EFFECT OF REMINERALIZATION ON COLOR CHANGE OF BLEACHED TOOTH

Miss Panita Suttisakpakdee

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By	Miss Panita Suttisakpakdee
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Thesis Advisor	Assistant Professor Sirivimol Srisawasdi, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Dentistry
(Assistant Professor Suchit Poolthong, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Mansuang Arksornnukit, Ph.D.)

.....Thesis Advisor
(Assistant Professor Sirivimol Srisawasdi, Ph.D.)

.....External Examiner
(Assistant Professor Sirichan Chiaraputt, Ph.D.)

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วัตถุประสงค์ของการศึกษานี้เพื่อที่จะทดสอบอิทธิพลของการคืนกลับของแร่ธาตุและเวลาต่อการเปลี่ยนแปลงของสีและความแข็งผิวของฟันที่ผ่านการฟอกสีฟันด้วย 10% คาร์บาไมด์เปอร์ออกไซด์ (10%CP) หรือ 40% ไฮโดรเจนเปอร์ออกไซด์ (40%HP) โดยใช้ชิ้นส่วนผิวฟันขนาด 6x6x2 มม^3 จำนวน 72 ชิ้น ที่ได้มาจากฟันกรามน้อยของมนุษย์ และได้ทำการวัดสีและความแข็งผิวเริ่มต้นก่อนทำการทดลอง (T0) ชิ้นส่วนฟันทั้งหมดจะถูกแบ่งออกเป็น 6 กลุ่ม โดย 2 กลุ่มจะถูกฟอกสีด้วย 10%CP แบบที่ไม่มีและมีการทาสาร CPP-ACP ตามมา (10%CP, 10%CP/CPP-ACP) อีก 2 กลุ่มจะถูกฟอกสีด้วย 40%HP แบบที่ไม่มีและมีการทาสาร CPP-ACP ตามมา (40%HP, 40%HP/CPP-ACP) หนึ่งกลุ่มจะไม่ได้รับการฟอกสีฟันและไม่ได้ทาสาร CPP-ACP เลย (CON) ส่วนกลุ่มสุดท้ายจะทำการทาสาร CPP-ACP เพียงอย่างเดียวโดยไม่ได้รับการฟอกสีฟัน (CON/CPP-ACP) การทาสาร CPP-ACP นั้นจะทำการทาวันละ 2 ครั้ง ครั้งละ 5 นาทีเป็นเวลา 7 วันภายหลังจากผ่านการฟอกสีหรือตามกำหนดของแต่ละกลุ่ม สำหรับการบันทึกค่าการเปลี่ยนแปลงสีฟันและความแข็งผิวแบบวิคเกอร์นั้น จะวัดเป็น 3 ระยะคือ ภายหลังฟอกสีฟัน 1 วัน (T1) ภายหลังฟอกสีฟัน 2 สัปดาห์ (T2) ภายหลังฟอกสีฟัน 1 เดือน (T3) ซึ่งได้พบว่าหลังจากฟอกสีฟันไปแล้วเกิดการเปลี่ยนแปลงของสีฟัน (ΔE)ในทุกระยะแต่พบว่ามีนัยสำคัญเพียงกลุ่ม 10% CP และ CON/CPP-ACP ($p<0.05$) ที่ระยะ T3 ส่วนความแข็งผิวฟันมีแนวโน้มที่จะลดลงจากระยะ T0 และที่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างช่วงเวลาคือกลุ่ม 40%HP, CON และ CON/CPP-ACP ในแง่ความสัมพันธ์ของการเปลี่ยนแปลงสีฟันและการเปลี่ยนแปลงความแข็งผิว พบเฉพาะในกลุ่ม 10%CP และ 10%CP/CPP-ACP บทสรุป ความขาวของฟันที่ผ่านการฟอกสีมีการลดลงจากระยะ T1 ถึง T3 ในกลุ่ม10%CP และสาร CPP-ACP สามารถป้องกันการคืนกลับของสีฟันได้ในกลุ่ม 10%CP/CPP-ACP และ CON/CPP-ACP ความแข็งผิวถูกพัฒนาขึ้นในกลุ่ม 10%CP/CPP-ACP เมื่อเปรียบเทียบกับกลุ่ม 10%CP ที่ระยะ T3 โดยทั้งนี้ความขาวของฟันที่ผ่านการฟอกสีและความแข็งผิวฟันจะมีความคงที่ได้อย่างน้อยเป็นเวลาหนึ่งเดือนเมื่อได้รับการทาสาร CPP-ACP ซึ่งพบในกลุ่ม 10%CP/CPP-ACP

สาขาวิชา ทันตกรรมบูรณะเพื่อความสวยงามและทันตกรรมรากเทียม ลายมือชื่อนิสิต .....

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The aim of this in vitro study was to investigate the effect of remineralization and time on the color and microhardness change of teeth bleached using 10% carbamide peroxide (10%CP) or 40% hydrogen peroxide (40%HP). Seventy-two 6x6x2 mm3 enamel slabs were prepared from human premolars. The specimens' color and Vickers' microhardness were recorded at baseline (T0). The specimens were divided into six groups, two groups were treated with 10%CP with or without casein phosphopeptides and amorphous calcium phosphate (CPP-ACP) (10%CP and 10% CP/CPP-ACP, respectively), two groups were treated with 40% HP with or without CPP-ACP (40%HP and 40%HP/CPP-ACP, respectively), one group received no bleaching (CON) and another group received only CPP- ACP treatment without bleaching, (CON/CPP-ACP). The CPP-ACP groups were treated with CPP-ACP twice daily for 5 minutes for seven days after completing their respective protocol. The color change and Vickers' microhardness were recorded at three time points after treatment; 1 day (T1), 2 weeks (T2), and 1 month (T3). After 1 month, tooth color changed (ΔE) in all groups at every time point, however, significant ΔE was found only at T3 for the 10% CP and the CON/CPP-ACP groups ($p<0.05$). Microhardness tended to decrease from the baseline (T0) value at each time point. There were significant differences between time points in microhardness (ΔVHN) in the 40%HP, CON, and CON/CPP-ACP groups. A relationship between ΔE and ΔVHN was found in the 10%CP and 10%CP/CPP-ACP groups. In summary, bleached teeth whiteness decreased in the 10%CP group from T1 to T3. The CPP-ACP can prevent color relapse in 10%CP/CPP-ACP and CON/CPP-ACP groups. Microhardness improved in the 10%CP/CPP-ACP group compared with 10%CP group at T3. Both tooth whiteness and microhardness were stable for at least one month when CPP-ACP was used as an intervention in 10% CP/CPP-ACP group.

Field of Study: Esthetic Restorative and Implant Dentistry Student's Signature .....
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Background and Rationale

Hydrogen peroxide (HP) and carbamide peroxide (CP) have been used for decades for in-office bleaching and home-used bleaching. However, the color of bleached teeth is not stable, returning to a darker or yellower color over time.(1, 2) Some studies have demonstrated that development of a darker or more yellowish tooth color after bleaching resulted from absorption of external organic pigments and stains from various foods into the tooth structure or dental restorations.(3-5) In previous study, extracted teeth were soaked in red wine, coffee or tea, showed higher color value, hue, and chroma. The color relapse is an important concern in dental field in terms of improving esthetics. Naturally, older patients are often found to have more yellowish teeth compared to younger patients.(6) These are suggestions that other factors might be involved in causing color change in bleached teeth. Teeth as well as bleached teeth can absorb external chromophores into the internal structure. In addition, tooth color may be showed up more color underneath by mechanical damages, such as tooth brushing, resulting in thinner enamel structure allowing yellow dentin to show through. (7) Nevertheless, thin enamel or other color changes requires a long period of time to develop. Currently, the color relapse process is not well understood, and there are not many studies regarding this issue. If the color relapse process were better understood, we could predict how tooth color of bleached teeth may change in shade overtime. It is very important to be clear that how this issue relevant. This issue is also able to help us to predict the appropriate time that dentist can start to restore the bleached tooth with the tooth color restoration or veneering without worrying of mismatching color causing

from structural changes. Although this is a small issue, we cannot overlook this point because it is an important aspect in esthetic dentistry.

Bleaching causes changes in morphology of enamel surface due to demineralization, resulting in reduced enamel surface hardness and fracture toughness (8, 9). The average calcium loss when carbamide peroxide contacting enamel surface for 6 hours was approximately 1.06 $\mu g/mm^2$.(10) Tooth shade changes after bleaching was related to the color of enamel and dentin with enamel demonstrating significantly higher changes in translucency and color change compared with dentin.(2) Tooth color becomes lighter after bleaching due to destruction of pigment by peroxide and demineralization.(11) Leading to the question that whether remineralization is related to the color relapse in bleached teeth. A study has reported that addition of remineralizing agents in hydrogen peroxide gel, to compensate demineralization process did not influence bleaching efficacy.(12) However, remineralization after bleaching may affect color relapse in bleached teeth because both of strength and color value are both change after bleaching.

The process of color regression should be well understood by dentists because this is a crucial part of esthetic dentistry. It is known that predictable bleached color stability is critical to the long-term success of bleached tooth, but regression of tooth color develops over time, and it may result from the rehydration process of bleaching, reduction in the amount of oxidized substances, increased deposition of external organic pigments or from remineralization. Longer color stability has been reported when the bleaching treatment was extended for 6 months.(1) Moreover, a higher rate of tooth color regression has been found after an in-office power bleaching compared with home-use bleaching at 2 weeks, 1 month and 3 months respectively. However,

there was no significant difference between either treatment in color regression for 6 months after bleaching.(1) Therefore, it can be assumed that, after 3 to 6 months of in-office and home bleaching, factors affecting the color stability disappeared. After 2 weeks, dehydrated teeth, by the dehydration effect of in-office bleaching with light application, would be completely rehydrated or re-stained; therefore, this may be one of the reasons that in-office bleaching treatment presents color relapses faster than home-applied bleaching.(13)

It is not currently known, whether tooth shade regression after bleaching treatment develops by the remineralization of enamel or dentin apart from rehydration and external pigments absorption. Nonetheless, after bleaching calcium and phosphate are released from the enamel, the tooth shade becomes lighter and whiter. Therefore, the objective of this study is to determine the correlation between the color regression of bleached teeth and remineralization of the tooth structure by two bleaching agents.

Literature Review

There are many methods for treating teeth to be lighter and whiter, such as whitening toothpastes, bleaching strips, professional bleaching or other OTCs bleaching. Chemical agents, which are very popular to whiten tooth is hydrogen peroxide and carbamide peroxide, have been used for many decades and considered quite safe for patients. The effect of hydrogen peroxide and carbamide peroxide to improve tooth colors result in hard dental structure is oxidizing organic pigments, decreasing pigments size, and reducing a number of pigments.(14)

Carbamide peroxide has more complex molecule more than hydrogen peroxide and use longer time to release peroxide molecule into tooth surface. As a result of carbamide peroxide function, it has considered to be used as a home-use bleaching agent instead of in-office bleaching agent because clinicians would reduce chair time of in-office bleaching, while home-use bleaching patients can spend a lot of time to use it at home without concerning about stability of agent and waste of time during the day. Although the effect of carbamide peroxide seems to be inferior to hydrogen peroxide, its stability of function during treatment has more advantages than hydrogen peroxide and less side effects to soft tissue. Therefore, carbamide peroxide has been used with the same desirable outcome as in-office bleaching to improve and maintain tooth color until at present. Carbamide peroxide was oxidized its molecule into hydrogen peroxide and urea to start working. Braun, et al, 2007 showed the results of their study that tooth color cloud change whiter after 7 days bleached with 10% carbamide peroxide treatment.(14) However, the variations of tooth colors could be indicated that there was a similar result of tooth colors change when treated with 10% and 17% carbamide peroxide gel for 1 week.(14)

In fact, both of hydrogen peroxide and carbamide peroxide agent whiten natural teeth via influencing carbonic rings in chromophore molecules, absorbing more light than unsaturated double-bond structure in linear chain.(15) Moreover, the colors of teeth can be whiter when facing a situation of demineralization and dehydration.(16)

The another reason bleached teeth have become whiter may result from the morphological changes which has an evidence with the use of the SEM or confocal laser scanning microscopy to observe sections in bleached teeth after treating with hydrogen peroxide or carbamide peroxide in previous studies.(8, 17)

According to tooth morphology, there are three main compositions of the tooth structure, which are enamel, dentin and pulp chamber. The two structures of enamel and dentin are classified as dental hard tissues, whereas pulp chamber is considered as soft tissue. In enamel part, G.Stein and P.E. Boyle demonstrated a histological observation, and found yellow pigment in ameloblast and the cell of stratum intermedium in granule form, and this pigment has resistant to 5% nitric acid.(18) Some authors stated that pigmentation on enamel due to nutritional deficiencies, hormonal disturbances and various intoxications.(19) For dentin area, it shows higher organic matter than enamel area. The results of one study also pointed out that when bleaching with 38% hydrogen peroxide for 20 minutes, the enamel surface morphology was significantly changed, but did not affect the dentin area.(8) Actually, dentist frequently found that root canal treated tooth could become darker after completed treatment without blood circulation inside as vital tooth; therefore, some studies mentioned that its discoloration caused by dental treatment or dental medications. However, after walking bleach treatment, the color relapse still occur as vital bleaching.

Pigmentation in tooth structure is essential for color perception and appearance. A study showed that CIE lab color scale, b* value was significantly correlated with patients' whiteness satisfaction. As a result of above mentioned study, changing yellow to blue scale is more important than other scales in terms of whiteness satisfaction.(20, 21)

Another impact factor of tooth color changes is food, drinks and pigment colors in humans consuming in today's world. This factor leads to a question that how we can control external pigmentation in clinical study. All of previous clinical study about the

rebounding of tooth color has not shown how they controlled the external pigments, so that the scientific evidence about tooth color relapse may not be reliable enough.

