EFFECT OF CHLORHEXIDINE GLUCONATE ON DENTIN CARIOUS LESION *IN VITRO*

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ผลของคลอร์เฮกซิดีนกลูโคเนตต่อรอยผุชั้นเนื้อฟันการวิจัยเชิงทดลองในห้องปฏิบัติการ

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พัชรนันท์ บรมปิยสวัสดิ์ : ผลของคลอร์เฮกซิดีนกลูโคเนตต่อรอยผุชั้นเนื้อฟันการวิจัยเชิง
ทดลองในห้องปฏิบัติการ. (EFFECT OF CHLORHEXIDINE GLUCONATE ON
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ศุกรวรรณ

วัตถุประสงค์: เพื่อเปรียบเทียบค่าความหนาแน่นแร่ธาตุเฉลี่ยและศึกษารูปแบบการคืนกลับแร่ธาตุของรอยผุ
หลังเตรียมโพรงฟันด้วยสารคลอร์เฮกซิดีนกลูโคเนตและบูรณะด้วยวัสดุกลาสไอโอโนเมอร์ซีเมนต์ในห้องปฏิบัติการ *วิธีการ
ทดลอง*: ฟันกรามน้ำนมที่ถูกถอนจำนวน 40 ซี่ ได้รับการกำจัดเนื้อฟันผุแบบเลือก นำตัวอย่างหาค่าความหนาแน่นแร่ธาตุ
เฉลี่ยก่อนบูรณะด้วยเครื่องเอกซเรย์คอมพิวเตอร์ระดับจุลภาค และแบ่งกลุ่มแบบสุ่มเป็น 4 กลุ่มดังนี้ กลุ่ม A (n=10); ทา
สารปรับสภาพเนื้อฟันและบูรณะด้วยวัสดุกลาสไอโอโนเมอร์ซีเมนต์ (Equia Forte™) กลุ่ม B (n=10); เตรียมโพรงฟันด้วย
สารคลอร์เฮกซิดีนกลูโคเนตร้อยละ 2 เป็นเวลา 1 นาที ก่อนทาสารปรับสภาพเนื้อฟันและบูรณะด้วยวัสดุกลาสไอโอโน
เมอร์ซีเมนต์ (Equia Forte™) กลุ่ม C (n=10); บูรณะด้วยวัสดุกลาสไอโอโนเมอร์ซีเมนต์ (Ketac Universal™) และกลุ่ม
D (n=10); เตรียมโพรงฟันด้วยสารคลอร์เฮกซิดีนกลูโคเนตร้อยละ 2 เป็นเวลา 1 นาที และบูรณะด้วยวัสดุกลาสไอโอโน
เมอร์ซีเมนต์ (Ketac Universal™) ภายหลังบูรณะนำตัวอย่างทั้งหมดหาค่าความหนาแน่นแร่ธาตุเฉลี่ยหลังบูรณะ และ
ผ่านกระบวนการจำลองสภาวะช่องปากด้วยความเป็นกรดด่าง 14 วัน หาค่าความหนาแน่นแร่ธาตุเฉลี่ยหลังจำลองความ
เป็นกรดด่าง สุ่มตัวแทน 1 ตัวอย่างจากแต่ละกลุ่มเพื่อศึกษารูปแบบการคืนกลับแร่ธาตุของฟันผุด้วยกล้องจุลทรรศน์
อิเลคตรอนแบบส่องกราด *ผลการทดลอง*: เปรียบเทียบค่าความหนาแน่นแร่ธาตุเฉลี่ยที่เพิ่มขึ้นหลังบูรณะระหว่าง 4 กลุ่ม
พบความแตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่ม Equia™ และ กลุ่ม CHX-Ketac™ (oneway ANOVA with Post
hoc (Tukey) test, *P* = 0.045) กลุ่ม A: ค่าความหนาแน่นแร่ธาตุเฉลี่ยที่เพิ่มขึ้นหลังบูรณะ (±SD) คือ 88.81 (±59.857),
กลุ่ม B; 168.29 (±100.899), กลุ่ม C; 165.54 (±72.366) และกลุ่ม D; 183.00 (±73.096) mgHA/ccm และเมื่อ
เปรียบเทียบค่าความหนาแน่นแร่ธาตุเฉลี่ยที่เพิ่มขึ้นหลังบูรณะระหว่างกลุ่มที่ใช้และไม่ใช้สารคลอร์เฮกซิดีนกลูโคเนตพบว่า
กลุ่ม Equia™ และ CHX-Equia™ มีความแตกต่างกันอย่างมีนัยสำคัญ (Independent t-test, *P* = 0.046) ในขณะที่ไม่
พบความแตกต่างระหว่างกลุ่ม Ketac™ และ CHX-Ketac™ ซึ่งผลของค่าความหนาแน่นแร่ธาตุเฉลี่ยที่เพิ่มขึ้นสอดคล้อง
กับรูปแบบการคืนกลับแร่ธาตุของฟันผุดังนี้ CHX-Ketac™ พบรูเปิดท่อเนื้อฟันขนาดเล็กที่สุดและมีความหนาของเนื้อฟัน
ระหว่างท่อมากที่สุดเมื่อเปรียบเทียบทั้ง 4 กลุ่ม นอกจากนี้กลุ่ม CHX-Equia™ มีความหนาของเนื้อฟันระหว่างท่อ
มากกว่ากลุ่ม Equia™ *สรุป*: กลุ่มที่เตรียมโพรงฟันด้วยสารคลอร์เฮกซิดีนกลูโคเนตร้อยละ 2 มีค่าความหนาแน่นแร่ธาตุ
เฉลี่ยที่เพิ่มขึ้นมากกว่าและมีความหนาของเนื้อฟันระหว่างท่อมากกว่ากลุ่มที่ไม่ได้ใช้สารเตรียมโพรงฟัน ดังนั้นการใช้สาร
คลอร์เฮกซิดีนกลูโคเนตร้อยละ 2 บนเนื้อฟันที่มีการสูญเสียแร่ธาตุสามารถช่วยส่งเสริมให้เกิดการคืนกลับแร่ธาตุใต้วัสดุ
บูรณะกลาสไอโอโนเมอร์ซีเมนต์ได้

สาขาวิชา	ทันตกรรมสำหรับเด็ก	ลายมือชื่อนิสิต ....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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KEYWORD: Chlorhexidine gluconate, Glass ionomer cement, Mineral density, Atraumatic restorative treatment, Micro-computed tomography

Patcharanun Borompiyasawat : EFFECT OF CHLORHEXIDINE GLUCONATE ON DENTIN CARIOUS LESION *IN VITRO* . Advisor: Assoc. Prof. WALEERAT SUKARAWAN, D.D.S., Ph.D.

