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Research Report

The effect of CPP-ACP containing fluoride on *Streptococcus mutans* adhesion and enamel roughness

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ABSTRACT

Background: *Direct contact between the bleaching agent and the enamel surface results in demineralization, alteration in surface roughness and bacterial adhesion. Many studies try to minimize this side effect through different way.* ***Purpose:*** *The aim of this study was to determined the effect of Calcium Phospho Peptide-Amorphous Calcium Phosphate (CPP-ACP) containing fluoride application before and after bleaching procedure on the adhesion of S. Mutans and enamel roughness.* ***Methods:*** *The samples were 6 teeth which were divided into 4 groups, and each tooth was cut into four pieces. Group A and C were treated with CPP-ACP after bleaching, while group B and D were treated with CPP-ACP before and after bleaching. CPP-ACP used in group C and D was the one that contain fluoride. After treatment, all samples were sterilized, immersed in steril human saliva for one hour, then immersed into S. mutans suspension of 108 CFU. Samples were incubated overnight. On the next day the samples were put into steril BHI and vortexed for one minute to detach the bacteria. Fifteen ml BHI containing bacteria was poured into TYS agar then incubated 37°Cfor 48 hours. Bacterial colony was counted with colony counter. The SEM examination was done on all samples.* ***Results:*** *Application of desensitizing agent reduced the S.mutans adhesion significantly among groups ($p<0.05$) except between group A and C. SEM evaluation revealed significant differences among groups.* ***Conclusion:*** *The application of CPP-ACP containing fluoride before and after bleaching was effective to reduce the accumulation of S.mutans colony and enamel roughness.*

Key words: *CPP-ACP, fluoride, adhesion, Streptococcus mutans*

ABSTRAK

Latar belakang: *Kontak langsung antara bahan bleaching dan permukaan enamel menyebabkan demineralisasi, perubahan kekasaran permukaan dan berpengaruh terhadap banyaknya bakteri Streptococcus mutans (S. mutans) yang melekat. Banyak peneliti mencoba meminimalkan efek samping ini dengan cara yang beragam.* ***Tujuan:*** *Penelitian ini bertujuan untuk meneliti efek aplikasi CPP-ACP mengandung fluor sebelum dan setelah bleaching terhadap adhesi S.mutans dan kekasaran enamel.* ***Metode:*** *Sampel penelitian adalah 6 buah gigi yang dibagi dalam 4 kelompok, kemudian masing-masing gigi dibelah menjadi 4 bagian. Kelompok A dan C diaplikasi dengan CPP-ACP setelah bleaching, sedang Kelompok B dan D diaplikasi CPP-ACP sebelum dan setelah bleaching. CPP-ACP yang digunakan pada kelompok C dan D adalah yang mengandung fluor. Setelah perlakuan, semua sampel disterilkan dan direndam dalam saliva steril, lalu direndam dalam suspensi S. mutans 108 CFU dan diinkubasi 24 jam. Hari berikutnya sampel dimasukkan dalam BHI steril, divortex 1 menit untuk melepaskan bakteri. Lima belas ml BHI yang berisi S. mutans tersebut diambil untuk dikultur dalam agar TYS dan diinkubasi 37°C selama 48 jam. Bakteri yang tumbuh dihitung dengan colony counter. Pemeriksaan SEM dilakukan untuk meneliti permukaan enamel.* ***Hasil:*** *Aplikasi CPP-ACP(F) menurunkan jumlah bakteri yang melekat pada enamel secara signifikan ($p<0,05$) pada semua kelompok, kecuali antara kelompok A dan C.* ***Simpulan:*** *Aplikasi CPP-ACP mengandung fluor sebelum dan sesudah bleaching efektif mengurangi akumulasi S. mutans dan kekasaran pada permukaan enamel.*

Kata kunci: *CPP-ACP, fluor, adhesi, Streptococcus mutans*

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INTRODUCTION

Information concerning esthetic dentistry that can be accessed easily make people more concerned to improve their performance. Brighter teeth are something that encourages people to see a dentist to bleach their teeth. Bleaching can be categorized into 2 groups: intracoronal bleaching and extracoronal bleaching. Intracoronal bleaching is performed to bleach non vital teeth, while extracoronal bleaching is indicated for vital teeth. Bleaching for vital teeth can be classified into at home bleaching (professionally dispensed) and in office bleaching (professionally administered).[1]

