosfat. **Tujuan:** Tujuan penelitian ini untuk mengetahui kadar kalsium enamel setelah pengulasan CPP-ACP pada permukaan enamel 2 kali sehari selama 3, 14 dan 28 hari. **Metode:** Sampel adalah gigi insisif sapi, usia 3 tahun. Pada penelitian ini sampel ( $n = 24$ ) dibagi menjadi 4 kelompok yaitu kelompok I sebagai kelompok kontrol, dan kelompok II, III, IV sebagai kelompok perlakuan dengan pengulasan CPP-ACP 2 kali sehari. Semua kelompok direndam dalam saliva buatan. Kelompok II direndam 3 hari, kelompok III direndam 14 hari, kelompok IV direndam 28 hari. Pengulasan CPP-ACP sejumlah 1 tetes diratakan pada seluruh permukaan labial gigi. Pengukuran kadar kalsium dilakukan dengan metode titrasi. Semua data dianalisa dengan uji One-Way ANOVA dengan taraf kemaknaan 5%. **Hasil:** Hasil penelitian menunjukkan perbedaan yang signifikan kadar kalsium enamel gigi di antara kelompok ( $p = 0,001$ ). Hasil penelitian menunjukkan perbedaan yang signifikan kadar kalsium gigi pada kelompok

kontrol dan perlakuan antara lama waktu pengulasan 3, 14, dan 28 hari ( $p = 0,001$ ). **Kesimpulan:** Pengulasan CPP-ACP selama 3, 14 dan 28 hari mampu meningkatkan kadar kalsium pada enamel.

**Kata kunci:** Kalsium, enamel, casein phosphopeptide-amorphous calcium phosphate

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## INTRODUCTION

There are four important factors that could cause caries, namely bacteria, carbohydrates, host, and time. Some kinds of bacteria have ability to perform fermentation of carbohydrate substrate in food (e.g glucose and sucrose) in order to form acid and to lower pH to below 5 or 4.5 within 1–3 minutes. To return to normal pH around 6-7, it takes about 30–60 seconds.<sup>1</sup>

Mineral component of enamel is hydroxyapatite with chemical formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . In normal condition, hydroxyapatite is balanced with saliva containing  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions. Hydroxyapatite is also reactive with hydrogen ions at pH <5.5 called as critical pH. Thus,  $\text{H}^+$  then reacts with  $\text{PO}_4^{3-}$  to  $\text{HPO}_4^{2-}$ . It is known as  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2(s) + 8\text{H}^+(aq) \rightarrow 10\text{Ca}^{2+}(aq) + 6\text{HPO}_4^{2-}(aq) + 2\text{H}_2\text{O}(l)$ .<sup>2</sup>

The process of dental caries is characterized by the presence of demineralization. Demineralization is caused by fermentation of organic acids from carbohydrate substrates caused by bacteria. The demineralization process of carbohydrates caused by bacterial colonies is often called as plaque that produces acids which damage hydroxyapatite minerals of teeth, therefore the releasing process of mineral in enamel is called demineralization.<sup>1</sup> Remineralization is a natural process for repairing dental tissue suffering from demineralization. Remineralization occurs if there are  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions in sufficient quantities generally obtained from saliva.<sup>3</sup>

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is material with the form of paste containing milk protein (casein). Casein phosphopeptide (CPP), has ability not only to bind and stabilize calcium and phosphate in solution, but also to bind dental plaque and tooth enamel. Calcium phosphate, which is in the form of the crystal structure is usually insoluble at neutral pH, but CPP keeps calcium and phosphate in the form of an amorphous form. In the amorphous form, calcium and phosphate ions can diffuse into tooth enamel. Thus, casein contains calcium and phosphate minerals. Casein even can improve the quality of the enamel surface to be more resistant to organic acids as a result of the fermentation of carbohydrate substrates caused by bacteria. Enamel that gets demineralization, therefore, can be improved by the provision of calcium and phosphate ions contained in CPP-ACP inserted to the inside of enamel in order to replace the dissolved minerals, so remineralization can occur.<sup>4</sup> Casein actually contains minerals, such as calcium and phosphate. The casein ability to stabilize

calcium phosphate and enhance mineral solubility and bioavailability confers upon CPP potential to be biological delivery vehicles for calcium and phosphate.<sup>5</sup> For those reasons, the purpose of this study was to determine the level of calcium in tooth enamel on which CPP-ACP is covered 2 times a day with a 12-hour interval for 3, 14 and 28 days.