Objective: The purpose of this study were to compare mean mineral density(MMD) and examine the remineralized pattern of carious dentin after cavity disinfectant with chlorhexidine gluconate (CHX) and restore with H-GIC *in vitro*. *Materials and Methods:* Selective caries removal to leathery dentin was performed in forty extracted primary molars. The samples were scanned using micro-computed tomography (micro-CT) as the MMD baseline and randomly divided into 4 groups: Group A (n=10) applied dentin conditioner and restored with H-GIC (Equia Forte™), Group B (n=10) disinfected the cavity with 2% CHX for 1 minute before applied dentin conditioner and restored with H-GIC (Equia Forte™), Group C (n=10) restored with H-GIC (Ketac Universal™) and Group D (n=10) disinfected the cavity with 2% CHX for 1 minute before restored with H-GIC (Ketac Universal™). After restoration, all samples were scanned micro-CT as the MMD after restoration. All samples were subjected to pH cycling process for 14 days and scanned micro-CT as the MMD after pH cycling. One sample from each group was randomly selected to analyze by the scanned electron microscope (SEM). *Results:* The comparison of MMD gain after restoration among 4 groups was a significant difference between Equia™ and CHX-Ketac™ group (oneway ANOVA with Post hoc (Tukey) test, P = 0.045). Group A: the MMD gain after restoration (±SD) was 88.81 (±59.857), group B; 168.29 (±100.899), group C; 165.54 (±72.366) and group D; 183.00 (±73.096) mgHA/ccm. Moreover, there was a significant difference of the MMD gain after restoration between Equia™ and CHX-Equia™ group (Independent t-test, P = 0.046). But between Ketac™ and CHX-Ketac™ group, there was no difference. From SEM, CHX-Ketac™ group had the smallest dentinal tubule orifices and the thickest intertubular dentin among 4 groups. CHX-Equia™ group had thicker intertubular dentin than Equia™ group. *Conclusion:* The groups with 2% CHX as a cavity disinfectant had higher MMD gain and thicker intertubular dentin than non-CHX group. Therefore, the application of 2% CHX on demineralization dentin enhances the remineralization of contacted dentin underneath the restoration.

Field of Study:	Pediatric Dentistry	Student's Signature	
Academic Year:	2020	Advisor's Signature	

ACKNOWLEDGEMENTS

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Chapter 1

Introduction

Background and rationale

At present, the management of dental caries is turned to the new preventive and restorative strategy called 'minimal intervention' (1), which promotes preserving tooth structure and emphasizing maximum tooth function. One of the management with evidence-based outcomes is selective caries removal that is used in deep caries without any signs and symptoms of pulpal degenerate (2). The concepts of selective caries removal are removing surrounded axial-wall caries and leaving pulpal wall caries in the cavity. The remaining bacteria in deep caries acts as an irritant that promotes the inflammatory process and subsequently induced odontoblast cells to form reactionary dentin. Nevertheless, the severe irritant leads to odontoblast cells death, which stimulates the dental pulp stem cells or progenitor cells to differentiate to odontoblast-like cells to form reparative dentin (3). Leaving deep caries nearly pulp motivates dentin to repair itself, in addition to avoiding pulpal exposure and maintaining pulpal vitality (4).

Selective caries removal is often used in atraumatic restorative treatment (ART), which is a method to manage deep caries lesions by hand instrument for less trauma (5). ART was developed mainly for treating caries in children living in under-served areas where resources are limited (6). High viscosity glass ionomer cement (H-GIC) is one of the materials that has been used in ART (7). ART using H-GIC helps dental treatment easier, faster, and more comfortable than the conventional restorative treatment (8). Besides reducing pulpal damage, ART leads to reduce pain experience and, gains a better attitude in dental treatment and is more cost-effective than the conventional treatment (6, 9, 10). Therefore, ART is suitable for childhood patients who have severe multiple caries, prevention programs, and controlled disease needs. Even if the selective caries removal method provides a high survival

rate, defective restoration, pulpal inflammation, and secondary caries can still cause the failure of ART (11).

There are several ways to improve the success rate of ART. First, the selection of proper restorative material is an important concern for ART. Several studies found that bond strength between the restorative material and affected dentin was different compared with normal dentin (12-15). Morphological, chemical, and physical characteristics of the affected dentin showed influences on the bonding structure between the affected dentin and material (12). A low evidence-based study showed that ART using H-GIC had more failure from the restoration in both primary and permanent teeth when compared with the conventional treatment (6). The survival rate in 2-year follow-up of single surface ART with GIC was high in both primary and permanent posterior teeth, while the multiple surface restoration showed a medium survival rate (16). Although H-GIC was not recommended for multiple surface restoration in primary molar in the past (17), currently a new generation of H-GIC such as Equia Forte™ (GC Corporation, Tokyo, Japan) and Ketac™ Universal Aplicap™ (3M ESPE Dental Products, St. Paul, USA) are claimed to be used with cavity class II. Hybrid technology in Equia Forte™ helps increase flexural strength (18), which prevents material deformation against chewing force (19).

Second, the hypermineralized zone of the adjacent dentin to the restoration helps decrease the progression of secondary caries (20, 21). The hypermineralized zone occurs from the exchange of charged ions between restorative materials and the tooth's structure. (20). The mean mineral density of dentin after restoring with GIC (Fuji VII, GC) is significantly higher than before restoration whereas the mean lesion depth is decreased (22). Comparing between GIC and amalgam restored cavities, teeth restored with GIC showed less recurrent carious lesions (20, 23). H-GIC contains several minerals that promote the hypermineralized zone underneath contacted dentin (24). Fluoride and strontium ions from H-GIC can penetrate deep into carious demineralized dentin and produce a remineralization process (24).

Lastly, antimicrobial agents help to reduce treatment failure in long term by inhibiting the growth of residual bacteria in deep caries (25). Several studies suggested using antimicrobial agents as cavity disinfectant before the restoration (26,

27). Chlorhexidine gluconate (CHX) is a well-known antimicrobial agent to be used in ART (26). CHX's property is to eliminate microorganism cell negative-charge membrane with the positive charge. CHX has been shown to reduce *E. faecalis* in deep caries lesion, which was difficult to eliminate and known to induce pulpal and periapical inflammation in long term (25, 28).

Besides the antimicrobial effect, several studies found that using CHX with polyacrylic acid helps to create desirable bond ability of GIC (29-32). CHX helps to neutralize the dentin surface that is applied by an acid conditioner (31) and also increases the surface energy of the dentin (31). However, evidence is not clear about the effect of CHX on the remineralization ability of the affected dentin after GIC restoration (33). This study aimed to investigate the effect of CHX as a cavity disinfectant on the dentin carious lesion and restore with H-GIC.

Research question

Does chlorhexidine gluconate affect the carious dentin after restoration with glass ionomer *in vitro*?

Research Hypothesis

In comparison with carious dentin using H-GIC, the chlorhexidine gluconate as cavity disinfectant affects the carious dentin using H-GIC *in vitro*.

H_0: there is no difference in mineral density of carious dentin after restoration with H-GIC with or without disinfecting with chlorhexidine gluconate.

H_1: there is difference in mineral density of carious dentin after restoration with H-GIC with or without disinfecting with chlorhexidine gluconate.

Research objectives

1. To compare mean mineral density of carious dentin after cavity disinfectant with chlorhexidine gluconate and restore with H-GIC *in vitro*.
2. To examine the remineralized pattern of carious dentin after cavity disinfectant with chlorhexidine gluconate and restore with H-GIC *in vitro*.

Limitation

pH cycling was performed as an artificial oral environment. Therefore, the results might not explain what occur *in vivo* or clinical condition.

Research design

This study was an experimental study in the laboratory.