Evidently, some studies mentioned about color of food staining which affected tooth whiteness changes. Villalta et al and Um, et al. investigated extrinsic factors, such as absorption of stains into dental restorations, finding that extrinsic staining by wine and coffee caused significant discoloration.(3-5) These results probably attributed external staining and caused tooth color relapse.

Tooth surface in each area also presents several various shades of itself because of different light reflected in each section. Moreover, pigmentation, mineral deposition and thickness of enamel and dentin layer in different part may be the reasons why the tooth cannot show homogenous color. More evidently, the incisal part of tooth has significant color change greater than middle and cervical regions, but color after bleaching in middle part compared with cervical part was not significant difference.(22)

Interestingly, in vivo of in-office bleaching study, Alomari Q and Daraa EE, 2010 had an agreement with other studies that if the tooth had higher in initial color lightening, tooth color relapse was more likely to occur after one month.(20, 23) This study also determined that degree of relapse corresponded with the degree of initial color change.(23) In addition, the tooth colors was stable for 6 months after using two in-office treatment sessions.(21) However, the effectiveness of in-office bleaching was considered to be mainly due to the effect of dehydration on color change; therefore, after in-office bleaching the tooth whiteness decrease within 1 hour.(16, 24)

In many situations, dentists can observe a whiter of tooth surface after demineralization progress. For example, in case of tooth color restoration, the tooth surfaces have to face acid etchant to prepare an appropriate tooth structure before

placing restoration material, chalky white appearance will be showed after air blowing. Meaning that the tooth surface has morphological changes from acid etchant, resulting from reflection of light was different. Another situation can observe in poor oral hygiene patient, which is a white spot lesion on the smooth surface of tooth. This lesion is also resulting from demineralization process, but it will be disappear when the remineralization process is developed.

Remineralizing agents, which are added into both types of bleaching agents do not affect the effectiveness of bleaching. It also improves strength of tooth surface and prevents demineralization situation.(9, 12, 25) However, the tooth surface after bleaching typically found that its strength was reduced after the treatment. Many previous studies have been reported the effect of bleaching that, both of in-office bleaching and home-applied bleaching initiated tooth demineralization, which was confirmed by SEM or microhardness or micro-CT or atomic absorption spectrophotometer measurement.(10, 11, 26, 27) CPP-ACP was proved by many previous studies about remineralization, sensitivity, flexural strength and staining topics after bleaching.(28-31) As a result of these studies, they stated that CPP-ACP is able to improve tooth surface roughness and acid resistance, reduce tooth sensitivity, increase microhardness and decrease tooth staining after bleaching treatment.(28-31) Therefore, CPP-ACP should be used to improve enamel properties after bleaching. However, some studies showed remineralization effect of CPP-ACP compared with natural saliva, which was found that there was no different between CPP-ACP and natural saliva of remineralization effect for 15 days.(32) Nevertheless, another one study showed the evidence CPP-ACP applied for 1 minute, significantly increased mineral deposition and decreased carious lesion depth.(33)

Hard Dental Tissue Characteristic

1. Enamel

Enamel contains 94-96% inorganic matrix by weight, 4 % organic matrix by weight and water. In high risks caries situation, enamel resists to the processing of caries via acquiring ions from oral fluids, increasing size of crystal and decreasing the pores between them. As a result of these mechanisms, enamel can reduce its permeability and the tooth becomes darker.

Enamel light reflection, in vitro study presented enamel reflectance and transmission of thin slabs at wavelengths between 220-700nm,(34) and also showed that enamel does not have a major role in scattering at wavelengths in the blue range. However, the enamel slabs can be reflected pale blue and transmits pale yellow color.(10)

Physical characteristic of enamel indicates that enamel is the hardest calcified tissue in the human body. Nevertheless, enamel also shows brittle characteristic and can act as a semi-permeable membrane, permitting complete or partial passage of certain molecules. It has compressive strength around 384 MPa, tensile strength about 10 MPa, 84 GPa of elastic modulus, and 350-430 of Knoop hardness.

2. Dentin

Dentin composes of 20% organic matrix, 10% water and 70% inorganic matrix by weight(35). The organic composition consists of approximately 90% by majority of collagen type I and trace amounts of collagen type III or IV and ground substance.(35) The intertubular dentin has crystallite form composing from a needle appearance with 2-5 nm in thickness and 60 nm in length.(35)

Dentin color can be used to identify the color of tooth because it shows the majority color of tooth through out of enamel and reflects light sources which incident tooth surface. Generally, dentin color is quite light yellow and become darker related with time and aging.

Physical properties of dentin are harder than bone and cementum, but softer than enamel. Dentin has less resistant to acid, abrasion, and attrition. Its compressive strength is about 297 MPa, tensile strength 52 MPa, elastic modulus 18 GPa and Knoop hardness approximately 68, which shows less radiolucency than enamel.(21, 35)

History of tooth bleaching

Whiteness of tooth is one of the most desirable characteristics in a lot of people for beauty and attractive smile to everyone. It seems to be an important social value for people who concern with tooth esthetic function. In 1864, bleaching agent had been described for the first time, a number of chemical agents such as chloride, sodium hypochlorite, sodium perborate and hydrogen peroxide were used to treat in non-vital tooth discolored by combination or one agent.(26) After that other dentists tried to improve tooth color in vital tooth. In 1961, pulpless bleaching was improved to "walking bleach technique", used sodium perborate mixed with water into pulp chamber during visits.(26) Moreover, the thermocatalytic technique was developed by Stewart,1965. This technique was recommended by using heating lamps or other heat generated devices after placing oxidizing agents in pulp chamber. Another technique is called "inside/outside technique" which uses the combination of 10% carbamide peroxide and custom tray treating in the pulp chamber simultaneously.(36)

Hydrogen peroxide and carbamide peroxide has been used as bleaching agents including in over the counter (OTC) products. All of these agents were divided into two groups in terms of approaching techniques, called in-office bleaching and home bleaching. Most of the in-office bleaching treating with hydrogen peroxide while home bleaching actually used low concentration of carbamide peroxide. After that, tooth bleaching has been developed for many decades until at present.

Etiology of Tooth Discoloration

1. Extrinsic Causes

Most of the extrinsic factors affect tooth color in post eruptive period. Chromogen can be derived from dietary sources by absorption, such as coffee, red wine, tea and color-rich vegetable that absorbed directly into the tooth.(24, 37, 38) Staining on tooth surface is also caused from extrinsic etiology and it can be removed easily by polishing with prophylaxis cups and abrasive paste.(38) Other sources of extrinsic factor are chemical staining that indirectly adhere and usually found in polyvalent metal salts or cationic antiseptics e.g. chlorhexidine.(39) The staining from chlorhexidine mouthwash causes black and brown on superficial teeth. In poor oral hygiene patients, their teeth can be changed to green, brown or orange color, which are produced by chromogenic bacteria. Another one of extrinsic stain causes by tobacco this is type of direct stain have dark-brown color in general and it is also able to remove by polishing like dietary stain.(40)

2. Intrinsic Causes

In period of tooth development, tooth has deposition of many organic and inorganic molecules. One of those is pigment or chromogen that created color shade on

tooth appearance being color yellow, brown, red, blue, gray or black.(39) Moreover, the aging is one of the factor causing discoloration originated from deposition of secondary dentin, tertiary dentin and pulp stone or enamel changes via thinning.(38-40) Drug deposition on tooth most often on tetracycline treatment and fluoride during tooth formation which causes abnormal color or surface appearance, calling this phenomenal of tooth discoloration as the same as the name of medicines causing color changes, such as tetracycline tooth or fluorosis tooth. Another cause of intrinsic factor can be occur on tooth completely erupted period, such as trauma associated with pulpal hemorrhage and necrosis, as well as dental caries establishing redder and darker tooth.(38-40)

Abnormal tooth structure is also internal etiology of tooth color abnormality. It may be due to hereditary, some diseases or congenital intrinsic discoloration such as amelogennesis imperfecta, dentinogenesis imperfecta, jaundice disorder, Günter's disease, etc.

For internal causes in non-vital teeth, there are many possible suggestions from authors; for example, first, tissue degradation during the necrotic process causing tooth color change. Second, hemolysis of red blood cells causing from trauma or leaves pulp remnants during treated root canal; iron in red blood cells may be defused into dentinal tubules as mentioned above. Third, intracanal medications, which are phenolics and iodoform-based causing dentin penetrated and oxidation in dentin. Forth, the corrosion from silver points in the root canals can cause black or grey colors inside the tooth. Endodontic cement can also cause color change. The last etiology is insufficient irrigation and debridement.

Type of External Bleaching

In external bleaching techniques, bleaching agent is applied to external surface of enamel, divided into 2 main groups.

A. In-office Bleaching

This technique basically involves application of 30 to 40% hydrogen peroxide concentration with and without heat and light or ultraviolet rays to the enamel surface. Commercial in-office bleaching as we know can be divided in two systems.

I. Power bleaching systems

II. Non-power bleaching systems

B. Home-applied Bleaching

This technique requires fabrication of trays to be used with bleaching gel. A lot of products are available in the markets, mostly compositions contain either 3 to 10% hydrogen peroxide or 10 to 35% carbamide peroxide, divided into two types as following:

I. Bleaching gel with professional (custom) tray

II. Over the counter (OTC): OTC tray, whitening strips, paint-on brushes, whitening toothpastes, etc.(41)

Tooth Mineral Changes

1. Demineralization

Demineralization of tooth is the process of minerals decalcified from hard tissues. This process is normally occurs in an acidic environment in oral cavity, releasing calcium and phosphate from tooth structure, then roughness and porosity on the tooth surface will be found as well as the reducing of microhardness. (9)

2. Remineralization

Remineralization is a mechanism of inorganic molecules reforming into the hard tissue structure, presented in tooth structure after retrieving fluoride molecules, calcium phosphate molecules from artificial saliva or from other remineralizing agents. Remineralization from natural saliva alone may not be enough to completely seal the dentinal tubule from demineralization.(42) The majority of topical remineralizing agents are divided into the following:

2.1 Fluoride-remineralizing agent: sodium fluoride (vanish, or gel; acidulated phosphate fluoride gel (APF)), fluoride mouth rinse, fluoride in dentifrice (stannous fluoride), etc. The fluoride ions promote remineralization process by forming fluoroappatite crystal or calcium fluoride in tooth structure, enhancing surface hardness of enamel.(42) Fluoride dissolve in saliva when pH dropping lower than pH 5.5 and presents in plaque fluid to adsorb into the tooth surface to prevent demineralization .(43) When pH increases above the pH 5.5, the fluoride adsorbs to the tooth surface and forms calcium fluoride layer or fluoroapatite.(43) In addition, fluoroapatite crystal has higher resistant to acidic environment than hydroxyapatite structure, and replacing the old inorganic matrix, resulting in the stronger tooth structure after the remineralization (42, 43).

On bleached teeth, sodium fluoride can promote increased enamel micro-hardness, and reduces the demineralization process on bleached teeth after 5 days when treated with 35% carbamide peroxide.(42)

2.2 Calcium phosphate remineralizing agent: a casein phosphopeptide stabilized amorphous calcium phosphate (CPP-ACP or Recaldent^{TM}), an unstabilized amorphous

calcium phosphate (ACP or Enamelon^{TM}) and a bioactive glass containing calcium sodium phosphosilicate (NovaMin^{TM})

The calcium phosphate is one of the tooth morphological structures in part of inorganic matrix, and can form with fluoride ion to crystals of fluroapatite, which are harder than normal tooth structure. The inorganic molecules in fluoroapatite are incoporated from ten molecules of calcium, four molecules of phosphate and two molecules of fluoride.(44) Remineralization process will develop as remineralizing process of fluoride, if there is adequate calcium phosphate level. Therefore, the calcium phosphate-based is a good alternative remineralizing agent for tooth remineralization. (44, 45).

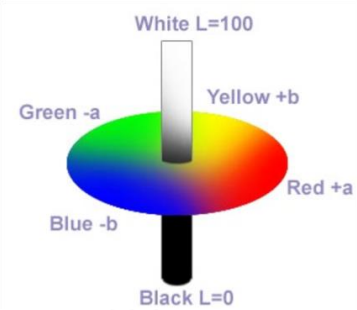
CPP-ACP is derived from a dairy product. It has been shown to have anticariogenic activity, increases the level of calcium and phosphate in suppragingival plaque to promote remineralization.(44, 45) Moreover, CPP-ACP is also believed to interferes the growth of bacteria and its adhesion.(45) For the commercial claim, Recaldent^{TM} claimed that CPP-ACP ingredient strengthens teeth by delivering calcium and phosphate to the tooth's surface.(44) The effect of CPP-ACP in bleached teeth which was studied showed that CPP-ACP helped increase flexural strength of hard dental structure and also reduced the absorption of stain in bleached teeth.(45)

Color Systems

1. CIELAB color system

This color space was developed with the use of three-dimensional spaces which are expressed as L* a* and b* values. L* is axis of graph, which explain the

luminance of the object; from black (100%) to white (0%). While a* and b* is represented red (+)

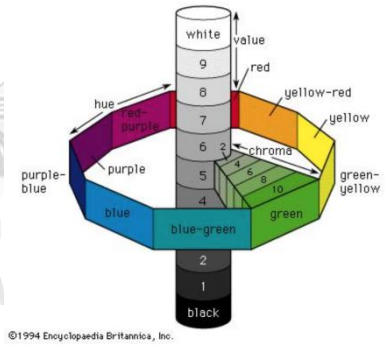


https://www.pce-instruments.com/english/measuring-instruments/test-meters/color-meter-colour-meter-kat_40051_1.htm

Figure 1. CIELAB color system

or green (-) and yellow(+) or blue(-) in parameter. This system is used in almost all of shade measurements, such as spectrophotometer, colorimeter and vita 3D-master.

