Analysisof CPP-ACP Complex in Combination with Propolis to Remineralize Enamel

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Abstract

The objective of the study was to continuous acid exposure can cause demineralization ofa tooth'senamel surface,creating white spot lesions. These lesions may be remineralized in the right environment. The casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) complex is a known remineralization agent. With the aim of this study was to analyze the ability of the CPP-ACP complex in combination with propolis to remineralize enamel. The methods used was 3 x 5 mm windows on the enamel surface of 12 caries-free human premolars weredemineralized using phosphoric acid 50%. The specimens were divided into four groups: Control, CPP-ACP complex, Propolis, CPP-ACP complex + Propolis 4%. The treatment gels were applied for five minutes, 26 times. The specimens were evaluated for microhardness, surface microstructure and element analysis. As the results showed no statistical differences in microhardness data after the geltreatment groups (p=0.1). The microhardness data between the after-demineralized and after-gel treatment group also showed no differences (p=0.16). An electron micrograph showed that CPP-ACP complex had irregular deposits at the enamel surface and CPP-ACP complex. Propolis 4% showed a homogeneous layer at the enamel surface. Element analysis showed no differences between the groups. It was concluded that a combination of the CPP-ACP complex and propolis might have the potentialto remineralize enamel. The CPP-ACP complex and propolis are promising as an anticariogenic.

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Introduction

Dental caries is a chronic multifactorial infectious disease. It has the highest incidence of oral disease in the world.[1-3] Susceptible tooth, bacterial plaque, and substrate are the three main factors that determine the development of a caries lesion. The physiologic process in tooth structure can be disrupted by unbalanced conditions between protective and pathologic factors.[4-7] The World Health Organization has developed a policy to support the preventive disease paradigm—Minimum Intervention Dentistry (MID)—to manage caries. One of the MID principles is the regeneration of tooth enamel using remineralizing agents.[3,8] Based on pathogenesis, dental caries can be managed by increasing host resistance and minimizing the predisposing factors to disease.[2,4]

The development of a caries lesion starts when the saliva/plaque pH at the enamel surface reaches the critical value of 5.5 and the organic acid from cariogenic bacteria diffuses into the enamel. At low pH values, the PO^{3-4} group has protonation, making calcium phosphate release from the enamel's surface, decreasing enamel micro hardness.[3,9,10] Visually, demineralized enamel appears as white spot lesions. These lesions can either develop into cavities or be remineralized. The remineralization process could repair demineralization if the environment provides an adequate calcium and phosphate ion concentration.[9-14]

Recently, the interest in the potential of CPP (casein phosphopeptide) as are mineralization agent has increased. Research has focused on finding or developing a new

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sequence or CPP formulation to increase oral health. CPP is a bioactive peptide made from milk casein. Cow milk was chosen as a primary source because it contains more casein than any other milk. To produce CPP, casein should be hydrolyzed enzymatically using a protease such as trypsin or papain.[3] In this study, we use the papain enzyme from papaya. The optimal CPP concentration to remineralize enamel is a 10% caseinate substrate. Calcium and phosphate were added to create the casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) complex. CPP binds calcium to the amino acid residue containing the phosphate group, stabilizing calcium and phosphate in the oral environment. The CPP-ACP complex acts as an ion reservoir, creating a supersaturation condition for the remineralization process.[3,6,15,16]

Propolis is a potent antibacterial agent.[17] Its therapeutic effect is because of the flavonoid agent in propolis.[17-19] Based on past research, an ethanolic propolis extract at ±4–5% is proven to have antimicrobial activity *in vitro*.

Mouthwash, toothpaste, gum, and gel are common preparations for the CPP carrier. A gel formulation was chosen in this study because it is easy to apply and to increase contact time between the active ingredients and the tooth enamel.[20]

Research about the CPP-ACP complex in combination with propolis has never been done before; the aim of this study is to evaluate its effectiveness in enamel remineralization.

Methods

This study was an analytic experimental laboratory and conducted in the Oral Biology Laboratory, Faculty of Dentistry, at the Universitas Indonesia. The specimens used were twelve freshly extracted caries-free premolars, covered with varnish, leaving a 3 × 5 mm window bare. The specimens were ground flat with SiC paper discs (400, 600, and 1200 grades) and polished with a one µm alumina suspension. For demineralization, the specimens were exposed to phosphoric acid 50% for 45 s.[9]

The specimens were randomly divided into four groups (n= 3), Control, CPP-ACP complex, Propolis, CPP-ACP complex + Propolis 4%. The CPP-ACP complex preparation was adopted from *World Intellectual Property Organization* (*WO Patent*) no. US 2005/0037948 about *Calcium Phosphopeptide Complexes*, by Eric C. Reynolds and modified using the papain enzyme from patent no. US7.060.472 by Holt in 2006.[3] Meanwhile, the propolis used in this study was collected from the Trigona species of bees in Indonesia.