Conceptual framework
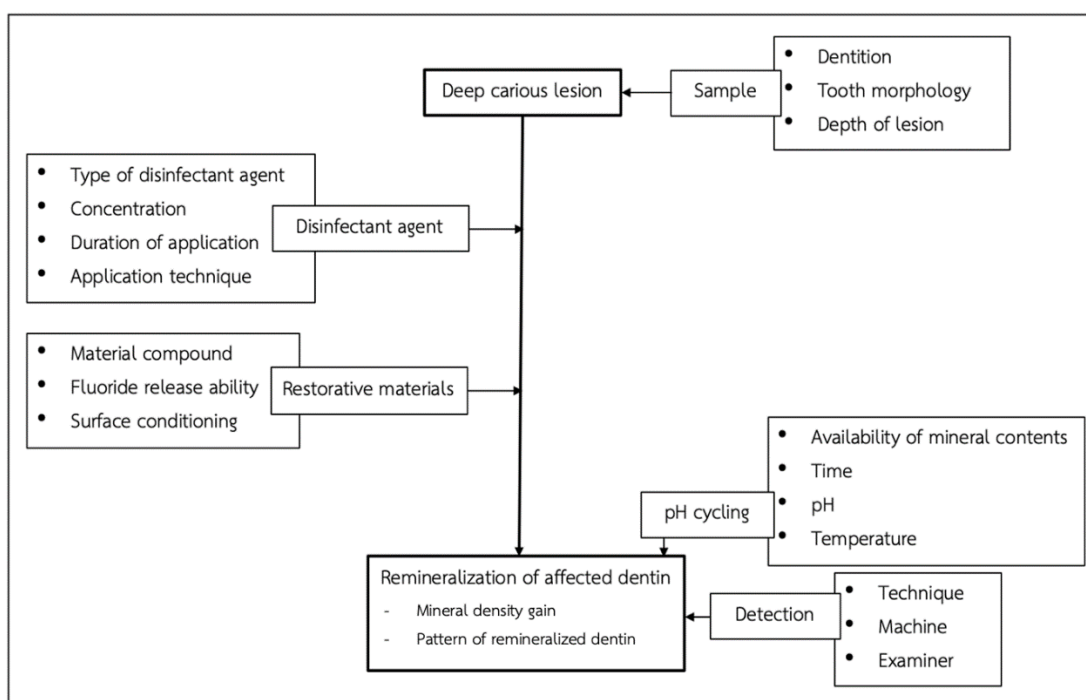
Figure 1. Conceptual framework

Assumptions

1. The sample teeth in this study were primary molar teeth with natural dentin caries. They were extracted following individual treatment plan of patients.
2. The mean mineral density which measured by micro-CT was compared with density of hydroxyapatite.
3. The remineralized pattern of carious dentin was investigated with SEM.

Operational Definitions

1. Atraumatic Restorative technique (ART)

   Atraumatic Restorative Technique (ART) was a less-trauma restorative method which used only hand instrument in removing carious dentin (5).

2. High Viscosity Glass Ionomer Cements (H-GIC)

   H-GIC was recommended for using in ART as a restorative material because of its antibacterial and physical property, fluoride releasing, less moisture sensitivity and biocompatibility (7).

Keywords

Remineralization

Chlorhexidine gluconate

Atraumatic restorative treatment

High viscosity glass ionomer cement

Mean mineral density

Ethical consideration

1. This study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU2020-043).

2. The sample teeth, which were used in this study, were primary extracted teeth following individual treatment plan of patients. All of the sample teeth were approved by the owner.

Biosafety consideration

This study protocol was approved by the Institutional Biosafety Committee of Faculty of Dentistry, Chulalongkorn University (DENT CU-IBC 032/2020).

Expected benefits of the study

The results have been used as a consideration of using chlorhexidine gluconate as a cavity disinfectant in ART. The CHX's properties can eliminate

microorganism. If it can promote the remineralization effect at residual carious dentin contacted to glass ionomer cement, it will reduce secondary caries incident rate. Moreover, the results from this study might be beneficial for others to continue researching the remineralization in the clinical trials.

Chapter 2

Literature Review

Minimal Intervention Dentistry (MID)

The minimal intervention dentistry (MID) is applied to the new theory of placement and replacement of dental restoration. This theory aims to prevent disease and intercept its process (34). Maximum conservation of demineralization, non-cavitated enamel and dentin are the main focus in this MID. Dental caries is classified as an oral disease and treated as infection control (35). There are 5 strategies of MID. First, Early detection of lesions and risk assessment. Second, Remineralization of demineralized enamel and dentin. Third, Optimal preventive or disease control strategies. Fourth, Minimal invasive dentistry. Fifth, Repair rather than replacement of defective restoration (35, 36).

American academy of Pediatric dentistry (AAPD) has recommended the patient examine their individual caries risk assessment based on child's age, social and biological factors, protective factors and clinical finding (37). Caries risk assessment mentions health providers with protocol and criteria for determining frequency of recall and type of dental treatment or management (37).

Atraumatic restorative technique (ART)

Caries removing method in the past focused on removing all the infected dentin but recently, the method has changed to selective caries removal method due to minimal intervention concept (38). The concepts of selective caries removal are removing surrounded axial-wall caries and leaving pulpal wall caries in cavity. Effect of existing deep caries bacteria promotes inflammatory process as an irritant. Following mild irritant to the pulp induces odontoblast cells forming reactionary dentin while the severe irritant leads odontoblast cells death (3). Leaving deep caries nearly pulp motivates dentin repairing itself, in addition to avoiding the pulpal

exposure and maintaining pulpal vitality (4). The selective caries removal is method of choice in atraumatic restorative technique (ART) (5).

ART was developed mainly for treating caries in children living in under-served areas where resources are limited (6). ART uses only hand instruments for less trauma in removing caries. Children are more comfortable during treatment with ART method. ART makes dental treatment easier and faster compared to the conventional treatment (8) and also leads to reduce pain experience, gain better attitude in dental treatment and cost effectiveness (6, 9, 10). The material which is usually used in ART is H-GIC (16). From systematic review and meta-analysis of de Amorim R.G. et al. (16), the 2-year survival rate of single surfaced ART with H-GIC in primary posterior teeth was 94.3% ($\pm$ 1.5) and for multiple surfaces was 65.4% ($\pm$3.9). In addition, the 3-year survival rate of single surfaced ART with H-GIC in primary posterior teeth was 85% ($\pm$ 5.7) and for multiple surfaces was 49.0% ($\pm$12.4). Though ART has high survival rate, the failure of ART was made up by secondary caries, pulpal inflammation and defective restoration (11).

Protocol for producing ART restorations with high-viscosity glass-ionomer cement (39).

1. Selective caries removal to firm at axial wall of cavity and selective caries removal to leathery at pulpal wall. All procedures apply by hand-instrument with a spoon excavator. Local anesthesia and electrical equipment are not provided in this technique.
2. Clean the cavity with wet and dry cotton pellet(s).
3. Apply a dentin conditioner around dentin and enamel in the cavity for 10-15 seconds.
4. Wash with a wet cotton pellet(s) for some 5 seconds.
5. Dry with cotton pellet(s) without contamination with saliva or blood. Shiny cavity will appear.
6. Isolate the teeth with cotton rolls.
7. Mix the GIC follow to the manufacturer's instructions. Encapsulated GIC and Hand-mixed GIC can be used both.

8. Insert GIC into cavity. Do not overfill much otherwise time wastes in removing overfilled material.

9. Coat the restoration with petroleum jelly or cocoa butter for balancing acid-based reaction of GIC.

Glass Ionomer Cements (GICs)

Glass ionomer cement is material of choice using in pediatric dentistry, there are desirable properties such as biocompatibility to tooth or soft tissue, fluoride releasing, antimicrobial activity, coefficient of material expansion which is similar to tooth expansion, and physio-chemical bond with the tooth structure (40). The other advantages of glass ionomer material are white color material and more tolerant to moisture than resin composite (40).

Several studies found that bond strength of the restoration on caries affected dentin was different compared to normal dentin (12-15). The morphological, chemical and physical characteristics of affected dentin had changed that caused effect to low bonded structure (12). Although, the bond strength of resin composite was superior to conventional GIC (41) and RMGIC (42) in sound teeth, RMGIC had higher micro-tensile bond strength than resin composite in caries affected primary molar teeth (43). H-GIC is suitable material for the situation that are obstacles to reach dental units because H-GIC is less moisture sensitive property than resin composite and RMGIC (44, 45). Therefore, H-GIC is also suitable for restoration on affected dentin.