People prefer their teeth to be bleached by a dentist (in office bleaching technique) than to do it by themselves because it does not take a long time to see the bleaching result. Beside that, they also feel much secured if the bleaching process is done by the dentist because of better gingival protection and better sensitivity control. This procedure is suitable for patient with bleaching tray intolerance although it result in higher tooth sensitivity than at home bleaching because of higher concentration used in this technique.[2]

The mechanism of bleaching discolored teeth still unclear. It differs according to the type of discoloration involved, the chemical and physical condition at the time of the reaction. Bleaching agent mainly oxidizers, slowly degrading organic structure into chemical product such as carbon dioxide that are lighter in color. The oxidation reduction reaction that occurs during bleaching is known as a redox reaction. The mechanism of bleaching involve the degradation of the extracellular matrix and oxidation of chromosphores located within enamel and dentin.[3,4]

Beside tooth sensitivity, there are several bleaching side effect that must be anticipated such as mineral loss, surface roughness and increasing bacterial adhesion such as *Streptococcus mutans* (*S. mutans*). *S. mutans* has ability to metabolize many sugars and produces various organic acid. *S. mutans* is strong acid producer hence cause an acidic environment. Consequently it lowers the pH of the surrounding environment that will lead to tooth demineralization.[5,6] According to Mithra and Moeny remineralization using desensitizing agent calcium phospho peptide-amorphous calcium phosphate (CPP-ACP) 3 minutes twice a day was achieved after 35 days.[7] Meanwhile the bleaching effect results in enamel porousity which enhances trans–enamel diffusion to reach deep area of dentin and pulp chamber.[8] In vitro studies have demonstrated that a high concentration of toxic components released from hidrogen peroxide 35% bleaching gels used for in office treatment are capable of diffuse through enamel and dentin and they significantly decrease the metabolism of pulp cells.[9,10]

Several studies have reported the effect of hydrogen peroxide on the structure of dental tissue such as pulp sensitivity, cervical resorption, release of selected components of dental materials and alteration of enamel surface.[4,8-10] However little has been reported about the adhesion of *S. mutans* and its relationship with the difference morphological alteration of the outer enamel surface. The aim of this study was to determine the effect of fluoride and non fluoride desensitizing agent that applied prior to and after bleaching procedure on the adhesion of *S. mutans* to enamel and enamel roughness.

MATERIALS AND METHODS

Six maxillary extracted premolar were used as sample of this study. Samples were cut into 4 pieces. Furthermore, the 24 pieces classified into 4 groups, each group contains 6 specimen. Group A was treated using non fluoride CPP-ACP after bleaching procedure was performed. Group B was treated using non fluoride CPP-ACP before and after in office bleaching was performed. Group C was treated using CPP-ACP containing fluoride after bleaching was performed, and Group D was treated using CPP-ACP containing fluoride before and after bleaching was performed.

Group A was bleached using hydrogen peroxide 40% for one hour, washed, dried, followed by the application of non fluoride CPP-ACP for 30 minutes, immersed into human saliva for one hour then immersed into *S. mutans* suspension of 10^8 CFU, incubated 37°C (24 hours). The day after the teeth were put into 3 ml sterilized Brain Heart Infusion (BHI), vortex for 1 minute.[11] After diluting $10,^3$ 0.1 ml BHI containing *S. mutans* were cultured in Trypticase-Soy-Sucrose-Bacitracin (TYS 20B) followed by incubating at 37°C for 48 hours. *S. mutans* colony were counted using colony counter. In group B, before in office bleaching procedure was performed, the non fluoride CPP-ACP was applied for 30 minutes then the samples were washed and dried. After bleaching, the non fluoride CPP-ACP was aplied again, followed by washing and immersed into human saliva for one hour, immersed into *S. mutans* suspension 10^8 CFU, incubated 37°C (24 hours). The day after the teeth were put into 3 ml sterilized BHI, vortex for 1 minute.[12] After diluting $10,^3$ 0.1 BHI containing *S. mutans* were cultured in TYS 20B followed by incubating 37 °C for 48 hours.[12] *S. mutans* colony were counted using colony counter. Group C was treated with the same procedure as group A, but in group C the CPP-ACP used was the one that containing fluoride. CPP-ACP (F) was also used in Group D, it was applied before and after bleaching procedure.