## MATERIALS AND METHODS

This study was considered as an experimental research with completely randomized design by using bovine incisors of  $\pm 3$  year old cows as samples. Those teeth were cleaned and stored in normal saline for  $\pm 1$  week with several criteria of eruption crown, without abrasion, without fracture/crack, and without caries.<sup>7</sup>

The study was conducted at the Laboratory of Chemistry University of Hang Tuah Surabaya. Samples ( $n=24$ ) were divided into 4 groups. Group I was control group, each sample was not covered with CPP-ACP. Group II, III and IV were treatment groups, each sample was covered with CPP-ACP. The surfaces of the tooth enamel then were covered with CPP-ACP, and left for 15 seconds. Next, they were washed with normal saline thoroughly and dried with a blower until they became white. Afterwards, those tooth enamel surfaces were covered with CPP-ACP 2 times a day with 12 hour interval, and then were immersed in 90 ml of artificial saliva. Artificial saliva used was made of 36.00 g NaCl, 1.69 g KCl, 0.96 g  $\text{CaCl}_2$ , 0.80 g  $\text{NaHCO}_3$  and added with 400 ml aquadestilata. This mixture can actually produce neutral pH (pH 7).<sup>7</sup>

Group II was immersed for 3 days, while group III was immersed for 14 days, and group IV was immersed for 28 days. CPP-ACP was used for about 1 drop, and then it was applied at the entire labial surface of the teeth after the teeth were washed with normal saline and dried for 10 minutes. Afterwards, the artificial saliva was replaced every time it was used. The measurement of calcium levels was then conducted by titration method. Samples were boiled with hydrochloride acid which would extract minerals and solve sample protein, and then were filtered. The calcium level of the filtrate was analyzed by using permanganometry titration. The procedures included the preparation of the ash, the preparation of ash solution/sample preparation, and the examination of dental calcium levels of Wistar rats' teeth. Data results were then tested by using statistical test, One-Way ANOVA.

**Table 1.** The mean and standard deviation of the calcium levels of tooth enamel based on cover times of CPP-ACP, namely for 3, 14 and 28 days

	3 Days		14 Days		28 Days	
	Mean	SD	Mean	SD	Mean	SD
CPP-ACP	40.1867	0.97558	44.8567	1.44750	47.3650	0.88090
Control	36.0217	0.75056	36.0217	0.75056	36.0217	0.75056

## RESULTS

The mean and standard deviations of calcium levels in enamel among the treatment groups covered with CPP-ACP were higher than the control group at the time of observation 3, 14 and 28 days (Table 1). The mean level of calcium in enamel of the group covered with CPP-ACP for 28 days was the highest compared to that in groups covered with CPP-ACP for 3 and 14 days. The mean and standard deviations of enamel calcium levels obtained were analyzed with Kolmogorov Smirnov test. The results indicate that all data were normally distributed ( $p > 0.05$ ), thus, parametric test can be used. Levene test was conducted on the observation of enamel calcium level covered with CPP-ACP. The results show that the probability value was  $> 0.05$ . It means that the homogeneous assumption was met, so parametric test could be conducted.

The results of One-Way ANOVA showed that there were significant differences of the enamel calcium levels ( $p < 0.05$ ), about 0.001 (Table 2), between the treatment groups covered with CPP-ACP for 3, 14, and 28 days and the control group. It was also known that there was significant difference of the calcium level between the treatment groups covered with CPP-ACP for 3 and 14 days with  $p$  about 0.001 ( $p < 0.05$ ). Similarly, there was significant difference of the calcium level between the treatment groups covered with CPP-ACP for 3 and 28 days with  $p$  about 0.001 ( $p < 0.05$ ). There was significant difference of the calcium level between the treatment groups covered

**Table 2.** The differences of the significant level of the calcium level of tooth enamel in treatment groups and control group based on cover times of CPP-ACP, namely for 3, 14 and 28 days

Variable	pValues Control – CPP-ACP		
	3 Days	14 Days	28 Days
Level of calcium	0.001*	0.001*	0.001*

Note: \* = Significance of difference

**Table 3.** The significant level of the calcium level of tooth enamel in treatment groups and control group based on cover times of CPP-ACP, namely for 3, 14 and 28 days

Variable	Cover time	pValues	
		Control	CPP-ACP
Level of calcium	3 days – 14 days	0.001*	0.001*
	3 days – 28 days	0.001*	0.001*
	14 days – 28 days	0.001*	0.003*

with CPP-ACP for 14 and 28 days with  $p$  about 0.003 ( $p < 0.05$ ) (Table 3).