The new GIC, Equia Forte™ (GC Corporation, Tokyo, Japan), claimed that it can use in cavity class II restoration (18). Equia Forte™ has hybrid technology which gains higher flexural strength than conventional H-GIC (18). Flexural strength represents the resistance of a material against deformation from chewing force (19). The more flexural strength, the more force that the restoration be able to withstand (19). Comparing with H-GIC like Fuji IX™ (GC Corporation, Tokyo, Japan), Equia Forte™ has a higher fluoride releasing rate (46). Ketac™ Universal Aplicap™ is claimed to continuously release fluoride for 24 months (47).

There are 2 phases of the fluoride releasing property. Fluoride in the early phase has a burst effect on the surface and continues release in low concentration (48). Owing to a less acidic environment, SrF_2 in H-GIC dissociates to higher fluoride release than CaF_2 whose electropositive charge causes less soluble (49). Fluoride and strontium ions from H-GIC can penetrate deep into carious demineralized dentin, they promoted a remineralization process (24). Mean mineral density of dentin after restoration with GIC (Fuji VII, GC) was significantly higher than before restoration whereas mean lesion depth was decreased (22). The hypermineralized dentin was significantly detected in dentin underneath to GIC (50). Hypermineralized zone is made from the exchange of charged ions between restorative materials and tooth's structure. Accordingly, the hypermineralized zone has a beneficial caries resistance effect (20). The progression of secondary caries is decreased by hypermineralized zone of adjacent dentin of the restoration (20, 21). Comparison between GIC and amalgam restored cavities, teeth restored with GIC showed less recurrent carious lesion (20, 23). The more fluoride uptake ability, the less recurrent carious lesions. Conventional glass ionomer cement, which has released higher fluoride than other fluoride releasing materials, has more depth of fluoride uptake into dentin (51).

Cavity conditioner is usually recommended to use before restoration with GIC especially when H-GIC from GC Corporation, Tokyo, Japan is performed. It is composed of polyacrylic acid and distilled water (52). The conditioner helps cleansing the dentin surface, removes smear layer and creates micro porosities. It improves interaction between acid and tooth structure as a result of increasing bonding ability of restoration (52-54). There were few studies of bond strength and microleakage, they showed higher bond strength and less microleakage compared to unconditioned tooth or other pre-conditioning solutions (52, 54). Even if there was no statistical difference, H-GIC with pre-conditioner was slightly higher survival rate of occlusal ART in 1 year follow-up (55). H-GIC from 3M ESPE Dental Products, St. Paul, USA claimed that their H-GIC was not necessary for pre-conditioning owing to its self-adhesive and self-curing properties (47). Polyacrylic acid as conditioner also showed a demineralized effect after application on dentin (56). The demineralization of adjacent dentin can increase risk of recurrent carious lesions. Due to the material

dehydration of conventional GIC after restoration, an interfacial gap was found (51). The interfacial gap promotes microleakage of restorative material and secondary caries (51).

Table 1: Restorative materials: manufactures, characteristics, general composition, and manufacturers' instructions (57-59).

Material (manufacturer)	Material type	Composition	Manufacturer's instructions
Equia Forte™ (GC Corporation, Tokyo, Japan)	High-viscosity glass ionomer cement (self-curing restorative material)	Powder: 95% strontium fluoroaluminosilicate glass, including the newly added highly reactive small particles, and 5% polyacrylic acid Liquid: 40% aqueous polyacrylic acid	1. Apply cavity conditioner for 10 seconds or Dentin conditioner for 20 seconds 2. Rinse with water and dry, do not desiccate 3. Shake well 4. Activate capsule with capsule applier once before mixing 5. Mix for 10 sec. Working time is 1 min. 15 sec. from start of mix 6. Insert on the applier again then click the capsule 7. Fill the material in cavity 8. Pack and contour please avoid moisture contamination and dry-out 9. Final finishing after 2 min 30 sec. from start of mix

			10. Finish the restoration by applying the EQUIA Forte Coat. Do not air blow 11. Light cure for 20 sec.
Polyacrylic acid dentin conditioner (Dentin conditioner®, GC Corporations, Tokyo, Japan)	A mild polyacrylic acid solution	10% polyacrylic acid 90% distilled water	1. After tooth preparation, apply dentin conditioner to the cavity surfaces for 20 seconds 2. Rinse thoroughly with water. Dry gently, do not desiccate.
Ketac™ Universal Aplicap™ (3M ESPE Dental Products, St. Paul, USA)	High-viscosity glass ionomer cement (Conventional glass ionomer restorative material)	Powder: Oxide glass Liquid: Water, Copolymer of acrylic acid – (Maleic acid, Tartaric acid and Benzoic acid)	1. After tooth preparation, clean tooth with water and dry with air. Moist dry (Dentin conditioner is not necessary) 2. Place the capsule on sturdy surface, after that insert in activator, firmly depress and hold it 2-4 seconds. 3. Mix 4,300 rpm with Capmix or Rotomix. 4. Insert the capsule in applier, then apply GIC in the cavity. 5. Protective coat is unnecessary. If desired,

			Ketac Glaze or Single bond adhesive can be used. 6. Setting time 3.40 minutes.

Figure 2. H-GIC Equia Forte™

(GC Corporation, Tokyo, Japan) (60)

Figure 3. Polyacrylic acid dentin conditioner

(Dentin conditioner®, GC Corporations, Tokyo, Japan) (60)

Figure 4. Ketac™ Universal Aplicap™

(3M ESPE Dental Products, St. Paul, USA)(61)

Chlorhexidine gluconate

Chlorhexidine gluconate (CHX) is a biguanide antimicrobial agent commonly used in dentistry which effects on Gram-positive (62). CHX suppresses the growth of *Streptococcus mutans* (62) and Gram-negative bacteria (63). There are differences concentration available, 0.12 to 0.2% mouth rinse, 2% cavity cleaning solution and 0.5 to 1% gel (63, 64).

In high concentration, CHX will act as bactericidal by precipitation and coagulation of the cytoplasmic content(65). But in low concentration, CHX affects as only bacteriostatic (66). The mechanism of action of CHX is cationic of CHX binding to negatively charge cell walls of microbes (66).

The previous study showed that using agents which have antimicrobial effect as cavity disinfectant reduced treatment failure in the long term due to bacterial caused pulpitis (25). Several studies used antimicrobial agents as cavity disinfectant before restoration (26, 27). Antimicrobial agent helps to inhibit deep caries residual bacteria from selective caries removal (67). CHX reduced *E. faecalis* that was found in deep carious lesions and difficult to eliminate (28). Persisting *E. faecalis* in dental pulp affected pulpal and periapical inflammation in the long term (25). CHX at lower concentration than 0.12% at any time of application could not eliminate *E. faecalis* (68). In Addition, CHX at concentration 0.2, 1, 2% in liquid solution killed the oral

microbes like *S. aureas, E. faecalis, C. albicans, P. endodontalis, P. gingivalis* and *P. intermedia* within 30 seconds (69). CHX at low concentration 0.2% in gel form used 2 hours to eliminate the microbes (69). Previous study demonstrated that ART combined with CHX at concentration 2% as cavity disinfectant reduced *S. mutans* and *Lactobacillus* sp. when applied on the dentin cavity for 60 seconds at 6 months follow up (70).