3. Munsell Color System



http://www.britannica.com/science/color/images-videos

. **Figure 2.** Munsell color system

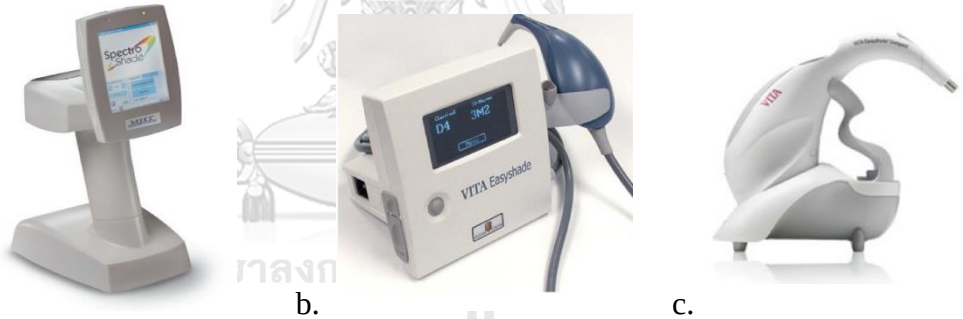
Munsell color system was developed by A.H.Munsell between 1905-1929. This color system is described into three scales values, which are hue, chroma, and value. Hue is the most characteristic of a color, which shows shade of the color, such as, red color, yellow color, blue color, etc. Saturation of color is called chroma. High saturation of color will provide color seem rich and full. Low saturation of color will make color

look dull and grayish. Value is described as brightness or luminosity, which provide the scale of light or dark color, white or black. All high saturation color is medium values rather than low or high value. This shade system is often used in laboratory communication.(46)

Shade Measurement

1. Spectrophotometer and Colorimeter

Spectrophotometer is a machine to measure color and shade matching both in vivo and in vitro.(1, 14, 26). Using spectrophotometer to evaluate the color of tooth was recommended in several studies as the highest reliable standard for color matching.(1, 14, 47)



a. b. c.

http://www3.unisi.it/dl2/20100304131719197/Corciolani.pdf

Figure 3. Spectrophotometers; a. Spectro Shade Micro(MHT), b. VITA Easyshade (spectrophotometer), c. Easyshade Compact with LED and wireless technology.

Some study revealed that an agreement of color between human eye and the machine was observed in the color dimension of lightness (K=0.6587), hue (K= 0.4337) and chroma (K=0.3578) respectively.(8) From the past, spectrophotometer has been developed together with digital color imaging. However, the cost of this device is high due to its advances and technology. Colorimeter was developed to reduce the cost and

more simple to use, but decreased in accuracy of measurement compared with the real spectrophotometer and smaller reading opening. Nowadays, both of them are mamufactured together with digital camera and digital color imaging in order to gain more effectiveness, quality, and convenience to the users.

Vita Easyshade (Vita Zahnfabrik, Bad säckingen, Germany) is a spectrophotometer device, which measures in small spot area approximately 5 mm diameter of optical tip. This device translates data into both of Vita shades and also into CILAB data from halogen light reflectance and scattered light. The advantage of Vita Easyshade compact is portable, small, battery operated, and contact type.(48)

2. Visual Shade Matching

a. b.

http://www.dentaleconomics.com/articles/print/volume-100/issue-11/features/shade-guides-and-color-visualization.html

http://www.dentalcity.com/product/12288/vident-vita-shade-guide

Figure 4. Two types of Vita Shade systems; a. Vitapan Classical, b. Vita 3D-Master

Visual shade matching is one of the color matching systems. Reliability of this system depends on accuracy of humans's eyes to detect differences of shade. The light source of a room has an influence on this method. Human's eye can limited receive and absorb light and have difficulty to divide in terms of sensitivity of color. However, an agreement with spectrophotometer is about 65% in lightness value.

This measurement is most often use dental shade guide as the color of ceramic systems, as well as aid dentists and patients to discuss about the results of the patient's tooth whitening during treatment plans. For example, the Vitapan Classical consists of 16 tabs and the Chromas is arranged by hue within the groups (A1 to D4). Using letter in each shade, the letter A means red-yellow, B means yellow, C means gray, D means red-yellow-gray, and the numbers 1-4 show differences of chromas and values.(49) Moreover, there are Vitapan 3-D and VITA Bleached shade for using in different systems and proposes. For VITA Bleached shades consists of shades 0M1, 0M2, 0M3 specially using for bleached teeth or patients who want extremely white teeth by veneering.

Remineralization Evaluations

For assessment the mineral changes in dental hard tissue, there are many methods to use and to evaluate in several terms as these following:

1. Microhardness test

The microhardness test is one of the most accurate evaluations for tooth remineralization, which are these following:

Vicker's hardness is the method for measuring small specimens using a diamond indenter to make a light loads indentation, which is raging from a few grams to one or several kilograms. The opposing indenter faces are set at a 136 degree angle from one another.(9, 15, 30)

Knoop hardness is a mechanical hardness, mostly used in very brittle specimens or thin sections. Knoop indentation is applied with a pyramid shaped diamond indenter

as Vickers, but the differences are the faces angle and width, the long faces is set at 172 degree and the another short faces are set at 130 degree.(50)

2. Micro-CT scanning

Micro computed tomography or micro-CT or microtomography is an x-ray imaging in 3D. The advantage of micro-CT is that the specimen is not destructed from this machine. It can measure longitudinal mineral changes several time in the same lesion.(40, 41) Moreover, micro-CT is able to investigate rate of remineralization and demineralization. The outcome data will show in 4 different depths of density and rate of remineralization or demineralization. The study of Lo EC and Itthagarun A. showed an advantage of evidence in the reliability of micro-CT that can be used to detect changes of demineralization and remineralization lesions.(51, 52) The principle of micro-CT is that x-ray penetrates through the specimens, and then the detector system will collect the data in order to reconstruct 2D or 3D images, or to transform the grey level values of images into true mineral density values.(52)

3. Scanning Electron Microscope

The scanning electron microscope is a device to detect changing surface of specimens. The purpose of the use of scanning electron microscope is to observe the superficial morphology of specimens or surface changes by showing in the images.(53) It is a type of electron microscope, using a focused beam of highly energy electron to scan a sample. EDS are used to detect chemical composition, and EBSD detector can determine the mineral and crystal structures. Wet samples are unsuitable for examination in SEM.(53)

4. Polarized Light Microscopy

Polarized light microscopy has been used to detect the mineralization and remineralization with high reliability of confidence as a standardized method. The images are shown in 2D and a thickness more than 100μm.(51)

5. Transverse Microradiography

Transverse microradiography (TMR) method used as a gold standard of demineralization or remineralization evaluation. It provides a monochromatic radiation to detect the thin object and then transforms the data to volume percentage of mineral.(54, 55) However, this method is not suitable for longitudinal changes because of its destructive tissue and the problem of time consuming.(52) The sections using in this method have to be dried and it will be difficult to preserve the normal structure of tissue because of losing water and shrinkages of tissue.(56)

6. Electron Probe Microanalysis

Electron probe microanalysis (EPMA) with energy-dispersive or wavelength-dispersive spectrometry has also been used for dental hard tissue analysis. The technique was found by Ngo et al to quantify the mineral changes in each element in hard tissue profile which was detected by percentage of molecular weight.(57)

Research Questions

1. Is there a correlation between microhardness and bleached color change?
2. Do remineralization time have an influence on bleached color change?

Research Objectives

1. To investigate the influence of tooth remineralization on color of bleached tooth.

2. To compare the effect of two types of bleaching on color change of bleached tooth.

3. To investigate the effect of remineralization times on color change of bleached tooth.

Statement of Hypothesis

Null Hypotheses

1. There is no correlation between microhardness change (ΔVHN) and bleached color change (ΔE).

2. There is no difference of bleached color changes (ΔE) at all periods of time (T1-3).

Alternative Hypotheses

1. There is a correlation between microhardness change (ΔVHN) and bleached color changes after remineralization.

2. There is a difference of bleached color changes (ΔE) at all periods of time (T1-3).

Conceptual Framework

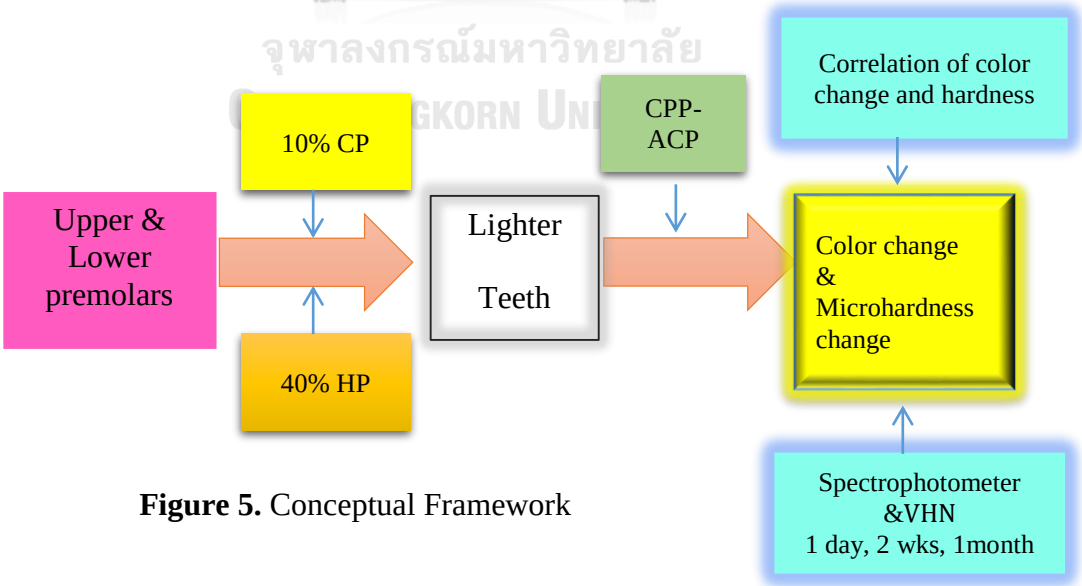


Figure 5. Conceptual Framework

Population: Humans' upper or lower premolar teeth.

Intervention: 10% Carbamide peroxide home bleaching agent was used for 4 hours a day for 2 weeks. 40% Hydrogen peroxide in-office bleaching was used only 20 minutes for two sessions, 1 day. CPP-ACP was used to apply in bleached area after complete bleaching treatment in two groups.

Outcome measurement: Color changes after bleaching, and microhardness (VHN) was measured with the use of spectrophotometer and Vicker's hardness after applying bleaching within 1 day, 2 weeks and 1 month respectively.

Key words:

Bleaching

Carbamide peroxide

Tooth color relapse/ rebounding/ regression

Tooth whitening

Remineralization

Sample size calculation

Sample size by is estimated following Polydorou O and Hahn P, 2008(50), which are 12 teeth/group.

For this study, 12 teeth per group would be followed as similar mentioned study. Therefore, total teeth were used in this study is 72 teeth, n=12/group.

Research methodology

Materials

1. 10% Carbamide peroxide gel (Opalescence PF 10%; Ultradent, South Jordan, UT, USA)

2. 40% Hydrogen peroxide gel (Opalescence Boost 40%; Ultradent, South Jordan, UT, USA)

3. Polishing cup (Disposable Prophy Cup for Low Speed Handpiece, Tianjin, Shanghai, China)

4. Pumice polishing powder without fluoride (Rite-Pumice X-Fine, Rilo Dent Mfg Corp, Hialeah, Florida, USA)

5. CPP-ACP (Tooth Mousse; GC Europe N.V., Leuven, Belgium)

6. Artificial saliva (Artificial saliva, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

7. 0.1% Thymol solution (M Dent, Bangkok, Thailand)

8. Fintrec transparent polyester matrix (PULPDENT m-ds Ultra-thin, ALPHA dental supplies ltd., ON, Canada)

9. Trephine drill diameter 5mm (Hu-friedy, Chicago, IL, USA)

10. Clear epoxy resin (SC2B clear, Super Silicone & Resin Art, Bangkok, Thailand)

Equipment

1. Spectrophotometer (VITA EASYSHADE V, VITA Zahnfabrik, Germany)

2. Microhardness Machine (FM-700e TYPE D, FUTURE-TECH, Japan)

3. Polishing machine (Nano 2000, Pace Technologies, USA)

Methods

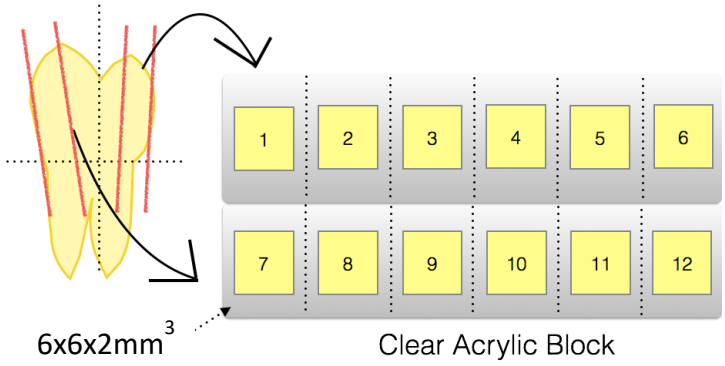
1. Sample description

The required sample size was determined after a pilot study. Extracted human upper and/or lower premolars were collected after informed consent, and obtained under a protocol approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, approval number HREC-DCU 2016-063. The collected teeth were disinfected in a 0.1% thymol solution (M Dent, Bangkok, Thailand) until the specimens were prepared.