RESULTS

The average of *S. mutans* colony accumulation was shown in Figure 1. The result showed that the the accumulation colony of *S. mutans* decrease when the CPP-ACP was used before and after bleaching. CPP-ACP (F) also reduced the amount colony of *S. mutans* (Figure 1).

The one way ANOVA showed there was a significant difference ($p<0.005$) of the accumulation colony of *S. mutans* among groups with once and twice CPP-ACP application, with and without fluoride (Table 1). The result of this research showed there was a significant difference between groups ($p<0.005$), except between group A and C ($p>0.005$). This may be caused by low fluoride dosage when the DA applied only once. There were Scanning Electron Microscope (SEM) evaluation for detecting enamel surface roughness with 2000 magnification.

In group A (CPP ACP after bleaching only): the dissolved periphery of enamel prism and the porosities could be seen. Areas of remineralization noted although at some places dissolved prism core and dissolved interprismatic substance are still evident (Figure 2). In group B (CPP ACP before and after bleaching) the configuration of the enamel was apparent with few porous defect. Areas of remineralization are evident clearly (Figure 3). In group C (CPP-ACP (F) after bleaching only) the porosities still could be seen clearly, but not as much as in group A. Certain areas of remineralization were evident (Figure 4). In group D (CPP ACFP before and after bleaching): the areas of mineralized deposits were more evident. The different pattern among group A, B, C and D maybe due to variation in crystallite orientation in the enamel prism (Figure 5). From this different morphological alteration point of view, SEM evaluation support the result

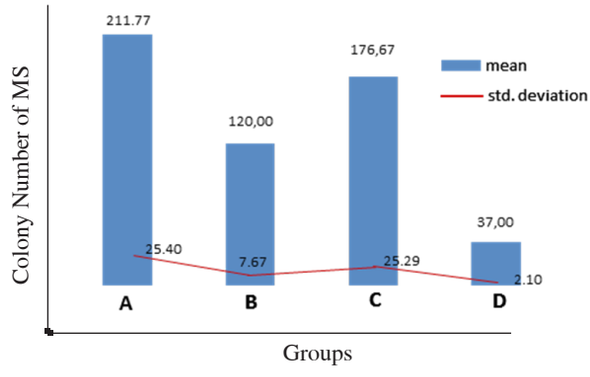

Figure 1. Average of *S. mutans* accumulation after once and twice DA application, with and without fluoride
Note:
A. Bleaching → CPP-ACP
B. CPP-ACP → bleaching → CPP-ACP
C. Bleaching → CPP-ACP (F)
D. CPP-ACP (F) → bleaching → CPP-ACP(F)

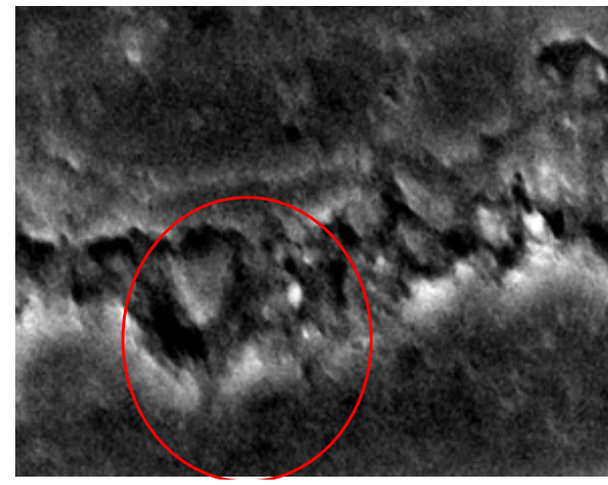

Figure 2. Group A (bleaching→CPP-ACP) the dissolve peripheral enamel prism and porosites apreared

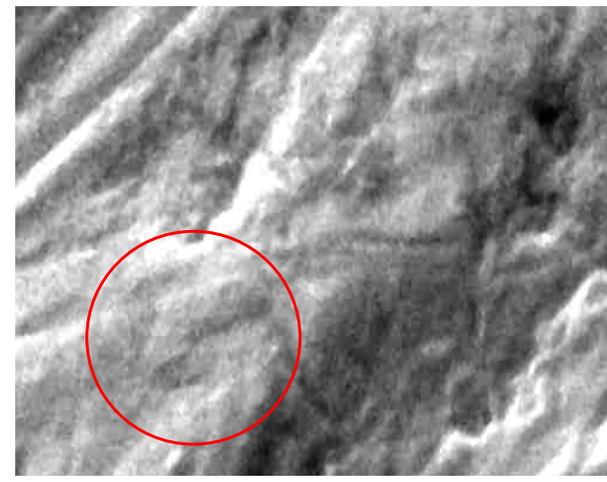

Figure 3. Group B (CPP-ACP→bleaching→CPP-ACP) the configuration of enamel revealed few porous defect