Several studies reported that 2% aqueous solution of CHX was biocompatible and toxicologically safe to the dental pulp (71, 72). Allergic reactions such as urticaria, Quincke's edema or dyspnea and very rarely severe anaphylactic reactions were reported after contact with CHX (73, 74). CHX eliminated mutans streptococci without trauma to odontoblast-like cell after indirect pulp treatment for 3 months (75)

The study between chlorhexidine gluconate and Glass ionomer cement

Due to the antimicrobial benefit of CHX, the combination between CHX and GIC was considered. It is not clear about the effect of CHX to GIC restoration (33). But several studies found desirable bond ability when used CHX with GIC (29-32). RMGIC plus 40% CHX varnish as cavity disinfectant and RMGIC alone was not different in bacterial coronal leakage (29). Shear bond strength of H-GIC was superior in chlorhexidine treated groups comparing with control group (32). Similar to the study of Equia™ RMGIC (GC Company; Tokyo, Japan), The CHX treated group showed higher shear bond strength than the control group (31). In contrast, the result of additional CHX in GIC was not satisfactory. H-GIC added with 0.5% CHX had compressive strength, tensile strength and shear bond strength not different to control H-GIC (30). The study of CHX which was incorporated with H-GIC during its manipulation found that at 2% CHX had decreased surface hardness and tensile bond strength, while lower concentration at 0.5% or 1% had not significantly decreased (76). CHX increased surface energy of dentin which resulted in higher wettability on the cavity surface. As its higher wettability, increased shear bond strength was found (31).

For remineralization property, CHX had a role to keep collagen cross-linking of tooth structure from collagen degradation by inhibiting the matrix metalloproteinase (MMP) activity through cation- chelating mechanism (77-79). The remineralization of dentin was encouraged from the remaining scaffold collagen fibrils that crystalize minerals (78, 80). Minerals and protein surrounding the collagen fibrils supported elastic behavior and helped resistant to demineralization. Elastic modulus was decreased in demineralized dentin due to deformable organic tooth structure (81-83). The previous study of remineralization showed elastic modulus of the CHX treated group on demineralized dentin block was more than the non-CHX group (78). The study found the more CHX concentration, the more elastic modulus of dentin blocks. The collagen fibril's structure had mechanical recovery themselves. When partial demineralized collagen fibrils are reincorporated with minerals, the remineralization is promoted again (83).

Moreover, there was electrostatic attraction between CHX and H-GIC. The protonated amine group of CHX was caught with the mineral phosphates from H-GIC (78). Then, the condensed minerals were attached to the dentin-restoration contact surface.

Micro computed tomography (Micro-CT)

Micro-CT is a method that is uses in scanning the structure of dental tissue. Radiographic projections are the major component that helps to diagnose due to their superimposition (84). The micro-CT plays useful in dental research because of its nondestructive method (85). Micro-CT demonstrates the microstructure parameters which were enamel thickness and tooth measurement for dental implant surgery or root canal shape for endodontics (85). Micro-CT is also used in analysis of craniofacial skeletal development and structure, biomechanics for finite element model of tooth and bone, tissue engineering, and mineral concentration of teeth (86). Furthermore, the mineral density change will be calculated by micro-CT compared with the hydroxyapatite density(87).

Scanning Electron Microscope (SEM)

SEM is used to scan the surface of the sample via electron beam (88). The scanning has high magnification (up to 150.000x) which can introduce the great resolution image (88). In dentistry, SEM is employed to find abnormalities and demonstrate the morphology of soft and hard tissue structure including dental materials (89). The mechanism of SEM is the scattered electrons on the surface and since dental tissue and dental material are unable to conduct themselves, in consequence sputtering of gold, gold-palladium or carbon is necessary (88).

Chapter 3

Materials and methods

Research design

This study was an experiment in the laboratory. Therefore, pH cycling was performed as an artificial oral environment.

Population and sample

1) Target population

Human primary teeth with dentin carious lesions

2) Sample

The sample teeth which were used in this study were primary molar extracted teeth following individual treatment plan of patients. All the sample teeth were approved by the owner and parents.

Eligibility Criteria

Inclusion criteria

A. Extracted carious primary molar with or without pulpal exposure.

B. If a carious lesion is exposed to the dental pulp, exposure size must less than 1x1 mm^2 after selective caries removal.

C. If a carious lesion did not expose to the dental pulp, a lesion must invade to the dentin from visual examination.

D. The remaining tooth structure must be more than 1/3 of the crown.

E. The roots of the teeth must be at least 1 mm. in length.

Sample size

The G*Power 3.1 program was employed to calculate sample size using the formula for two-way ANOVA. Variance explained by special effect and error variance of the mineral loss of dentin around the restoration after restoration with glass

ionomer cement and other materials from previous study were used for the sample size estimation (90). This study determined Variance explained by special effect = 4254318.76, error variance = 12223205.57, 95% confidence interval, type-I error (α) = 0.05, power of the test (1-β) = 0.80, numerator df = 1, effect size = 0.5899598 and number of groups = 4. The sample size obtained from the calculation was total 25 sample size or 6.25 per group. Additional 10% each group, the sample size which was used in this study was 7 samples per group (total 28 samples).

According to the *in vitro* micro computed tomography assessment of remineralization on dentin, 10 samples per group was employed in the study (90). Owing to the reliability of the study, sample size per group in this study was 10 (total 40 samples).

Intervention

The materials used in the ART technique in this study were:

1. Control group

   H-GIC (Equia Forte™ (GC Corporation, Tokyo, Japan) and Ketac Universal Aplicap™ (3M ESPE Dental Products, St. Paul, USA))

2. Intervention group

   2% Chlorhexidine gluconate (Department of pharmaceutical, faculty of dentistry, Chulalongkorn university, Bangkok, Thailand) and H-GIC (Equia Forte™ GC Corporation, Tokyo, Japan) and Ketac Universal Aplicap™ (3M ESPE Dental Products, St. Paul, USA)

Research materials and instruments

   <u>Instruments</u>

1. Rubber cup
2. Low-speed handpiece and prophy head
3. Slow speed cutting machine (Isomer1000, Buehler Ltd., LakeBluff, Illinois, USA)
4. Controlled refrigerator (Canon Ball manufacturing, Thailand)
5. Vortex mixer (Labnet VX 100, MO BIO laboratories, USA)

6. Abrasive paper

7. Timer

8. Micro computed tomography (μCT35, SCANCO, Switzerland)

9. Scanning Electron Microscope (JSM-6510A, JEOL, Japan)

Materials

1. Tooth storage

2. 0.9% Sodium chloride solution

3. 10% formalin solution

4. 2% Chlorhexidine gluconate (Department of pharmaceutical, faculty of dentistry, Chulalongkorn university)

5. Demineralized solution: 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM acetic acid, pH 4.8 (Preparation from Department of Biochemistry, faculty of dentistry, Chulalongkorn university)

6. Remineralized solution: 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.15 mM KCl, pH 7 (Preparation from Department of Biochemistry, faculty of dentistry, Chulalongkorn university)

7. Deionized water

8. Glass bottle 1 liter

9. Double sided tape

10. Spoon excavator

11. Fissure bur

12. Plastic syringe

13. Plastic bowl

14. Resin acrylic (clear)

15. Permanent marker pen

16. Pink wax

17. Microbrush

18. Polyacrylic acid dentin conditioner (Dentin conditioner[®], GC Corporations, Tokyo, Japan)

19. Artificial saliva (Preparation from Department of Biochemistry, faculty of dentistry, Chulalongkorn university)

20. H-GIC (Equia Forte™ (GC Corporation, Tokyo, Japan) and Ketac Universal Aplicap™ (3M ESPE Dental Products, St. Paul, USA))

Research Method

1. Tooth storage

The extracted primary molar teeth with carious lesions were stored in 0.9% sodium chloride solution and 10% formalin solution at room temperature at least 2 weeks following the study of Nawrocka A. et al. (91). The samples, which were sterilized and stored in sodium chloride solution and 10% formalin solution, were unharmful to the connective tissue and neutral to enamel and dentin microstructure. The 10% formalin solution was a suggested bactericidal solution in numerous studies (91).