2. Preparation of samples

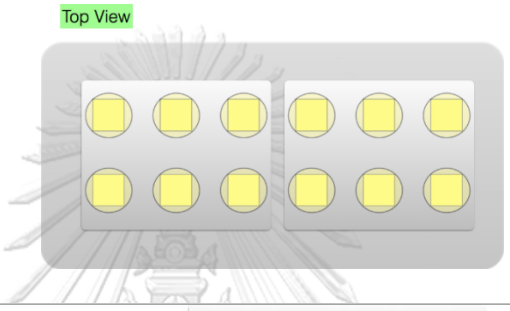
External staining was removed by polishing each tooth with pumice and rubber cup. Any teeth with visible cracks on the enamel surface, caries lesions, pathologic lesions or restorations were excluded from this study. Teeth with A and B shades (Vita shade) were used in this study. Seventy-two teeth were cut at the CEJ using a diamond disc 104918.190 HP (Diaswiss S.A., Nyon, Switzerland), and the crowns were separated buccolingually. The buccal crown portions were prepared with a diamond disc generating a 6x6x2mm3slabs. Subsequently, a polishing machine (Nano 2000, Pace Technologies, USA) with abrasive papers (800-2000 grit, Leco Corporation, MI, USA) and aluminum oxide paste was used to polish the surface of each specimen.

a.



b.

c.



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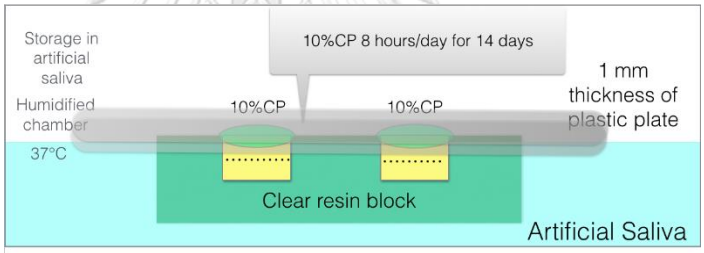
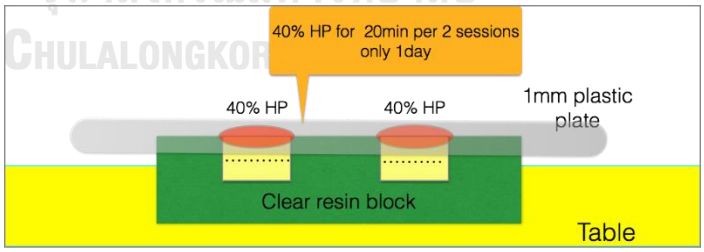


Lateral View

e.



Lateral View

f.

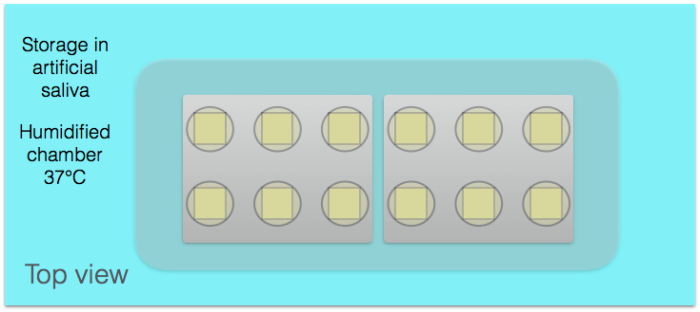
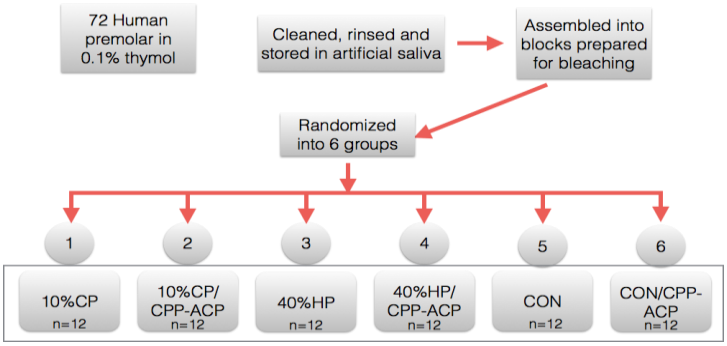


Figure 6.
a . Buccal and lingual side of upper premolar was cut into tooth slab with size 6x6x2mm^3.
b. 1mm plastic plate with 6mm diameter, 6 holes/plate (n=72).
c. Punched-plastic plate covers the teeth block.
d. Tooth blocks were treated with 10% carbamide peroxide with humidified chamber of artificial saliva.
e. 40% hydrogen peroxide was used under dry environment, 20minutes for 2 sessions.
f. CPP-ACP was applied on tooth blocks with in the hole of plastic plate under humidified chamber, 37°C.

3. Model preparation

A 6 mm diameter trephine bur (Hu-friedy, Chicago, IL, USA) was used to create two rows of 6 mm holes (6/plate) 5 mm apart in the plastic plates for the tooth specimens to be placed in. Twelve plates were prepared. The specimens (n = 6) were placed in the cavities of each block. Clear epoxy resin and accelerator liquid (SC2B clear, Super Silicone & Resin Art, Bangkok, Thailand) were mixed and poured into the cavities up to the level of the specimen and was allowed to set for 24 h.

4. Allocation technique



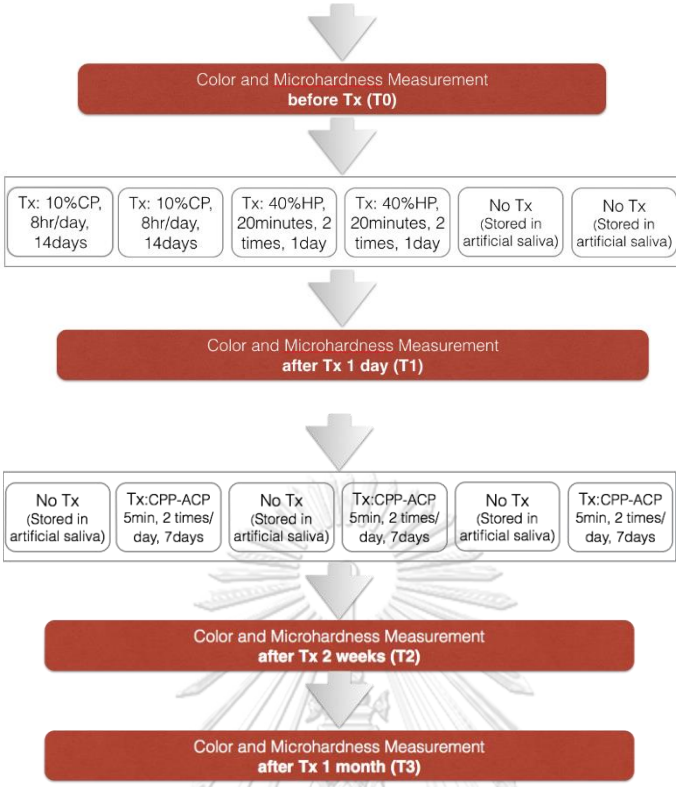


Figure 7. The procedure sequence of this study

All samples were divided into six groups. Initial shade and initial microhardness in all tooth specimens were performed with spectrophotometer (Vita Easyshade V, VITA Zahnfabrik, Germany), and microhardness machine (Fm-700e Type D, Future-Tech, Japan) as a baseline.

Group 1: **10% CP**--teeth are bleached with 10%carbamide peroxide 8 hours a day for 2 weeks without remineralizing agent. All teeth were stored in artificial saliva all period of time. Both of tooth shade and microhardness test were determined before and after 2 weeks of bleaching at 1 day, 2 weeks and 1 month.

Group 2: **10%CP/CPP-ACP**--teeth are bleached with 10% carbamide peroxide 8 hours a day for 2 weeks. Afterward, CPP-ACP was applied at bleached

surface for 5 minutes twice daily for 7 days. Both of tooth shade and microhardness test were measured after 2 weeks of bleaching at 1 day, 2 weeks, and 1 month.

Group 3: **40%HP**--teeth are treated with 40% hydrogen peroxide (Opalescence Boost 40%; Ultradent, South Jordan, UT, USA) for 20minutes, 2 sessions for 1 day without remineralizing agent. All teeth were stored in artificial saliva all period of time. Both of tooth shade and microhardness test were determined before and after bleaching at 1 day, 2 weeks, and 1 month.

Group 4: **40%HP/CPP-ACP**--teeth are treated with 40% hydrogen peroxide (Opalescence Boost 40%; Ultradent, South Jordan, UT, USA) for 20 minutes, 2 sessions only 1 day. Afterward, CPP-ACP was applied on bleached surface for 5 minutes twice daily for 7 days. Both of tooth shade and microhardness test were determined after bleaching at 1 day, 2 weeks, and 1 month.

Group 5: **CON (Negative control)**--teeth were stored in artificial saliva all the time in an incubator with 37ºC. Both of tooth shade and microhardness were measured before stored in artificial saliva, after stored in artificial saliva 2 weeks at 1 day, 2 weeks, and 1 month.

Group 6: **CON/CPP-ACP (Positive control)**--teeth were stored in artificial saliva all the time in an incubator with 37ºC. Both of tooth shade and microhardness were measured before stored in artificial saliva. CPP-ACP was applied on bleached surface for 5 minutes twice daily for 7 days. Afterward, all specimens were stored in artificial saliva and measured again at 1 day, 2 weeks, and 1 month.

5. Bleaching procedure

10% Carbamide peroxide (Opalescence PF 10%; Ultradent, South Jordan, UT, USA) was applied on the surface of tooth slabs equally to the dimension of plastic hole. Afterward, cement spatula was used to spread out bleaching gel equally to plastic template. Another plastic plate was used to cover teeth block until 8 hours of treatment time a day, within closed-box filled with artificial saliva.

In-office bleaching group, 40% hydrogen peroxide was applied on the surfaces of tooth with punched plastic plate to control in-office bleaching thickness. Steps for using 40% hydrogen peroxide is that two sides of in-office bleaching syringe were pressed to mixed red and white gel together. A minimum pressing time to mix the contents is 25 times on each side as manufacturing instruction. The mixed-bleaching gel was applied into center of the hole in plastic plate on the tooth surface until completely filled the hole. Cement spatula was used to spread out the bleaching gel. Treatment time is 20 minutes per session for twice sessions. Therefore, overall in-office treatment time is 40 minutes. After completed bleaching, tap water and microbrush were used to rinse and remove the agents. All blocks were stored in artificial saliva again, waiting for further procedure and analysis at each time point.

6. Remineralization Procedure

CPP-ACP (Tooth Mousse, GC Europe N.V., Leuven, Belgium) was used for remineralization procedure (group 2, 4, 6). The use of punched plastic plate was performed to monitor the volume of remineralizing agent. CPP-ACP was placed onto the holes on tooth slabs. Cement spatula was used to spread out again. After 5 minutes, CPP-ACP was renewed and left for another 5 minutes again. Then CPP-ACP on tooth

block was rinsed off with tap water and stored in artificial saliva. All of remineralization procedures were repeated every day for 1 week. The artificial saliva was refreshed every 1 to 2 days up to 1 month.

7. Outcome measurement

7.1 Tooth shade measurement

Spectrophotometer is a color measurement device. This device has reliability and color sensitivity better than human's visual. The shade measurement was repeated three times for each specimen, at the center of the specimen. Tooth shade measurement by spectrophotometer would be analyzed into number of L*a*b* values and used the average value of the three times measurement for further analyses. Shade measurement would be performed in bleached controlled (after bleaching within 1 day) group and every group to analyze and find the correlation between time and microhardness values each group.

Bleached teeth were evaluated shade at 1 day before bleaching, after bleaching within 1 day, at 2 weeks and 1 month, respectively.

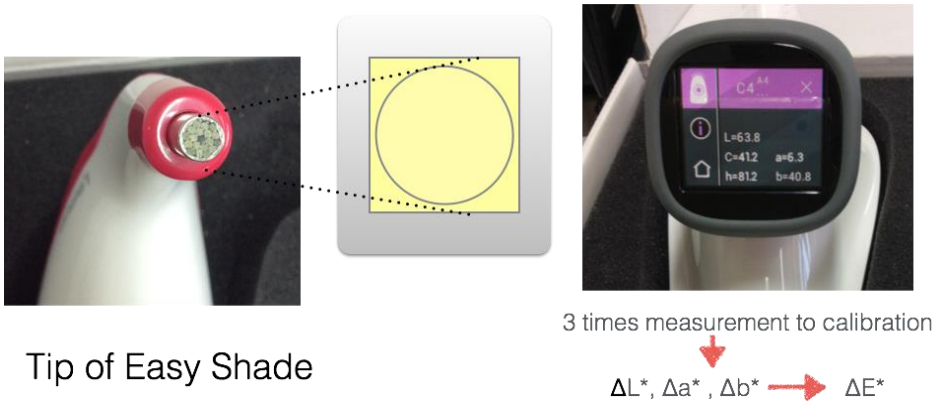


Figure 8. Shade measurement with the use of Easy Shade V Spectrophotometer

7.2 Microhardness evaluation

The Vickers hardness test was used in the study. The force load was fitted at 50g load for 15 seconds, which was used to evaluate microhardness on enamel surface as ISO28399: 2011 recommended. Three loading times were tested for obtaining microhardness on each specimen with distance of 100μm between them. According to the FM-800 micro-hardness machine manufacturer, the distance from each indentation shall be 4 times of diagonal length from center of indentation. The distance of indentation from margin of specimen shall be minimum at 2.5 times of diagonal length. The depth of indentation should be 1/7 time of the length of diagonal. Therefore, the thickness of specimens at least should be 1.5 times of diagonal length.