The propolis was extracted by ethanol; then the wax was separated from the extract. This was followed by heating and freezing the substance overnight before decanting it.[18] Both ingredients were combined into a gel formulation made from a base of guar gum and other suitable ingredients. First, guar gum and Na-CMC were dissolved in distilled water at the temperature of 90^{o}C. This was mixed constantly until homogeneity was achieved before adding glycerin.

The CPP-ACP complex and distilled water were adjusted at a ratio of 1:1 and then mixed with the gel solution. When the solution temperature fell below 40^{o}C, then propolis was added. To evaluate the gel's hydrophilic properties, it was dissolved in distilled water with a ratio of 1:6, stirred until homogeneity was achieved, and then centrifugated at 500 rpm for 10 min. The solution was then incubated for 24 h.

The treatment procedures were done according to past research. Demineralized specimens were exposed to the gel according to their group and put in the incubator for five minutes. This cycle was repeated 26 times to optimize the remineralization process.[9,21,22]

Evaluation based on enamel remineralization parameters, including an enamel surface micro hardness test (measuring baseline, after demineralization, and after gel treatment) using the Vickers micro hardness tester (Buehler ASTM E384) by evaluating the Vickers Hardness Number (VHN); an enamel surface microstructure test using the scanning electron microscope (SEM) (FEI S5); and calcium and phosphate content in weight % were measured using energy dispersive X-ray analysis (EDX).[9,23] Data analysis was performed using one-way analysis of variance (ANOVA).

Results

One liter of skim cow milk produces ±160.32gr CPP-ACP complex powder. All the gel has a low viscosity and successfully passed the hydrophilic test.

Table 1. VHN values for enamel surface microhardness

Group	Baseline	After Demineralization	After Treatment
Control	375.2±9	306.8±7	304.7±55
CPP-ACP	395±14.1	313.7±12.7	286.7±62
Propolis	376.6±9.3	303.9±5.8	231.9±9.1
CPP-ACP + Propolis 4%	384.3±9.3	307±9.6	312.5±9.3

No statistically significant differences were observed among the groups for baseline VHN values ($p > 0.05$). After demineralization, all groups showed lower VHN values in comparison with the initial values. Furthermore, the VHN after demineralization and after the gel treatment showed no statistically significant difference ($p = 0,16$). The VHN values for the after gel treatment group also showed no statistically significant difference ($p = 0.1$)

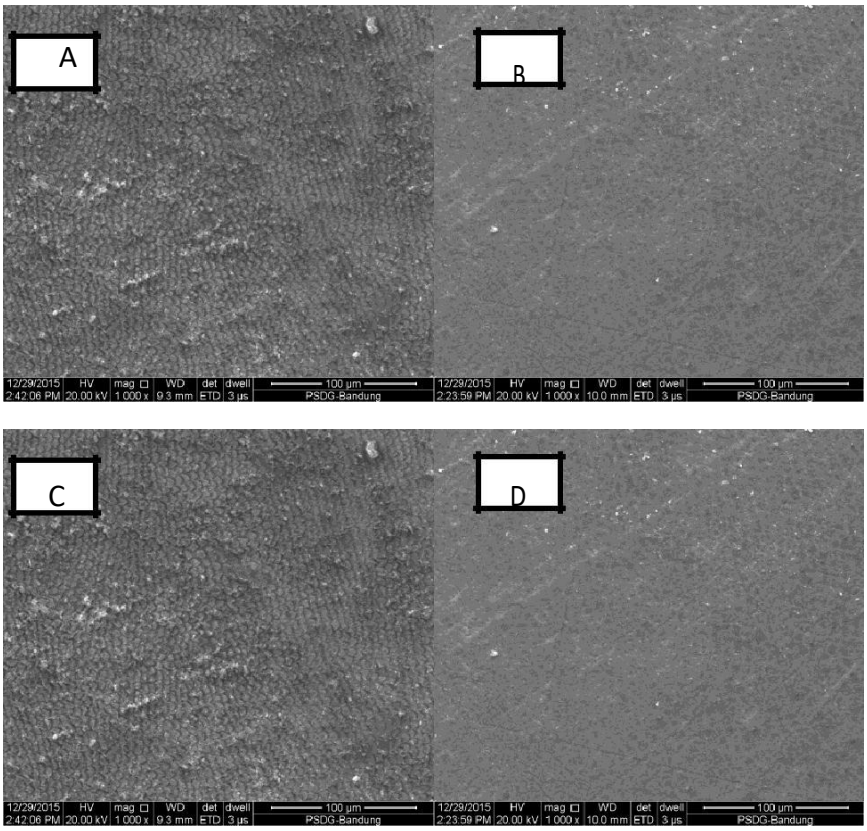

Figure 1. Enamel surface microsurface electron micrograph. Group 1. Control (A) 2. CPP-ACP complex gel (B) 3. ropolis 4% gel (C) 4. CPP-ACP + Propolis 4% gel (D)

Figure 1 shows SEM images after the gel treatment procedure. Figures 1A and 1C show areas of demineralized enamel with a honeycomb-like structure and a rough surface without a protective layer. The CPP-ACP gel group (Figure 1B) shows an organized enamel structure surface interspersed with microparticle deposits. The CPP-ACP complex + Propolis gel group (Figure 1D) indicates that the interprismatic and prismatic enamel structures appear to be covered by a thick, uniform homogeneous layer.