2. Sample preparation

a) The teeth were cleaned with pumice, rinsed in the deionized water and dried with tissue paper.

b) For the horizontal guide plane, all the teeth were prepared by cutting the cusp of teeth to a flat occlusal surface with a slow speed cutting machine. Then, teeth were embedded in a resin block and attached with dental pink wax.

c) All the teeth were removed caries with ART method by using only a spoon excavator, rinsed with water and dried with sterile cotton pellets. Removing carious lesion followed by the selective caries removal to leathery.

d) The teeth were measured the mean mineral density of dentin by micro-CT as the mean mineral density baseline.

3. Sampling method

The teeth were allocated to 4 groups of 10 samples each. Teeth were labeled numbers before sampling. Sampling methods were calculated with Permuted block randomization. The sequence of the sampling followed.

1. ABCD	2. ABDC	3. ACBD	4. ACDB	5. ADBC	6. ADCB
7. BACD	8. BADC	9. ACBD	10. BCDA	11. BDAC	12. BDCA
13. CABD	14. CADB	15. CBAD	16. CBDA	17. CDAB	18. CDBA
19. DABC	20. DACB	21. DBAC	22. DBCA	23. DCAB	24. DCBA

Random number table was used for collecting the sequence of sampling. The last two digits were used for sampling but if the numbers were more than 24, they were repeated 1-24 again. For example, 25 were derived to 01, 26 were derived to 02, 64 were derived 16, 96 were derived 24 but 97, 98 ,99, 00 were denied.
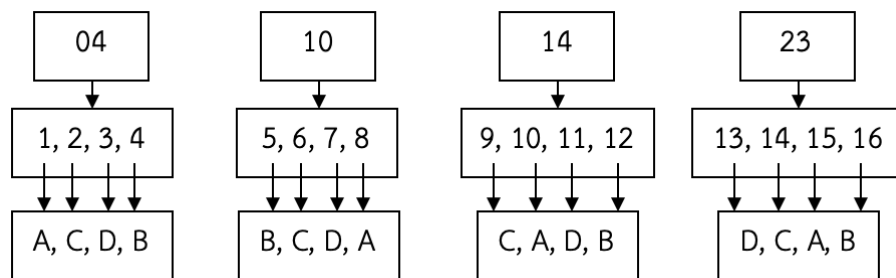
Figure 5. Translation of the random number

1. Group A; H-GIC (Equia Forte™)

   The samples in group A were treated with a dentin conditioner for 20 seconds, rinsed with water, restored with Equia Forte™ and coated with petroleum jelly.

2. Group B; 2% chlorhexidine gluconate and H-GIC (Equia Forte™)

   The samples in group B were prepared in the cavity with 2% chlorhexidine gluconate liquid for 1 minute by microbrush according to previous studies (26,

70, 78). Then, the cavity was treated with a dentin conditioner for 20 seconds, rinsed with water, restored with Equia Forte™ and coated with petroleum jelly.

3. Group C; H-GIC (Ketac Universal Aplicap™)

The samples in group C were restored with H-GIC (Ketac Universal Aplicap™).

4. Group D; 2% chlorhexidine gluconate and H-GIC (Ketac Universal Aplicap™)

The samples in group D were prepared in the cavity with 2% chlorhexidine gluconate liquid for 1 minute by microbrush. Then, the cavity was restored with H-GIC (Ketac Universal Aplicap™).

All samples were stored in artificial saliva in 37 Celsius degrees 24 hours. Then, all samples were scanned micro-CT as the mean mineral density after restoration

4. pH cycling

To establish the DES-RE cycle following Dias G.F. et al. (92), which derived from Ten Cate (93), all samples were immersed in demineralized solution for 8 hours and remineralized solution for 16 hours per day. The samples were separately stored in each tube. The cycle was performed 14 days in room temperature without stirring. After the pH cycling process, all samples were soaked with water before scanned micro-CT as the mean mineral density after pH cycling. The solutions used in the pH cycling were manipulated by the Biochemistry department, Faculty of Dentistry, Chulalongkorn university. Demineralized solution (pH 4.8) was composed of 2.2 mM calcium chloride ($CaCl_2$), 2.2 mM sodium phosphate (Na_2PO_3), 50 mM acetic acid. Remineralized solution (pH 7.0) was composed of 1.5 mM calcium chloride ($CaCl_2$), 0.9 mM sodium phosphate (Na_2PO_3), and 0.15 mM potassium chloride (KCl).

5. Measurement

All samples were scanned by the micro-CT at baseline, after restoration and after pH cycling process. Mean mineral density of each sample was calculated by Micro-CT programs (Micro-CT Ray Version 4.2 and Micro-CT Evaluation Program

Version 6.6). Micro-CT scanned programs were set at resolution of 1024 x 1024 megapixels compared with hydroxyapatite mineral density 1200 mg per cm^3, 70 kVp and 57 μA.

Before scanning micro-CT, the image was shown in program as 2D sagittal plane. The scanned area was chosen with the green lines. The upper green straight line showed the upper scanned limit, the lower green dash line showed the lower scanned limit (figure 6(A.)). Scanned The area of interest included the area from the first slide of the occlusal surface to the first slide of the roof of pulp chamber. Results of scanning were shown as slides in horizontal plane (figure 6(B. and C.)). The multiple slides of the area of interest were drawn anti-clockwise around the outer surface of the tooth for all tooth surface selection.

To analyze the mean mineral density, 3D bone morphology analysis program was selected. The contrast setting for the analysis was determined from the after-restoration samples because the contrast resolution between H-GIC and dentin in the after-restoration samples were differentiated easier than the dentin alone in the baseline samples (figure 6(D. and E.). The contrast resolution was derived from the lowest mean values between 2 examiners identified the difference contrast excluding restoration of 40 samples. Therefore, the micro-CT contrast value setting was performed at -1,000 for lower threshold and +550 for upper threshold on micro-CT 3D program. After setting the contrast, the 3D image was constructed from the interested area slides at baseline, after restoration and after pH cycling (figure 7(A.-C.)).
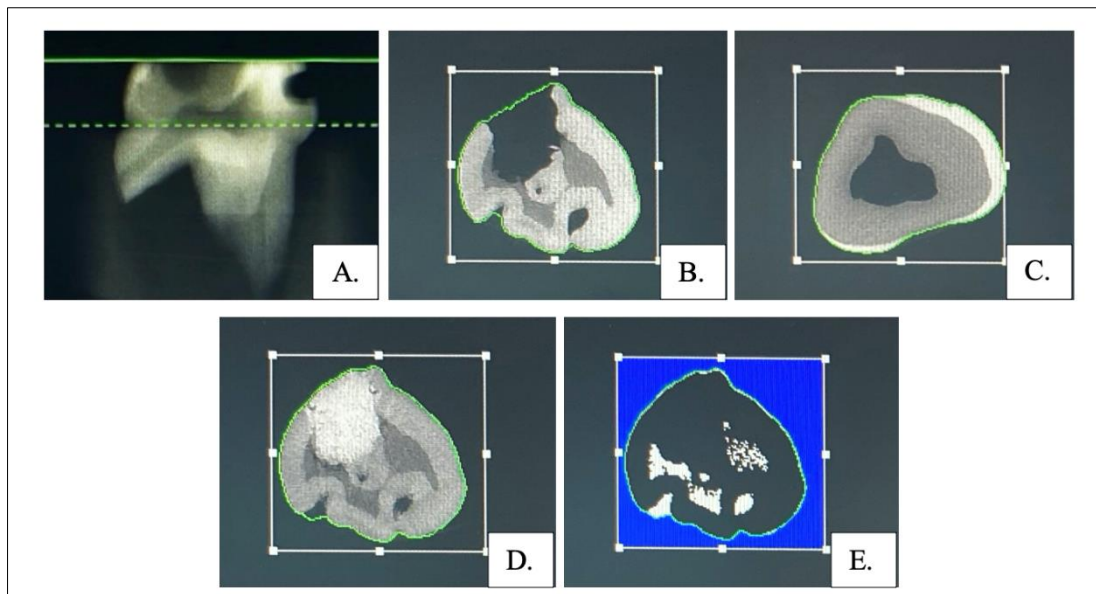
Figure 6. Representative micro-CT scanned images