The area in which was used to microhardness eavaluation was at four angles of slab according to point of time, and outer surface of the shade measurement. At each point of time measurement, the specimen was mark after complete evaluation. Three indentations per time per angle were performed.

1st measurement = VHN was obtained and analyzed before bleaching treatment.

2nd measurement = VHN was obtained and analyzed after 1 day of completed bleaching (2 weeks for home-use bleaching, 1 day for in-office bleaching).

3rd measurement = VHN was obtained and analyzed after 2 weeks of completed bleaching (2 weeks for home-use bleaching, 1 day for in-office bleaching).

4th measurement = VHN was obtained and analyzed after 1month of completed bleaching (2 weeks for home-use bleaching, 1 day for in-office bleaching).

Correlation between surface microhardness and color change were observed.

a. 

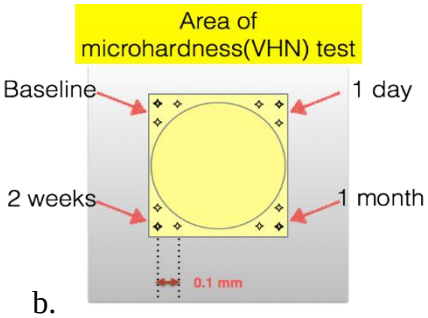
b. 

Figure 9. Microhardness device (Fm-700e Type D, Future-Tech, Japan) and area of microhardness indentation at the arrows point

8. Data collection

Shade Collection

For the color relapse data were collected using calculating the variation of ΔE according to this following equation:

$$\Delta E = [(\Delta L)^2+(\Delta a)^2+(\Delta b)^2]^{1/2}$$

where; ΔL= L final – L initial; Δa= a final – a initial; Δb= b final – b initial.

ΔE1 represents the difference of color between after treatment and within 24 hours.

ΔE2 represents the difference of color between after treatment and at 2 weeks.

ΔE3 represents the difference of color between after treatment and 1 month.

Microhardness Collection

For the data represents the remineralization were collected from Vickers hardness tests. The impression can be read repeatedly by computer measuring. The computer systems are able to read automatically, providing the automatically data collection. The average of Vicker's hardness at each point of time was compared.

9. Data Analysis

All data were input to a computer and the software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) were used in the statistical analysis.

1. Descriptive statistics of ΔE, ΔL, Δa, Δb values and microhardness values at each time point were conducted including mean, median and standard deviation of tooth shade and microhardness.

2. Repeated measure ANOVA analysis was used to analyze mean of shade changes between two types of bleaching groups and control groups at 3 periods of times.

3. Regression analysis was used to determine the correlation each period of time between tooth shade change (ΔE) and microhardness (ΔVHN) of bleaching groups and with / without CPP-ACP application groups.

Ethical consideration

All teeth are collected following ethical approval from the Ethics Committee, the Faculty of Dentistry, Chulalongkorn University, approval number HREC-DCU 2016-063.

Expected Benefits

1. To pave the way for clinicians to gain more information about tooth color change or relapse.

2. To add more scientific knowledge regarding timing and relationship of color relapse and remineralization.

3. To understand the effect of CPP-ACP on bleached tooth.

4. To compare two types of bleaching agents on color changes after mineral deposition.

5. To use the information for esthetic restorative dentist especially in anterior teeth.

Study Limitations

1. Only 10% carbamide peroxide and 40% hydrogen peroxide was used in the experiment.

2. Only microhardness (VHN) was performed for evaluating remineralization.

3. Only one remineralizing agents (CPP-ACP (Tooth Mousse)) was used.

Results

Tooth color change is presented in Table 3 and Figure 4. The majority of color change in the 10%CP and 10%CP/CPP-ACP groups were due to ΔL. Whereas Δb caused the majority of the color change in the 40% HP, 40%HP/CPP-ACP, and two control groups (Fig 4).

ΔE was decreased in the 10%CP, 40%HP and CON groups at T3 compared with T1. In contrast, the groups treated with CPP-ACP (10%CP/CPP-ACP, 40%HP/CPP-ACP and CON/CPP-ACP) tended to demonstrate increased ΔE at T3 compared with T1 (Table 3). However, there were significantly different color changes only in the 10%CP and CON/CPP-ACP groups at T3 ($p = 0.039$ and $p = 0.000$, respectively).

Mean microhardness (VHN) was presented in Table 4. The VHN of 10%CP group was significantly decreased at T3 ($p<0.05$) (Table 4). The others groups were not significantly changed in VHN at final time point T3 ($p<0.05$) (Table 4).

The correlation between ΔVHN and ΔE for each group is presented in Table 5. Only the 10%CP, and the 10%CP/CPP-ACP groups demonstrated a correlation between ΔVHN and ΔE ($p = 0.002$ and $p = 0.003$, respectively) (Table 5).

Table 1. The experimental groups based on treatment agent and application protocol

Group	Treatment agent + Application protocol		Remineralizing agent + Application protocol	
1. 10%CP	10% Carbamide peroxide	8 h x 14 d	None	None
2. 10%CP/CPP-ACP	10% Carbamide peroxide	8 h x 14 d	CPP-ACP	2 x 5 min x 7d
3. 40%HP	40% Hydrogen peroxide	2 x 20 min x 1 d	None	None
4. 40%HP/CPP-ACP	40% Hydrogen peroxide	2 x 20 min x 1 d	CPP-ACP	2 x 5 min x 7d
5. CON (Negative Control)	Immersion in artificial saliva	14 d	None	None
6. CON/CPP-ACP (Positive Control)	Immersion in artificial saliva	14 d	CPP-ACP	2 x 5 min x 7d

Abbreviations: CP = 10% Carbamide peroxide treatment, CP/CPP-ACP = 10% Carbamide peroxide treatment follow by CPP-ACP application, HP = 40% Hydrogen peroxide treatment, HP/CPP-ACP = Hydrogen peroxide treatment follow by CPP-ACP application, CON = Negative control (no treatment), CON/CPP-ACP = Positive control (no treatment with CPP-ACP application), h = hours, d = days, min = minutes)

Table 2. Composition of materials used in this study and manufacturer

Product Name	Materials/Type	Composition	Manufacturer
Opalescence PF	10% carbamide peroxide gel	10% Carbamide peroxide, 0.5% Potassium nitrate, 0.11% Fluoride ion, carbopol, glycerin, flavour.	Ultradent, S Jordan UT, USA
Opalescence Boost PF	40% hydrogen peroxide gel	Gel: 40% Hydrogen peroxide Activator: Potassium hydroxide, 1.1% Sodium fluoride and 3% Potassium nitrate	Ultradent, S Jordan UT, USA
GC Tooth Mousse	CPP-ACP cream	Pure water, Glycerol, CPP-ACP, D-sorbitol, CMC-Na, Propylene glycol, Silicon dioxide, Titanium dioxide, Xylitol, Phosphoric acid, Flavoring, Zinc oxide, Sodium saccharin, Ethyl p-hydroxybenoate, Magnesium oxide, Guar gum, Propyl p-hydroxybenzoate, Butyl p-hydroxybenzoate	GC Europe N.V., Leuven, Belgium
Artificial Saliva	Spray	Xanthan gum, Sodium carboxymethylcellulose, Potassium chloride, Sodium chloride, Magnesium chloride, Calcium chloride, Potassium dihydrogen orthophosphate, Methyl p-hydroxybenzoate	Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

Table 3. Mean and standard deviation in tooth color change (ΔE) in the groups at T1, T2 and T3 (T1=1 day, T2=2 weeks, T3=1month) after bleaching.

Type of Treatment	**Tooth Color Change (**ΔE) at 3 times (p<0.05)		
	Mean ±SD T1	Mean ±SD T2	Mean ±SD T3
10% CP	8.83 ±0.60[a]	8.15 ±0.84[a]	7.53 ±0.76[g]
10% CP/CPP-ACP	10.06 ±0.57[b]	10.34 ±1.09[b]	11.07 ±0.71[b]
40% HP	9.75 ±1.23[c]	9.24 ±0.92[c]	8.31 ±0.91[c]
40% HP/CPP-ACP	7.58 ±0.93[d]	7.05 ±0.89[d]	8.10 ±0.62[d]
CON (Negative Control)	2.98 ±1.04[e]	3.78 ±0.63[e]	2.56 ±0.31[e]
CON/CPP-ACP(Positive Control)	3.60 ±0.64[f]	3.10 ±0.74[f]	7.31 ±0.87[h]

The same letter indicates no significant difference each groups in the same rows at p<0.05.

Table 4. Mean and standard deviation (SD) in microhardness (VHN) between the groups at 4 time points: T0= baseline/ original stage, T1=1 day after bleaching, T2=2 weeks after bleaching, T3=1 month after bleaching.

Type of Treatment	**Microhardness Value (**VHN) at 4 time points			
	Mean(T0) ±SD	Mean(T1) ±SD	Mean(T2) ±SD	Mean (T3) ±SD
10% CP	309.28[a] ±35.39	284.21[a] ±46.61	279.39[a] ±16.48	278.17[g] ±22.35
10%CP/CPP-ACP	287.96[b] ±51.26	296.88[b] ±35.79	300.91[b] ±32.64	297.13[b] ±27.23
40% HP	272.36[ch] ±39.81	317.66[ci] ±33.00	327.90[i] ±4.35	272.78[h] ±20.45
40%HP/CPP-ACP	280.69[d] ±45.47	301.40[d] ±36.04	313.66[d] ±22.80	304.73[d] ±18.61
CON (Negative Control)	265.03[e] ±34.70	313.49[j] ±24.42	280.22[e] ±19.95	269.50[e] ±31.95
CON/CPP-ACP (Positive Control)	295.33[f] ±36.29	304.98[f] ±31.94	307.92[f] ±52.41	298.84[f] ±22.53

The same letter indicates no significant difference each groups in the same rows at p<0.05.

Table 5. Repeated measures analysis of shade color change (ΔE) at three periods.

	Group	Test	TypeIII Sum of Squares	df	Mean Square	F	Sig.
ΔE	10% CP	Greenhouse-Geisser	10.086	1.212	8.323	4.914	.039*
	10%CP/CPP-ACP	Sphericity Assumed	3.475	2	1.737	.541	.590
	40% HP	Sphericity Assumed	12.773	2	6.387	2.734	.087
	40%HP/CPP-ACP	Sphericity Assumed	6.535	2	3.268	.992	.387
	CON	Sphericity Assumed	9.232	2	4.616	.922	.358
	CON/CPP-ACP	Sphericity Assumed	126.993	2	63.497	31.839	.000*
	Group	Between-Subjects Effects	990.157	5	198.031	10.945	.000*
	Error	Between-Subjects Effects	1194.212	66	18.094		
***Statistically significant difference for 3 periods at p-value < 0.05**							

Table 6. Repeated measures analysis of microhardness change (ΔVHN) at three periods.

	Group	Test	TypeIII Sum of Squares	df	Mean Square	F	Sig.
ΔVHN	10% CP	Sphericity Assumed	245.091	2	122.546	.150	.861
	10% CP/CPP-ACP	Sphericity Assumed	122.368	2	61.184	.069	.934
	40% HP	Sphericity Assumed	20629.796	2	10314.898	20.387	.000*
	40% HP/CPP-ACP	Sphericity Assumed	963.910	2	481.955	.629	.542
	CON	Sphericity Assumed	12628.599	2	6314.299	11.122	.000*
	CON/CPP-ACP	Sphericity Assumed	514.377	2	257.189	.204	.817
	Group	Between-Subjects Effects	88235.803	5	31626.363	5.801	.019*
	Error	Between-Subjects Effects	359809.408	66	5451.658		
***Statistically significant difference for 3 periods at p-value < 0.05**							

Table 7. Regression analysis presented a relationship between ΔVHN and ΔE of in each group.

Group	Standardized Coefficients	t	p-value
	Beta		
10% CP	0.493	3.303	.002*
10% CP/CPP-ACP	-0.479	-3.179	.003*
40% HP	-0.279	-1.691	.100
40% HP/CPP-ACP	0.008	0.047	.963
CON (Negative control)	0.108	0.634	.531
CON/CPP-ACP (Positive control)	0.015	0.087	.931

*** Significant relationship between ΔVHN and ΔE in each group at p < 0.05**
Dependent Variable: ΔVHN, Predictor: ΔE

Figure 10. Means of Δ of L, a, b at three time points (T1= 1 day, T2 = 2 weeks, T3 = 1 month) in all groups.