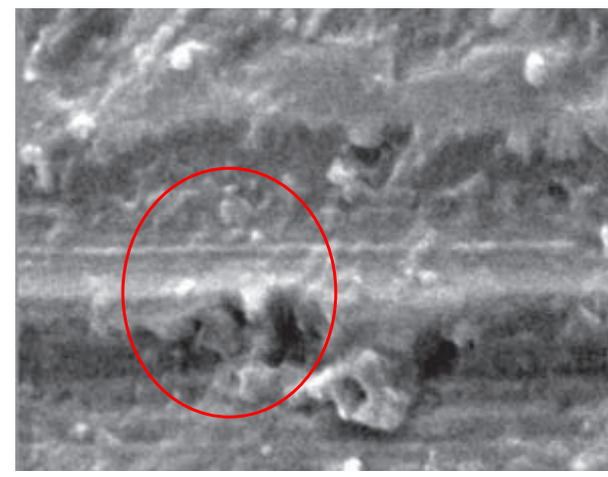

Figure 4. Group C (bleaching→CPP-ACP (F)) the porosities could be seen and certain area of remineralization were evident

Table 1. One way ANOVA of the accumulation colony of *S. mutans* after once and twice desensitizing agent application, with and without fluoride

	Sum of Squares	df	Mean square	F	Sig
Between groups	104163.792	3	34721.264	103.028	.000
Within groups	6740.167	20	337.008		
Total	110903.958	23			

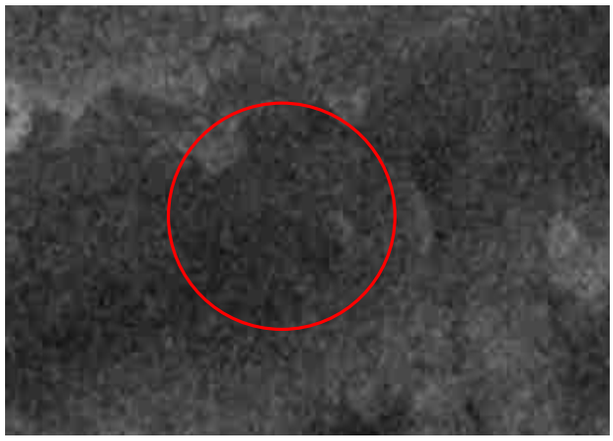

Figure 5. Group D (CPP-ACP (F)→bleaching→CPP-ACP (F) the areas of mineralized deposit were more evident

of this research. The higher the topography irregularities (roughness) of a material, the more the *S. mutans* colony could be counted.

DISCUSSION

The result showed there were significant difference occured on the accumulation of *S. mutans* colony among group A and B, group A and D, group B and C. This fenomena has suggested that there is a relationship between the accumulation of S. mutans with different surface roughness among group A and B, group A and D, group B and C as a result of bleaching treatment with tooth bleaching containing 40% H_2O_2 (Figure 2-5). Tooth bleaching releases reactive free radicals that will influence the reducing agent, so the yellowish pigment (xanthopterin) will be oxidated and become a whitening pigmen (leucopterin).[13] Bleaching agent can produce undesirable effect such as hypersensitivity, gingival irritation, micromorphological defect due to demineralization and effect on restorative material.[1] The result of this research shown that highest mean value of *S. mutans* colony can be noticed in group A, followed by group C, group B and group D. This means that application CPP-ACP (either with or without fluor) before and after bleaching were effective in reducing *S. mutans* colony. Mean value of *S. mutans* colony in group C below the mean value of *S. mutans* in group A. This means that fluoride containing DA showed better result in reducing *S. mutans* colony.