(A.) The micro-CT scanned area. Upper green straight line showed the first occlusal limit which was included the first occlusal slide, lower green dash line showed the lower limit including first slide of roof of pulp chamber

(B.) The first slide of the occlusal surface from the baseline sample

(C.) The first slide of the roof of pulp chamber from the baseline sample

(D.) The after-restoration slide showed the difference contrast between H-GIC and dentin

(E.) The preview selecting area of slide with restoration in contrast management, black area showed the excluding area such as restoration and enamel which had high resolution like the restoration and white area showed the including dentin area for mineral density calculation
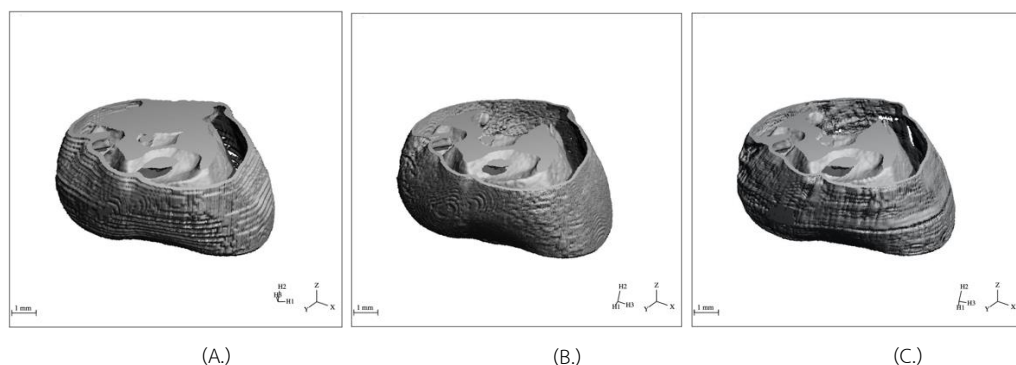
Figure 7. The reconstructed 3D images from micro-CT; (A.) at baseline, (B.) after restoration, (C.) after pH cycling

One sample of each group was selected by sampling method to demonstrate the SEM measurement. Sampling method was random number. The number was 1 to 10 in each group and if one number was selected, the samples of that number in all groups were also selected.

Four selected samples were cut in a horizontal plane with a slow speed cutting machine and prepared to air dry for SEM analysis (figure 8). These air-dried samples were sputtered with a thin layer of gold and attached to the aluminum stubs. Then, the surface morphology of the sample was observed under SEM with the magnification of 60X and 5000X. SEM results represented the morphology of contacted dentin underneath GIC restoration with or without chlorhexidine gluconate treated.
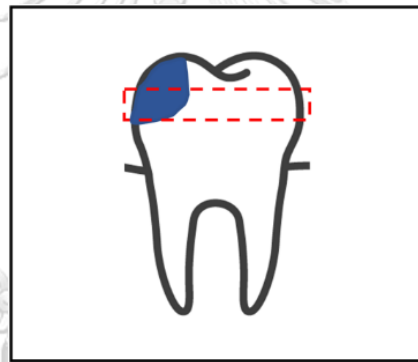
Figure 8. Representative the cutting plane of selected samples for SEM. The red rectangular showed scope area of sample observing under SEM
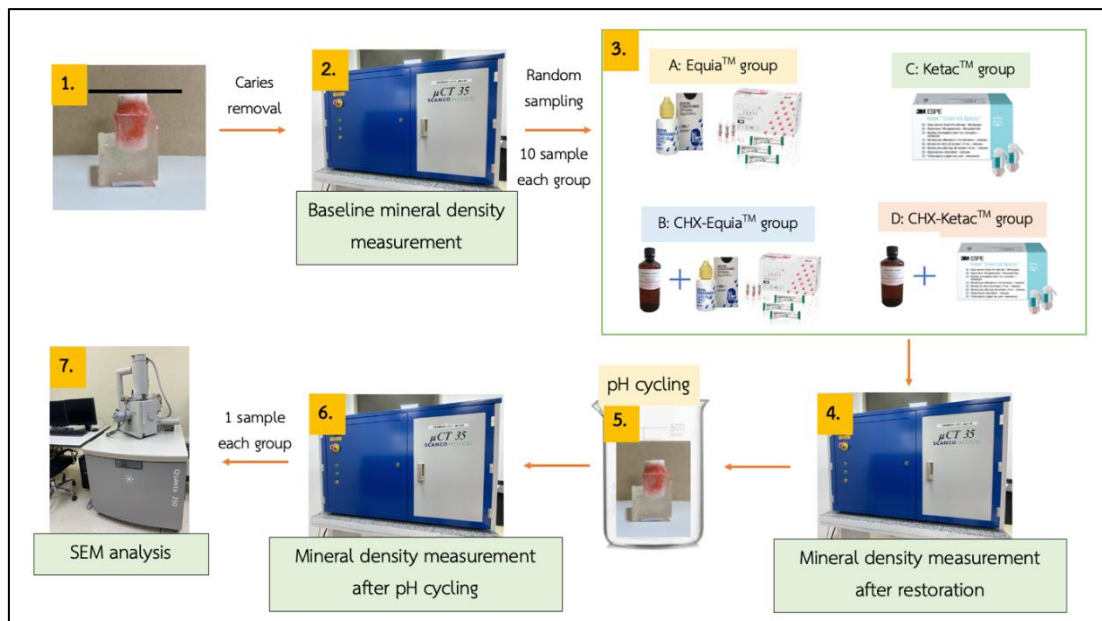
Figure 9. The flow chart of experimental design

(1) The sample with flat occlusal surface in a resin block
(2) After caries removal, all samples were scanned the micro-CT at baseline
(3) After scanning and random sampling, all samples were treated followed their group, A: Equia[TM] B: CHX-Equia[TM] C: Ketac[TM] and D: CHX-Ketac[TM] group
(4) The samples were scanned the micro-CT after restoration
(5) All samples were immersed in Demineralized/Remineralized solution following pH cycling process
(6) The samples were scanned the micro-CT after pH cycling
(7) One sample from each group was analyzed SEM

6. Statistical analysis

Descriptive statistics described the pattern of mineralization on dentin carious lesions under restoration from SEM of each group.

Shapiro-Wilk test and Levene's test were performed to test the normality and homogeneity of variance of the mean mineral density of carious dentin.