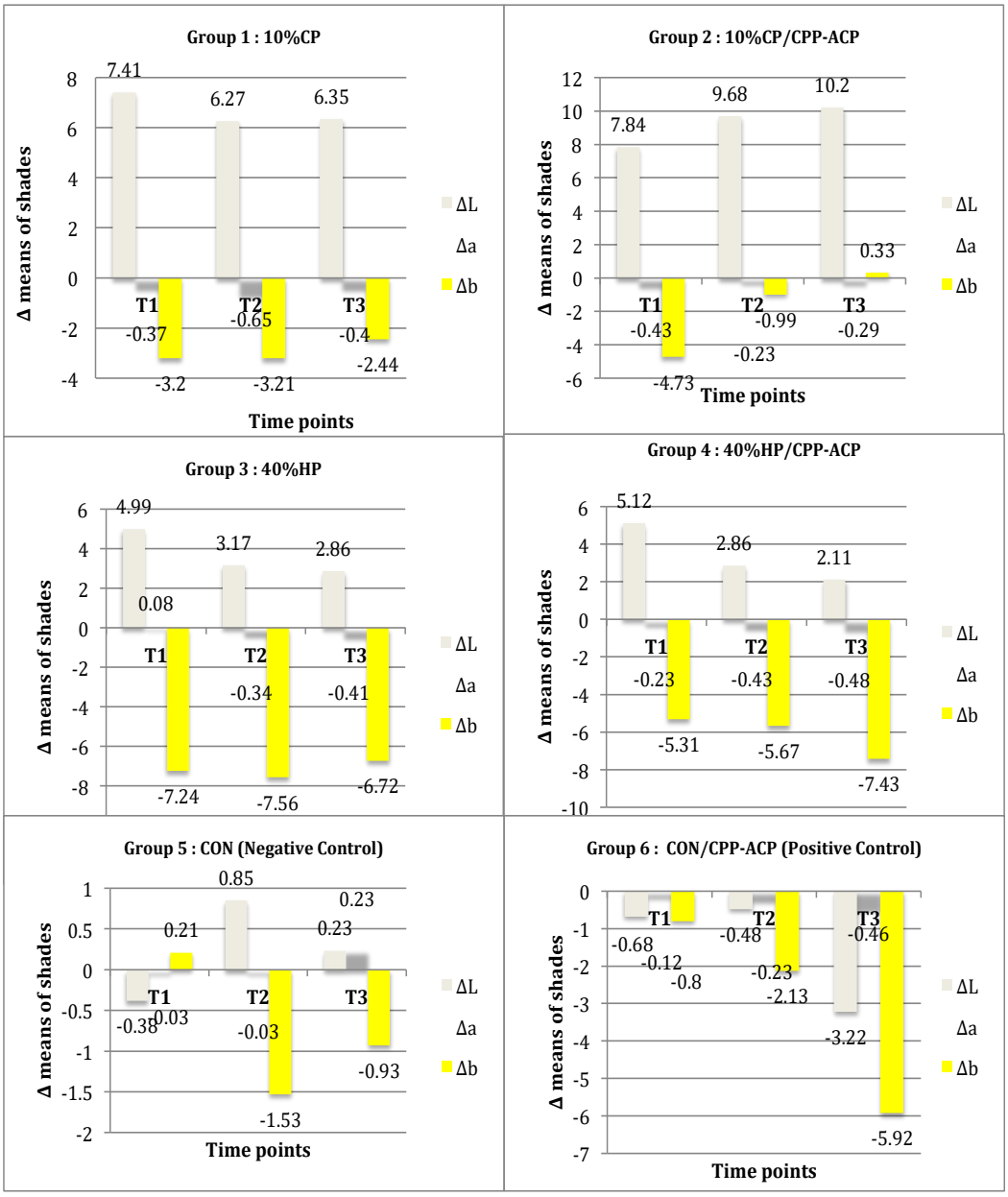


Figure 11. Summary of mean of shade changes (ΔE) in all groups for 3 periods; (T1=1 day, T2= 2 weeks, T3=1month) after completing treatment to baseline (T0).

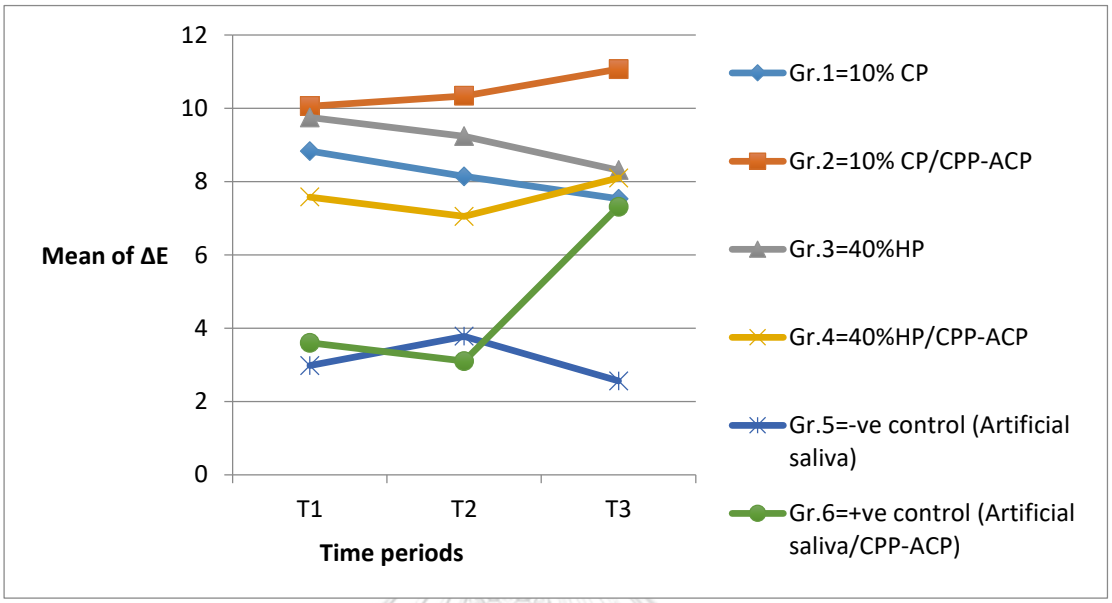
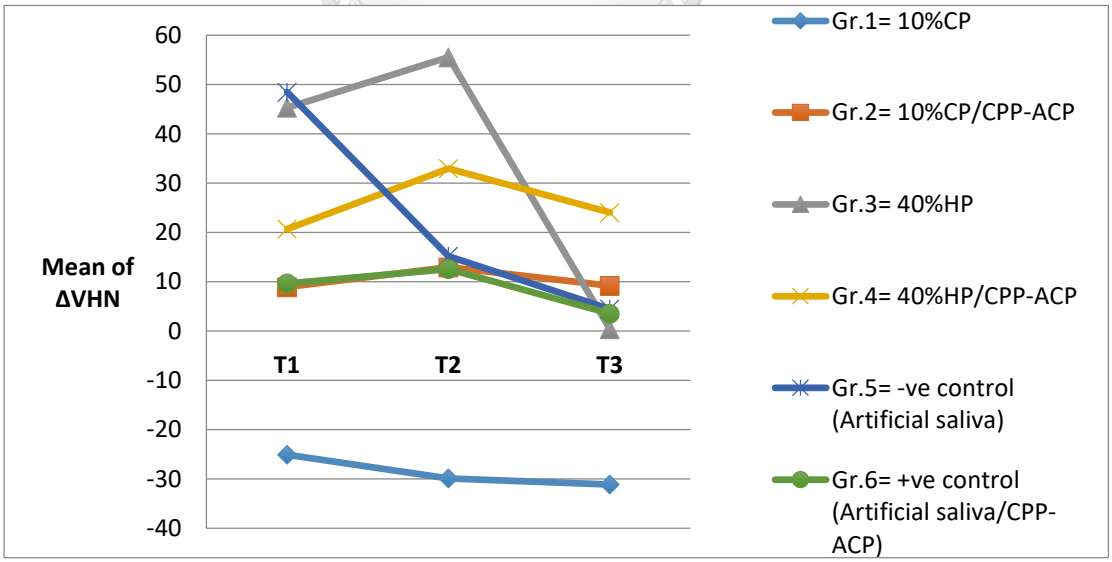


Figure 12. Mean of ΔVHN in all groups for 3 periods (T1=1 day, T2=2 weeks, T3=1month), after completing treatment to baseline (T0).



Discussion

Previous studies found that carbamide peroxide used for home-applied bleaching, and hydrogen peroxide for in-office bleaching caused tooth surface demineralization. (10-12, 58) Therefore, the present study investigated whether tooth remineralization resulted in color relapse of a bleached tooth. The microhardness results in the present study were similar to those of other studies, where the use of hydrogen peroxide (in-office bleaching) did not result in a change in microhardness.(15, 59) Moreover, there were no significant difference in microhardness of the 40%HP and 40%HP/CPP-ACP groups after 1 month (T3). These results agree with another study that where surface treatment with fluoride and CPP-ACP did not improve surface remineralization compared with fluoride treatment, suggesting that fluoride may hinder the effect of CPP-ACP.(60)

In contrast to the in-office bleached teeth results, the microhardness in 10%CP group decreased significantly compared with baseline; however, there was no significant difference in microhardness in the 10%CP/CPP-ACP group. This may be because CPP-ACP increased the surface microhardness of the home-bleached teeth compared with the in-office bleached teeth. The microhardness in the two control groups were also not significantly different after 1 month (T3). The microhardness in the 10%CP and 40%HP groups were different, the microhardness in the 10%CP group decreased over time, while the microhardness in the 40%HP group increased from T0 to T2, but decreased at T3. This may be because the higher percentage of fluoride containing in the 40%HP bleaching gel was more effective in remineralizing the tooth surface.(61, 62) However, a recent study found that the use of 37.5% hydrogen

peroxide for 60 minutes resulted in greater morphology change than that for 30 minutes and 16% carbamide peroxide for 14 and 28 hours.(63)

The pH of the home-applied bleaching gel used in the present study was lower than that of the in-office bleaching gel at 6.5 and 7, respectively. Some studies found that bleaching gels with a pH less than 5.2 caused demineralization.(64, 65) Although the pH of the bleaching gels were not less than the critical pH of approximately 5.3-5.5, which can cause tooth structure demineralization, (43,66) the microhardness in the home-bleached groups without CPP-ACP decreased over time.(10) This may be because the longer exposure times of the home-applied bleaching (8 hour/day for 14 days) compared with that of the in-office bleaching resulted in more demineralized tooth surface.

In the present study, tooth whiteness of the 10%CP group was reduced (decreased ΔE) at 1 month after bleaching, whereas, the 10%CP/CPP-ACP group did not demonstrate color relapse at any time point. These results indicate that, the use of CPP-ACP might have an effect on preventing color relapse in home-bleached teeth. For the in-office bleaching groups, the tooth color of the 40%HP group was not significantly different compared with the tooth color of the 40%HP/CPP-ACP group at 1 month. Therefore, remineralization using CPP-ACP minimally affected the color of in-office bleached teeth. In the control groups, CON/CPP-ACP group were whiter compared with the CON group after 1 month, and this change could be detected visually (ΔE = 3.71). After 1 month (T3), groups treated with CPP-ACP, increased in whiteness (increased ΔE) by increasing ΔL or reducing Δb. Thus, CPP-ACP, in addition to its remineralization effect, may improve tooth whiteness and prevent tooth color relapse for at least 1 month in either normal or home-bleached teeth. This may be due to the

whiteness and opacity of CPP-ACP that is deposited in the porous tooth structures during remineralization.

CPP-ACP was used in this study as a remineralizing agent. CPP-ACP, made from dietary products such as milk, and cheese, has been shown to possess an anticariogenic effect. Several studies showed that tooth remineralization using CPP-ACP strengthened enamel surface.(28-30, 44, 60) The use of CPP-ACP also prevented re-staining of the bleached teeth.(31) Other remineralizing agents, such as topical fluorides, were not used in the current study because they are for professional use only and their toxicity is not conclusive. Artificial saliva is a remineralizing agent that mimics the properties of human saliva. A recent study concerning storage conditions found that natural saliva was only storage media that showed similar behavior to in situ.(66) However, the use of both artificial saliva and natural saliva were efficient in enamel remineralization.(66) This study also present the result of remineralizing with artificial saliva as showed in microhardness result (Table 4).

Regression analysis demonstrated that microhardness change and color change in the present study were correlated only in the home-applied bleaching 10%CP and 10%CP/CPP-ACP groups (10%CP: $p = 0.002$, 10%CP/CPP-ACP: $p = 0.003$). Thus, the first null hypothesis was rejected. For the 10%CP group, beta was 0.493 indicating that tooth color and microhardness correspondingly reduced. For the 10%CP/CPP-ACP group, beta was -0.479, thus, when microhardness increased the tooth color became darker, or when microhardness decreased the tooth color became whiter. These findings contradicted the color results because microhardness was reduced at T3 the opposite of the effect observed for color. The final microhardness in the other groups were also reduced (T2 to T3). However, duration of microhardness reduction was different from

another study where none of the remineralizing products maintained microhardness 14 days post-bleaching.(67) In addition, there was no relationship between color change and microhardness change in the in-office bleaching groups and control groups ($p>0.05$).

The 10%CP and CON/CPP-ACP groups demonstrated a significant color change (ΔE) between time points (T1-T3)($p<0.05$). Therefore the second null hypothesis was rejected. The number of samples treated did not allow them all to be evaluated immediately. Thus to eliminate differences based on time of evaluation, the color and microhardness evaluations in the present study were performed 1 day after bleaching. The spectrophotometer and microhardness tester were calibrated before each measurement. Both evaluations were performed in the same investigator. The color measurement was performed with the Vita Easyshade V spectrophotometer for all groups using the same neutral grey background. The spectrophotometer tip abutted the specimens to eliminate light reflection from the environment into the sensor. Because Δb is the most visually perceptible parameter, (68) a significant improvement in Δb was found in the positive control group, which received CPP-ACP treatment. The use of a high concentration of hydrogen peroxide for only one day may reduce enamel microhardness less than a low concentration of carbamide peroxide at high frequency. Other bleaching gels may generate different results due to the different compositions and percentage of bleaching agents contained in each bleaching gel. However, CPP-ACP application reduced enamel demineralization after frequent bleaching. The clinical implication from this study is that CPP-ACP can be used to prevent tooth color relapse. The use of CPP-ACP in non-bleached tooth for at least 7 days may enhance ΔE or lighter color. In-office bleaching (Opalescence Boost 40%) may be suitable for

reducing yellow scale (b*). Home-applied bleaching (Opalescence 10% PF) may be suitable for reducing black scale (L*). The other remineralizing materials such as tri-calcium phosphate, bioactive glass, etc. should be evaluated in further studies to compare their effects on color change and remineralization. CPP-ACP should be used to longer than 7 days the microhardness result in further studies.