EDX element analysis showed no significant differences in the element content for Calcium and Phosphate in the superficial enamel layer.

Table 2. Calcium and Phosphate (%) at the enamel surface.

Group	Calcium (%)	Phosphate (%)
Control	40,99	21,21
CPP-ACP	42,72	19,79
Propolis	38,8	18,47
CPP-ACP + Propolis 4%	39,12	18,12

Discussion

Activities to prevent dental caries must be carried out.[1-3,8,17] In the past three decades, the clinical use of calcium and phosphate ions for remineralization was not significantly effective because of their insoluble properties, the effort required for application, and the low concentration of calcium and phosphate ions found in saliva/plaque. This treatment could not be localized on the tooth's enamel surface and could not create an effective concentration gradient to remineralize the enamel's surface. CPP-ACP complex technology was made to counteract these limitations. The CPP-ACP complex could stabilize a high concentration of calcium and phosphate ions on the tooth's surface, creating supersaturation conditions. In acidic conditions, the CPP-ACP complex acts as buffer and dissolves into diffusible calcium and phosphate ions. These ions are deposited into the enamel's subsurface, remineralizing enamel lesions.[6,7,15,24-26]

Previous research stated that propolis is a natural substance that effective against cariogenic microbiota. However, until now, few formulations containing propolis extract were used in dentistry.[17] So, in this work, we developed pharmaceutical formulations containing the CPP-ACP complex and an Indonesian propolis extract.

In this study, microhardness baseline tests showed that all the specimens had a similar microhardness number of ±380 VHN, indicating that all specimens had homogeneous physical properties. After the demineralization treatment, the microhardness numbers dropped to ±305 VHN. Demineralization affected the enamel's surface, creating an 'enamel tag' and losing microhardness.[27] Gel treatment after the demineralization step could be used as an anticariogenic agent especially for the CPP-ACP complex + PROPOLIS 4% gel treatment, even though statistically the groups showed no differences before and after treatment, and no other statistical differences between the groups were found. This finding showed that CPP-ACP complex + PROPOLIS 4% had the same result as control positive group or SDF.

The research from past studies suggests that *in vitro* remineralization could not simulate natural remineralization.[28] Enamel mineral deposition could be affected by many factors, such as contact time, preparation, contamination, treatment procedure, storage and laboratory transfer.[29] Other research suggests that the gel formulation may not release the active ingredient constantly.[17]

There are contradictions in CPP-ACP complex research.[3,21] In 2013, Klaric et al. indicated that combination ACP + Natrium Fluoride gels efficiently helped mineral stabilization in oral cavities.[30,31] Other research indicates that, until now, no remineralizing agent has been more effective than fluoride and sealant for tooth protection.[32] Clinical research by Ogata suggests that the remineralization effects from the CPP-ACP complex were ineffective and that fluoride supplementation is still the gold standard in the reduction of lesion depth.[21] These differences in remineralization research might be because of the differences in remineralization methods, and it is hard to evaluate the efficacy because there is no standard protocol for enamel remineralization.[3,21]

Propolis supplementation resulted in no differences in the microhardness numbers. This may be because the inorganic substances in propolis are only 5% and cannot increase enamel microhardness significantly.[33,34] Other research

suggests that propolis can decelerate calcium phosphate crystal formation.[35]

The enamel surface microstructure of the CPP-ACP complex gel application showed an organized enamel crystal structure similar to healthy enamel. It also showed interspersed microparticle deposits, indicating that calcium ions might have deposited at the surface and enamel remineralization was starting to begin. The enamel surface microstructure of the propolis gel group showed areas of demineralized enamel with a honeycomb-like structure. It indicated that the propolis gel could not initiate enamel remineralization.

Furthermore, the enamel surface microstructure of the CPP-ACP complex + propolis gel showed a smooth and homogeneous film at the enamel surface. It suggested that the resin in the propolis might have bound with the CPP-ACP complex. This result was similar to research done by Franca in 2014.[17]

Calcium and phosphate concentration at the enamel surface showed minimal differences between groups, but this is not statistically significant because enamel remineralization might have just begun.[36]

Conclusion

A combination of the CPP-ACP complex and an ethanolic propolis extract in gel formulation may have the potential to remineralize enamel.

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Conflict of interests

Declared none.

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