SEM evaluation showed that group A display the highest roughness value, followed by group C, B and D. Surface roughness can be measured by qualitative method such as SEM or quantitative method such as profilometry. SEM is a powerful magnification tool that utilizes focused beams of electron to obtain information while profilometer is a simple tool to measure surface's profile in order to quantify its roughness. It determines line roughness, in either vertical or horizontal direction, but it can not penetrate certain micro irregularities. This tool is easy to operate and rapid to obtain the measuring result. SEM and profilometry have limitation in defining surface topography. The electron beam techniques in SEM does not allow visualization of three dimensional surface texture.[14,15]

According to Katsikogianni, the first stage of bacterial adhesion consist of the initial attraction of the cells to the surface followed by absorption and attachment. At the second stage, molecular-specific reaction between bacterial surface structure and substratum surface become predominant.[16]

There was no significant difference occured between group A and C. This fact can be studied from three perspectives. First, it was suggested that *S. mutans* can adapt to fluoride because of either widespread or longterm use of fluoride. There are few alteration could be detected in fluoride resistant *S. mutans*, one of them is the fatty acid membrane. Membrane of fatty acid plays an important role in maintaining normal physiological cell function, tolerance of physiological stressor including oxidative stress.[17,18] Cell membranes, which are structurally made of large amount of polyunsaturated fatty acid are highly susceptible to oxidative attacks. Oxidative attack will result in oxidative stress when the balance between the existace of reactive oxygen species and antioxidant defence is lost. The free radical mediated oxidative stress result in oxidation of membrane of lipoprotein, glycoxidation, oxidation of DNA, subsequently cell death result.[19]

In fluoride resistance *S. mutans*, the unsaturated/ saturated ratio during the stationary phase was higher than the wild-type strain. A significant difference in the amount of long chain mono saturated fatty acids between fluoride resistant strain and wild-type strain was detected in acidic condition. Previous study conducted by Zhu *et al*.[17] showed that the level of gene that is responsible for biosynthesis fatty acid (*fab*M) RNA in the fluoride resistant-strain was significantly higher than that of the wild- type strain in the acidic condition as well although the sequence of the *fab*M gene was the same in the fluoride resistant strain as

the one reported for the wild-type strain. The *fab*M gene sequence from the fluoride resistant strain was 100% homologous to the wild-type strain. A single gene product of *fab*M in *S. mutans* is responsible for the synthesis of monounsaturated fatty acid and is necessary for survival in acidic environment. Alteration in the lipid content of membranes of an organisme is the major importance in response to environmental stress. These findings are consistent with Zhu *et al.*[17] If that fluoride-resistant *S. mutans* exhibit greater ability to resist acid stress compare to wild-type *S. mutans*.

Another possibilities that influence this result is the concentration of the CPP-ACP containing fluoride in group C is to low (900 ppm). If the CPP-ACP only applied once after bleaching it means the fluoride delivered still below the traditional treatment as in mouth rinse or in tooth paste (1500 ppm). Many researcher still confuse to formulate the precision consentration of fluoride, so debate around fluoride consentration still persist. Treatment with 5000 ppm fluoride significantly enhanced remineralization and inhibit demineralization when compared with treatment with 1500 ppm.[19] In group D, CPP-ACP containing fluoride was delivered twice before and after bleaching. It means the total fluoride delivered was 1800 ppm. Present findings showed lower surface roughness value and lower *S. mutans* colony accumulation (data not shown).

Our results indicated that fluoride combinated with CPP ACFP that was delivered before and after bleaching (group D) showed lower *S. mutans* colony accumulation than CPP ACP without fluoride that delivered in the same manner (group B) This is because fluoride can inhibit glucocyltranferase produced by *S. mutans* by inhibiting enolase that has an important role in glycolysis. Beside that, fluoride was involved in developing complex metal-fluor, usually aluminium floride (ALF4) that account for inhibiting glucan formation from glucose 6–phosphatase that result in inhibiting glucosyltransferase activity.[20]

On the colony counting method, the difference between alive and death *S. mutans* could not be seen, so it is possible to have false positive. The surface roughness value of group A seems to be the same as in group C. This condition could be as a result of contaminant such as residual of the desensitizing agent used that can not be rinsed because of the certain retentive topography on the enamel surface. The variation of enamel topography it self has wide diversity. Every part of enamel surface has diferent surface roughness value that can influence its agent retention capability. In this study the surface roughness value of human enamel before treatment are almost above 1 µm. It means that the starting point of surface roughness value was above critical value for bacterial colonization (0.2 µm).[21]

The study suggested that the application of CPP-ACP containing fluoride before and after bleaching was effective in reducing *S. mutans* accumulation and enamel roughness.

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