Conclusion

Within the limitations of this study, it can be concluded that tooth color relapse was found within 30 days in the groups treated with 10%CP ($p<0.05$). The final microhardness significantly decreased in the 10%CP group after 1 month. Applying CPP-ACP on home-bleached teeth prevents tooth color relapse and tooth strength reduction for at least 30 days.

Conflict of Interest

The authors do not have any product, service, or financial interest in the companies whose materials are presented in this study.

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APPENDIX

Table 1. Data of ΔL, Δa, Δb and ΔE from the 10%CP group at T1, T2 and T3 (T1=1 day, T2=2 weeks, and T3=1month) after bleaching

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	3.5	-0.6	-2.2	4.18	0.6	-0.7	-3	3.14	2.9	-0.6	0.5	3.00
2.	7.8	-0.7	-2.1	8.11	4.7	-1.1	-4.8	6.81	5.7	-0.9	-2.7	6.37
3.	9	-0.7	-1.9	9.22	5.7	-1.1	-4.7	7.47	5.5	-0.8	-2.9	6.27
4.	8.9	-1.2	-4.1	9.87	8.8	-1.2	-2.7	9.287	7.9	-1	-3.3	8.62
5.	4.6	-0.1	-10	11.01	6.3	0	-8	10.18	6.8	0.4	-6.7	9.55
6.	5.7	0	-5.5	7.92	3.2	-1.6	-6.9	7.77	3.8	0	-6	7.10
7.	10.9	0	2.3	11.14	14.6	-0.2	3.9	15.11	12.8	-0.2	3.9	13.38
8.	8.7	-0.8	-1.6	8.88	8.4	-1	0.1	8.46	7.4	-0.8	-0.7	7.47
9.	6.7	0	-2.8	7.26	5.3	-0.1	-3.4	6.30	6.3	-0.1	-2.9	6.94
10.	7.6	-0.9	-4.8	9.034	5.5	-1	-1.3	5.74	3.6	-1.1	-3	4.81
11.	3.7	0	-6.5	7.48	2.8	-0.2	-7.7	8.20	3.7	-0.1	-6	7.05
12.	11.8	0.6	0.8	11.84	9.3	0.4	0	9.31	9.8	0.4	0.5	9.82
Mean	3.5	-0.37	-3.2	8.83	6.27	-0.65	-3.21	8.15	6.35	-0.4	-2.44	7.53
SD	2.67	0.52	3.28	2.08	3.66	0.61	3.53	2.89	2.88	0.53	3.10	2.63

Table 2. Data of ΔL, Δa, Δb and ΔE from the 10%CP/CPP-ACP group at T1, T2 and T3 (T1=1 day, T2=2 weeks, and T3=1month) after bleaching.

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	3.2	-2.7	-5.9	7.23	7.5	-0.7	-3.5	8.306	9.6	-0.8	0.4	9.64
2.	8.5	0.7	1.9	8.74	9.5	0.5	2.6	9.86	9.2	0.3	5.3	10.62
3.	6.6	0.6	-7.3	9.86	2.3	0.6	-4.4	5.00	9.1	0.7	-0.3	9.13
4.	9.6	0.2	-7.7	12.31	10.5	-0.1	-3.1	10.95	10.9	-0.3	-1.1	10.96
5.	9.3	-1.2	-5.8	11.02	11.7	-0.8	1.2	11.79	10.8	-0.8	1	10.88
6.	12.4	-0.4	-1.8	12.54	13.2	-0.2	0.1	13.20	14.1	-0.4	0.9	14.13
7.	5.8	-0.6	-5.6	8.08	3.2	-0.8	-0.7	3.37	6.3	-0.9	2.7	6.91
8.	10.3	-1.5	-4.9	11.50	16.7	-1	3.1	17.01	15.6	-1.2	1.4	15.71
9.	3.3	0.3	-11.5	11.97	10.3	0.1	-7.9	12.98	8.5	0.5	-3.8	9.32
10.	6.2	1.2	-2.5	6.79	9.8	1.4	0.8	9.93	11.3	1.4	1.6	11.50
11.	10.7	-0.5	0	10.71	13.3	-0.6	0.8	13.34	12.3	-0.5	2.6	12.58
12.	8.2	-1.2	-5.6	10.00	8.2	-1.2	-0.9	8.34	4.7	-1.5	-6.8	8.40
Mean	7.84	-0.43	-4.73	10.06	9.68	-0.23	-0.992	10.34	10.2	-0.29	0.325	11.07
SD	2.88	1.10	3.63	1.97	4.08	0.77	3.19	3.78	3.04	0.86	3.15	2.45

Table 3. Data of ΔL, Δa, Δb and ΔE from the 40%HP group at T1, T2 and T3 (T1=1 day, T2=2 weeks, and T3=1month) after bleaching.

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	4	0.1	-11.2	11.89	2.4	-0.3	-10.3	10.58	2.7	-0.2	-9.6	9.97
2.	10.4	0.4	-8.4	13.37	4.3	-0.3	-8.2	9.26	2.9	0	-6.8	7.39
3.	0.6	-0.4	-10.6	10.62	0.7	-0.6	-11.5	11.54	-0.6	-0.7	-10.8	10.84
4.	11.1	-0.4	-12.2	16.50	11.8	-0.9	-11.8	16.71	7.7	-1	-11.3	13.71
5.	1.6	-0.5	-8.4	8.57	1.6	-0.8	-8.1	8.30	0.2	-0.7	-8	8.03
6.	11.2	0.5	-5.8	12.62	9.1	0.3	-6.8	11.36	8.4	0.1	-6.8	10.81
7.	-3	-0.3	1.3	3.28	-5.9	-0.6	-2.3	6.36	-2.6	-0.8	-0.3	2.74
8.	6.4	0.6	-8	10.26	7	0.3	-6.9	9.83	9.7	0.2	-6.2	11.51
9.	1.6	-0.2	-4.8	5.06	-0.5	-0.6	-6.8	6.84	-0.7	-0.7	-5.7	5.79
10.	1.4	-0.1	-1.7	2.20	-0.2	-0.7	-4.1	4.16	-2.1	-0.7	-4.3	4.84
11.	7.9	0.7	-8.3	11.48	3.2	0.3	-6.6	7.34	4.7	0.1	-4.1	6.24
12.	6.7	0.5	-8.8	11.07	4.5	-0.2	-7.3	8.58	4	-0.5	-6.7	7.82
Mean	4.99	0.08	-7.24	9.75	3.17	-0.34	-7.56	9.24	2.86	-0.41	-6.72	8.31
SD	4.66	0.44	3.93	4.26	4.70	0.44	2.76	3.20	4.18	0.42	3.06	3.16

Table 4. Data of ΔL, Δa, Δb and ΔE from the 40%HP/CPP-ACP group at T0=baseline, T1=1 day, T2=2 weeks, and T3=1 month after bleaching.

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	3.9	-0.5	-4.4	5.90	-3.4	-0.6	-9.8	10.39	-1.7	-0.3	-9.5	9.66
2.	11.4	-1.4	-8.2	14.11	6.9	-1.7	-9.6	11.94	6.5	-0.8	-6.9	9.51
3.	5.8	0	-4.3	7.22	1.6	-1.9	-7.9	8.28	1.5	-1.3	-6.9	7.18
4.	8.7	0.6	-7.6	11.57	5.5	0	-7.7	9.46	2	-0.1	-6.6	6.90
5.	3.4	-0.2	-2.7	4.35	3.7	-0.9	-7.3	8.23	1.1	-1.1	-10	10.12
6.	4.7	0.1	-3.3	5.74	0.4	-0.2	-3.9	3.93	-0.9	-0.4	-5.3	5.39
7.	5.8	-0.2	-3.8	6.94	1.9	0	-4.3	4.70	1.7	-0.1	-6.9	7.11
8.	3	-0.6	-5.9	6.65	3.3	-0.5	-4.5	5.60	0.9	-0.9	-8.6	8.69
9.	4.7	0.3	-7.8	9.11	4.3	0.3	-5	6.60	4.8	0.1	-9.4	10.56
10.	1.3	-0.5	-1.5	2.05	-0.8	-0.4	-0.1	0.9	0.7	-0.8	-3.2	3.372
11.	7	-0.1	-7	9.9	8.5	0.5	-2.8	8.96	6.6	0.4	-7	9.63
12.	1.7	-0.2	-7.2	7.40	2.4	0.2	-5.1	5.64	2.1	-0.4	-8.8	9.056
Mean	5.12	-0.23	-5.31	7.58	2.86	-0.43	-5.67	7.05	2.11	-0.48	-7.43	8.10
SD	2.90	0.50	2.26	3.23	3.30	0.75	2.87	3.09	2.62	0.51	1.96	2.16

Table 5. Data of ΔL, Δa, Δb and ΔE from the CON group at T1, T2 and T3 (T1=1 day, T2=2 weeks, and T3=1month)

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	2.1	-0.1	-0.6	2.19	-1.8	0.2	5.1	6.04	3.8	-0.3	1.4	4.06
2.	-3.5	-0.1	-0.8	3.59	-1.4	0.2	4.9	5.14	-3	-0.4	-0.8	3.13
3.	2	-0.1	0.3	2.02	-2.1	-0.3	3.6	4.21	1.6	-0.3	2.2	2.74
4.	-1.8	-0.1	-1	2.06	0.9	0	1.3	1.59	-1.9	-0.3	-1.9	2.70
5.	-2.8	0	0.4	2.83	-0.3	0.5	2.3	2.61	-2.6	0.1	-0.9	2.75
6.	-2.7	0.3	2.2	3.50	-1	0.1	3.6	3.76	-0.1	-0.3	0.7	0.77
7.	4.9	-0.1	-0.6	4.94	-3.6	-0.2	0.8	3.74	2.6	-0.2	1.8	3.17
8.	1.1	-0.1	0.5	1.21	-0.9	-0.8	-9	9.17	0.7	-0.2	2.3	2.41
9.	2.4	0	-1.1	2.64	-1.2	-0.5	2	2.35	0	-0.6	2.3	2.38
10.	3.6	0.1	0.9	3.71	1.9	0	0.8	2.39	0.6	-0.2	0.4	0.75
11.	2.9	0.3	0.3	2.93	1	0.1	1.1	1.57	-1.6	0	0.8	1.79
12.	-3.6	0	2	4.12	-1.7	0.1	1.9	2.82	-2.9	-0.1	2.9	4.10
Mean	0.38	0.03	-6.40	2.98	-0.85	0.03	1.53	3.78	2.11	-0.23	0.93	2.56
SD	3.05	0.15	1.10	2.18	1.52	0.35	3.64	2.18	2.22	0.18	1.51	1.07

Table 6. Data of ΔL, Δa, Δb and ΔE from the CON/CPP-ACP group at T1, T2 and T3 (T1=1 day, T2=2 weeks, and T3=1month).

	T1				**T2**				**T3**			
	ΔL	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**	**ΔL**	**Δa**	**Δb**	**ΔE**
1.	4.4	-0.7	-5.5	7.08	3.7	-0.6	-6	7.075	3.3	-0.5	-8.2	8.85
2.	-4.3	-0.6	-4.4	6.18	-2.9	-0.7	-3.5	4.60	-8.7	-0.7	-9.4	12.83
3.	-3.7	-0.9	-6.1	7.19	-4.5	-0.8	-7.5	8.78	-7.3	-1	-9.7	12.18
4.	-1.4	-0.5	-2.9	3.26	-0.2	-0.2	-1.2	1.23	-3.3	-0.7	-9.1	9.7
5.	-1.4	-0.2	-2	2.45	-2.1	-0.2	-2.1	2.98	-6.2	-0.1	-4.8	7.84
6.	-0.5	-0.2	-1.9	1.97	-1.4	0	-0.3	1.43	-4.7	0.1	-4.9	6.79
7.	1.4	0.5	3.8	4.08	1.1	0.1	1.4	1.78	-2.7	-0.5	-4.7	5.44
8.	-1.6	0.3	3	3.41	-1.6	0.1	0.7	1.75	-4.3	-0.1	-3.7	5.67
9.	-1.1	0	1.3	1.70	1	0	-0.9	1.35	-2.3	-0.5	-4	4.64
10.	0.4	0	0.4	0.57	0.1	0.1	-0.7	0.71	-3	0	-3.4	4.53
11.	-1.2	0.8	3.7	3.97	0.5	-0.1	-1.5	1.58	0.2	-0.5	-3.9	3.94
12.	0.8	0.1	1	1.28	0.6	-0.5	-3.9	3.98	0.4	-1	-5.2	5.31
Mean	-0.68	-0.12	-0.8	3.60	-0.475	-0.23	-2.125	3.10	-3.22	-0.46	-5.92	7.31
SD	2.30	0.51	3.51	2.22	2.16	0.33	2.65	2.56	3.40	0.37	2.43	3.00