## DISCUSSION

Bovine fresh extracted teeth were used referring a previous study because bovine teeth are easily obtained in large quantities and in good condition (rarely getting any caries).<sup>8</sup> In this study, samples of the study were covered with 37% phosphoric acid in order to obtain surface roughness of enamel. The samples consisted of 4 groups: 3 treatment groups covered with CPP-ACP for 3 days, 14 days, 28 days, and control group. Each group then consisted of 6 samples.

Each sample was then covered with acids made of  $H^+$  ion reacting with  $PO_4^{3-}$  and altering into  $HPO_4^{2-}$ . This  $HPO_4^{2-}$  compound, however, did not play a role in the balance of hydroxyapatite since hydroxyapatite contained more  $PO_4^{3-}$  than  $HPO_4^{2-}$ , so hydroxyapatite crystals would dissolve and cause demineralization.<sup>2</sup> Acid, dissolved  $\pm 10 \mu m$  of the enamel surface (enamel rods), so porosity was formed about 5-50  $\mu m$ .<sup>9</sup>

This study took 3 days, 14 days, and 28 days to compare dentin tubule porosities formed since according to the result of the research conducted by Oshiro *et al.*,<sup>13</sup> using SEM with those cover times was effective enough to determine the effect of the use of CPP-ACP. Besides, according to the factory rules the use of CPP-ACP was supposed to be 2 times a day. Therefore, the total of the use of CPP-ACP was 6 times for 3 days, 28 times for 14 days, and 56 times for 28 days.

The average level of calcium in enamel of the treatment groups covered with CPP-ACP for 28 days was the highest of all. The levels of calcium were different between the treatment groups and the control group, and there was significant difference of the calcium levels among those treatment groups with different cover times. This is because in CPP-ACP there is a complex amalgamation of nano, amorphous calcium phosphate (ACP), diffusion into dental plaque and tooth surface. CPP has an important role as carrier of ACP to tooth surface location where the solubility level of calcium phosphate is very high. This localization then will maintain the high concentration gradient of calcium and phosphate ions below the surface of the enamel, so remineralization can easily occur.<sup>10</sup>

CPP-ACP is a material with pasta form shape derived from casein, a milk protein containing calcium and phosphate minerals. In the study conducted by Reynolds

and Johnson proved that the use of milk containing casein may play a role in preventing caries in oral environment. It is because CPP-ACP can bind to plaques, provide large amounts of calcium, and then slow diffusion of free calcium. Thus, it can restrict mineral loss during cariogenic episode and provide calcium to be used to remineralization process later.<sup>11</sup>

CPP can keep calcium and phosphate in the form of amorphous non-crystalline. In amorphous form, calcium and phosphate ions can diffuse into tooth enamel.<sup>11</sup> The use of CPP-ACP on the surface of tooth enamel can accelerate the process of remineralization compared to that without the use of the CPP-ACP. It occurs because CPP-ACP does not only contain calcium and phosphate minerals, but also creates both physical-chemical bond between  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions, and complex compound,  $\text{CaHPO}_4$ , decomposed in demineralization process of tooth enamel and then forming strong bond with calcium, phosphate, and fluoride ions which later forms fluoroapatite crystals  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})\text{F}]$ . These crystals are more resistant to acid ion with pH above 4.5 compared to pure hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$  with critical pH about 5.5.<sup>2</sup>

Once calcium and phosphate ions diffused into the carious lesion through dissociation, the activity of calcium and phosphate ions will be increased, so the adhesion of bacteria on the surface of the enamel can be reduced and remineralization can be increased.<sup>12</sup>

Similar study conducted by Kumar<sup>4</sup> showing that there was not only a high efficiency of CPP-ACP in remineralization of early lesions in enamel, but also a high potential of CPP-ACP in remineralization when applied topically after tooth brushing with toothpaste containing fluoride. Some studies even suggest that CPP-ACP will be more effective when mixed with saliva in mouth.<sup>13</sup>

In conclusion the use of CPP-ACP for 3, 14 and 28 days can increase the levels of calcium in tooth enamel. The longer the use of CPP-ACP, the higher the level of calcium in tooth enamel.

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