Table 7. Data of VHN from the 10%CP group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	275.71	295.82	297.85	289.79	204.96	208.50	214.61	209.36	257.58	270.34	268.59	265.50	240.44	266.85	274.80	260.69
2	274.80	262.57	272.11	269.83	184.51	222.96	210.31	205.93	281.24	284.07	288.88	284.73	255.13	251.93	265.98	257.68
3	282.18	291.83	295.82	289.94	280.31	299.91	285.03	288.42	291.82	273.89	290.84	285.52	288.88	297.86	295.83	294.19
4	349.66	334.60	327.43	337.23	312.67	315.99	310.48	313.05	297.85	318.22	310.48	308.85	274.80	265.12	250.35	263.42
5	271.22	279.38	275.51	275.37	298.88	275.71	272.11	282.23	300.94	293.82	284.07	292.94	266.85	259.22	283.12	269.73
6	357.58	333.39	336.42	342.46	352.27	347.08	332.18	343.84	290.84	320.49	280.31	297.21	292.82	268.58	295.83	285.74
7	267.71	257.58	260.06	261.78	236.06	287.91	234.63	252.87	259.23	255.13	265.98	260.11	317.10	288.88	309.40	305.13
8	283.12	285.98	290.84	286.65	256.76	261.73	253.53	257.34	297.85	273.01	272.11	280.99	264.27	232.51	239.70	245.49
9	330.98	335.82	330.99	332.60	260.89	269.46	295.82	275.39	244.94	266.85	254.32	255.37	315.98	294.82	284.08	298.29
10	401.51	369.97	361.64	377.71	343.26	349.66	335.82	342.91	290.84	274.8	295.82	287.15	322.78	322.78	314.87	320.14
11	328.63	327.45	322.80	326.29	300.94	292.82	295.82	296.53	265.12	276.62	273.90	271.88	274.80	273.00	269.46	272.42
12	319.36	318.22	327.43	321.67	349.66	349.67	328.61	342.65	255.95	262.57	268.58	262.37	262.57	255.95	276.62	265.05
Mean	311.87	307.72	308.25	309.28	204.96	208.50	214.61	284.21	277.85	280.81	279.49	279.39	281.37	273.13	280.00	278.17
SD	42.90	34.07	30.71	35.39	55.18	46.44	44.32	47.61	19.91	20.48	15.39	16.48	26.43	24.32	22.27	22.35

Table 8. Data of VHN from the 10%CP/CPP-ACP group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	251.93	257.58	250.35	253.29	313.77	317.10	301.98	310.95	248.79	227.66	232.50	236.32	338.27	301.98	327.43	322.56
2	294.82	241.93	291.83	276.19	349.66	343.26	333.39	342.10	322.78	319.35	354.91	332.35	311.57	295.82	313.76	307.05
3	255.13	258.40	258.40	257.31	296.84	290.84	296.84	294.84	321.63	321.63	301.98	315.08	299.91	285.98	300.94	295.61
4	319.35	303.02	301.98	308.12	277.54	264.26	262.57	268.12	314.81	335.81	306.18	318.933	288.88	289.86	265.12	281.29
5	306.19	309.40	308.33	307.97	224.28	241.18	251.14	238.87	308.32	319.35	321.63	316.43	297.86	300.94	286.94	295.25
6	365.77	363.01	338.27	355.68	270.34	274.80	288.88	278.01	277.55	286.94	322.78	295.76	271.22	254.33	263.42	262.99
7	198.15	207.31	200.95	202.14	307.25	305.12	299.90	304.09	229.02	249.57	260.06	246.22	322.78	311.57	315.98	316.78
8	357.58	339.51	368.55	355.21	334.60	344.53	348.37	342.50	311.57	319.35	333.39	321.44	349.66	349.66	364.38	354.57
9	215.86	218.39	210.91	215.05	249.56	250.35	264.27	254.73	285.98	265.98	271.23	274.40	248.79	270.34	269.46	262.86
10	395.19	335.82	320.49	350.50	348.37	350.96	354.92	351.42	325.09	317.10	297.85	313.35	265.98	263.42	272.11	267.17
11	286.94	283.12	268.58	279.55	281.24	273	276.62	276.95	353.59	320.49	344.53	339.54	311.57	317.10	301.97	310.21
12	296.83	292.83	293.81	294.49	307.25	294.81	297.86	299.97	299.91	284.08	319.35	301.11	288.88	292.82	285.98	289.23
Mean	295.31	284.19	284.37	287.96	296.73	295.85	298.06	296.88	299.92	297.28	305.53	300.91	299.61	294.49	297.29	297.13
SD	59.42	48.99	49.32	51.26	38.37	37.22	33.27	35.79	34.73	34.18	35.84	32.64	29.50	25.59	29.99	27.23

Table 9. Data of VHN from the 40%HP group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	216.49	224.28	225.62	222.13	347.08	348.37	339.51	344.99	314.87	314.87	308.32	293.80	291.83	278.46	289.86	286.72
2	232.50	230.41	231.10	231.34	272.11	285.03	265.98	274.37	339.51	320.49	311.57	294.06	264.27	260.06	250.36	258.23
3	297.85	299.91	297.85	298.54	317.10	318.22	298.88	311.40	315.98	310.48	320.49	291.63	260.07	270.34	269.47	266.63
4	293.81	293.82	295.88	294.50	288.88	297.86	285.99	290.91	316.53	312.66	301.97	294.34	248.79	249.57	231.10	243.15
5	339.52	349.67	335.82	341.67	312.66	312.66	337.04	320.79	305.12	312.66	311.57	292.94	311.57	300.94	297.85	303.45
6	328.61	298.88	318.23	315.24	264.27	255.95	265.12	261.78	301.98	312.66	296.84	292.62	291.83	297.85	291.82	293.83
7	204.39	229.72	213.37	215.83	313.76	307.25	308.32	309.78	330.98	328.61	328.61	287.51	267.71	277.54	273.01	272.75
8	232.5	220.34	276.62	243.15	308.32	280.31	300.94	296.52	318.22	314.87	289.85	281.81	269.46	270.34	263.42	267.74
9	283.12	278.46	265.12	275.57	348.37	350.97	347.08	348.81	291.83	308.33	285.98	273.82	321.63	281.24	288.88	297.25
10	256.76	251.14	251.14	253.01	357.58	349.66	343.26	350.17	273	278.46	270.34	268.47	272.11	289.86	293.82	285.26
11	284.07	329.80	296.84	303.57	375.68	378.59	354.91	369.73	335.82	343.26	347.09	264.00	236.78	260.89	249.57	249.08
12	255.94	277.53	287.91	273.79	333.39	349.67	314.87	332.64	253.52	267.71	267.71	285.00	260.06	238.97	248.79	249.27
Mean	268.80	273.66	274.63	272.36	319.93	319.55	313.49	317.66	308.11	310.42	303.36	285.00	274.68	273.01	270.66	272.78
SD	42.98	42.95	38.14	39.81	34.16	36.39	31.21	32.10	25.37	20.03	23.15	10.73	25.01	18.67	22.12	20.45

Table 10. Data of VHN from the 40%HP/CPP-ACP group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	230.40	231.10	224.95	228.82	283.12	327.43	306.18	305.58	312.66	313.76	318.22	314.88	328.61	289.38	326.26	314.75
2	224.29	210.92	231.10	222.10	268.59	286.94	271.22	275.58	318.22	295.83	303.02	305.69	300.94	343.26	326.26	323.49
3	328.61	305.12	318.22	317.32	294.82	294.82	287.91	292.52	282.18	279.38	268.59	276.72	261.73	277.54	274.80	271.36
4	298.88	309.40	319.35	309.21	288.88	290.85	292.81	290.85	298.87	305.13	303.02	302.34	300.94	329.79	284.07	304.93
5	212.13	202.09	217.76	210.66	328.61	364.39	348.37	347.12	334.60	312.66	300.94	316.07	236.78	252.73	299.9	263.14
6	245.70	284.07	288.88	272.88	274.80	280.31	273.90	276.34	350.97	345.80	310.48	335.75	315.98	323.93	327.43	322.45
7	229.02	227.65	233.91	230.19	368.56	354.91	349.67	357.71	322.77	320.09	332.08	324.98	318.32	314.87	290.85	308.01
8	299.82	290.34	296.62	295.59	345.80	326.25	349.67	340.57	339.51	306.18	322.78	322.82	309.40	303.02	308.32	306.91
9	311.57	310.48	308.32	310.12	310.48	306.19	288.88	301.85	348.37	348.37	348.37	348.37	312.67	295.83	314.87	307.79
10	318.22	311.57	319.35	316.38	271.22	282.18	288.88	280.76	330.99	329.8	326.26	329.02	311.57	322.78	292.82	309.06
11	332.19	304.07	309.40	315.22	220.99	229.71	236.06	228.92	317.10	317.10	320.49	318.23	304.07	303.02	317.10	308.06
12	340.76	340.76	337.86	339.79	319.36	313.77	323.93	319.02	274.80	259.23	273.00	269.01	315.98	312.66	321.63	316.76
Mean	280.97	277.30	283.81	280.69	297.94	304.81	301.46	301.40	319.25	311.11	310.60	313.66	301.42	305.73	307.03	304.73
SD	48.54	46.42	43.87	45.57	39.52	36.28	35.55	36.04	24.24	25.33	23.01	22.80	26.12	24.73	18.13	18.61

Table 11. Data of VHN from the CON group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	263.42	260.89	262.57	262.29	282.19	278.46	288.89	283.18	290.84	268.59	296.84	285.42	192.21	193.80	200.38	195.46
2	277.54	294.82	285.98	286.11	310.49	318.22	314.87	314.53	295.82	275.71	298.88	290.14	317.10	286.94	308.32	304.12
3	259.23	251.12	251.93	254.09	325.09	310.48	338.27	324.61	283.12	291.83	296.84	290.60	299.91	260.89	290.85	283.88
4	267.71	265.12	270.34	267.72	296.83	306.18	298.87	300.63	244.94	293.82	267.71	268.82	305.13	260.06	314.87	293.35
5	328.61	339.51	328.61	332.24	344.53	329.79	344.54	339.62	248.01	260.06	245.71	251.26	320.49	248.79	178.20	249.16
6	300.94	309.40	306.18	305.51	314.87	309.40	315.98	313.42	269.45	243.43	241.93	251.60	213.98	283.12	309.40	268.83
7	234.63	230.41	222.96	229.33	278.45	296.83	307.25	294.18	295.82	271.23	277.53	281.53	278.45	224.95	293.81	265.74
8	217.76	222.95	215.86	218.86	327.43	310.48	347.08	328.33	300.94	291.82	270.34	287.70	266.84	301.98	319.36	296.06
9	214.62	214.62	216.49	215.24	348.37	350.97	349.66	349.67	282.18	295.82	251.93	276.64	285.02	314.87	293.81	297.90
10	273.00	271.22	281.25	275.16	330.98	304.07	318.22	317.76	283.12	247.24	265.12	265.16	236.06	209.10	238.96	228.04
11	290.84	283.12	274.80	282.92	347.08	326.26	321.63	331.66	327.43	318.22	328.61	324.75	268.59	281.24	265.12	271.65
12	255.13	251.14	246.47	250.91	268.58	260.06	264.26	264.30	289.86	294.82	282.18	288.95	262.57	287.91	288.88	279.79
Mean	265.29	266.19	263.62	265.03	314.57	308.43	317.46	313.49	284.29	279.38	276.97	280.22	270.53	262.80	275.16	269.50
SD	33.11	36.51	35.17	34.70	27.70	23.52	25.59	25.60	22.50	22.22	25.34	19.95	40.00	37.53	46.06	31.95

Table 12. Data of VHN from the CON/CPP-ACP group at T0, T1, T2 and T3 (T0= baseline, T1=1 day, T2=2 weeks, and T3=1month).

	T0				T1				T2				T3			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	265.98	267.70	295.83	276.50	270.34	284.07	280.31	278.24	255.14	239.70	263.42	252.75	310.48	311.57	308.32	310.12
2	310.48	307.26	307.25	308.33	303.02	305.13	284.07	297.41	272.11	266.84	271.22	270.06	317.10	310.49	297.86	308.48
3	272.11	267.71	240.44	260.09	343.27	344.53	347.08	344.96	265.12	293.81	301.98	286.97	285.90	309.40	306.18	300.49
4	293.82	285.03	293.82	290.89	248.79	275.71	275.71	266.74	299.91	234.63	296.84	277.13	289.86	276.62	278.46	281.65
5	335.82	305.12	315.98	318.97	311.57	300.94	345.80	319.44	293.81	288.88	317.10	299.93	281.24	299.9	326.26	302.47
6	354.91	360.28	333.39	349.53	262.57	248.01	255.95	255.51	281.24	330.99	337.04	316.42	285.03	259.23	292.82	279.03
7	319.35	299.91	328.61	315.96	363.01	322.19	337.04	340.75	358.92	339.51	314.87	337.77	343.27	356.24	347.08	348.86
8	272.11	287.91	258.40	272.81	347.09	333.39	354.91	345.13	317.10	276.62	308.32	300.68	278.46	273.90	274.80	275.72
9	320.49	339.51	300.94	320.31	291.83	266.84	250.35	269.67	300.94	301.97	285.02	295.98	326.26	313.76	307.25	315.76
10	321.63	303.02	320.49	315.05	292.82	304.07	300.97	299.29	492.86	340.75	544.34	459.32	296.84	297.85	333.39	309.36
11	297.86	322.77	293.81	304.81	314.87	318.22	319.35	317.48	294.82	301.97	285.02	293.94	265.13	256.76	273.90	265.26
12	224.95	204.39	202.66	210.67	328.61	328.61	318.23	325.15	303.02	305.12	304.07	304.07	292.82	290.84	283.12	288.93
Mean	299.13	295.88	290.97	295.33	306.48	302.64	305.81	304.98	311.25	293.40	319.10	307.92	297.70	296.38	302.45	298.84
SD	35.74	39.54	38.77	36.29	35.37	29.15	36.48	31.94	63.15	34.92	73.81	52.41	22.40	27.59	23.80	22.53

VITA

Name-Surname Panita Suttisakpakdee

Date of Birth June 21, 1987

Place of Birth Buriram, Thailand

Work Experiences

2011 Banmai Chaiyapoj Hospital,Buriram, Thailand

2013 Chamni Hospital, Buriram, Thailand

Education Background

2005 Doctor of Dental Surgery, DDS, Srinakarinwirot University, Bangkok, Thailand

2014 Esthetic Restorative and Implant Dentistry Program, Chulalongkorn University, Bangkok, Thailand