Because there were 2 interventions in this study which were non-CHX/CHX and two systems of H-GIC materials. The statistics, which was used to find the effect of two factors, was the two-way ANOVA.

Comparison the mean mineral density

- between baseline/after restoration or baseline/after pH cycling in same group by paired t-test
- between group by one way ANOVA with Post hoc (Tukey) test

Comparison the mean mineral density gain

- between group was analyzed by one way ANOVA with Post hoc (Tukey) test
- between EquiaTM/CHX-EquiaTM group or KetacTM/CHX-KetacTM group were analyzed by independent t-test

For all statistical analyses, the test was performed at the 95% confidence interval and SPSS statistic 22.

Chapter 4

Results

Mean mineral density

Mean mineral density at baseline, after restoration and after pH cycling was shown in Table 2. There were no significant differences in mean mineral density at baseline, after restoration and after pH cycling among groups (oneway ANOVA with Post Hoc (Tukey) test, $P = 0.356$, $P = 0.299$ and $P = 0.419$, respectively) (Table 2, Graph 1). Mean mineral density in all groups (after restoration and after pH cycling) are significantly increased compared with the baseline (Pair t-test, $P < 0.001$) (Graph 1).

Mean mineral density gain was shown in Table 3. The mean mineral density gain after restoration among groups was significantly different (oneway ANOVA, $P = 0.045$) (Table 2, Graph 2). The Post Hoc (Tukey) test showed a significant difference between the Equia™ group and CHX- Ketac™ group ($P = 0.049$).

In contrast, the comparison of mean mineral density gain after pH cycling among 4 groups was not significantly different (oneway ANOVA, $P = 0.065$) (Table 2, Graph 2).

There was no statistically significant difference (two-way ANOVA, $P = 0.217$) in terms of mean mineral gain after restoration between two systems of H-GIC (Equia™ VS Ketac™) either with/without CHX (table 4). Interestingly, there was a significant difference in mineral gain after restoration between the Equia™ group and CHX-Equia™ group (Independent t-test, $P = 0.046$) (Graph 3).

Table 2. The mean mineral density difference between 4 groups

GROUP	A: Equia™ (mgHA/ccm)	B: CHX-Equia™ (mgHA/ccm)	C: Ketac™ (mgHA/ccm)	D: CHX- Ketac™ (mgHA/ccm)	P
Mean mineral density baseline	782.74±71.238	735.31±119.479	766.99±59.361	717.10±94.508	0.356
Mean mineral density after restoration	871.55±54.160	903.60±63.015	932.54±87.099	900.10±71.427	0.299
Mean mineral density after pH cycling	881.94±77.213	904.52±68.776	933.31±73.769	922.26±68.670	0.419

Table 3. The mean mineral density gain difference between 4 groups

GROUP	A: Equia™ (mgHA/ccm)	B: CHX-Equia™ (mgHA/ccm)	C: Ketac™ (mgHA/ccm)	D: CHX- Ketac™ (mgHA/ccm)	P
Mean mineral density gain (after restoration)	88.81±59.857	168.29±100.899	165.54±72.366	183.00±73.096	0.045[*]
Mean mineral density gain (after pH cycling)	99.20± 77.240	169.21±99.199	166.32± 74.182	205.15± 91.678	0.065

[*]Comparison the mean mineral density gain after restoration of 4 groups, significant difference ($P < 0.05$)

Table 4. The effect of H-GIC and CHX on the mean mineral density gain after restoration

Source	Sum of squares	Degree of freedom	Mean square	F	P
H-GIC	20903.454	1	20903.454	3.435	0.072
CHX	23489.905	1	23489.905	3.860	0.057
H-GIC*CHX	9619.882	1	9619.882	1.581	0.217
Error	219092.614	36	6085.906		
Total	1190164.702	40			
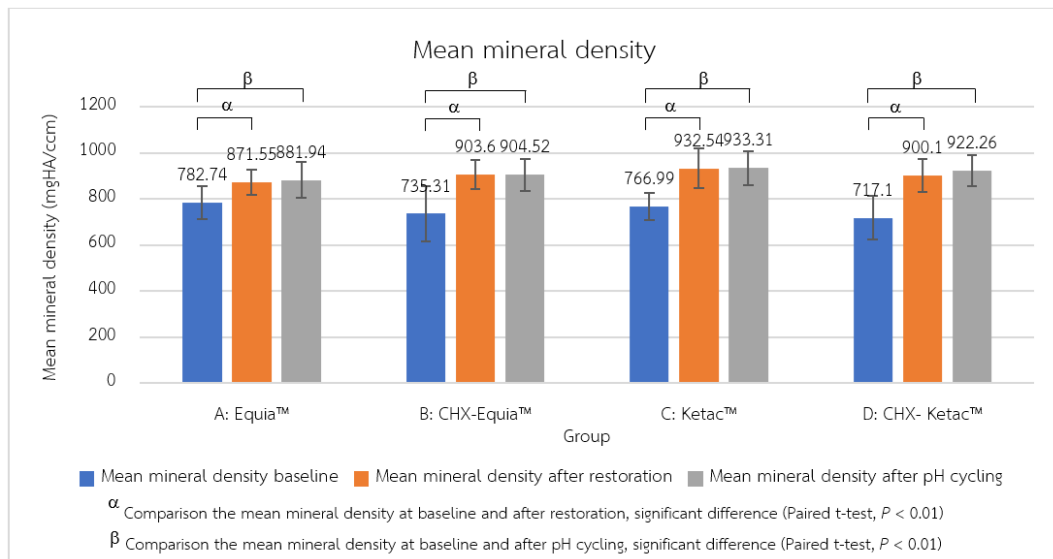
Graph 1. The comparison of mean mineral density of all groups
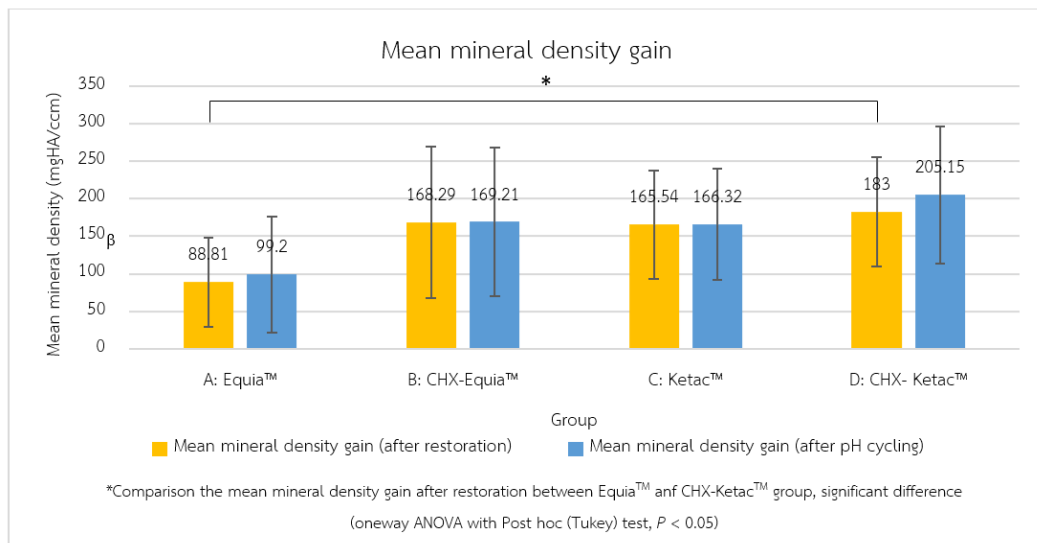
Graph 2. The comparison of mean mineral density gain of all groups
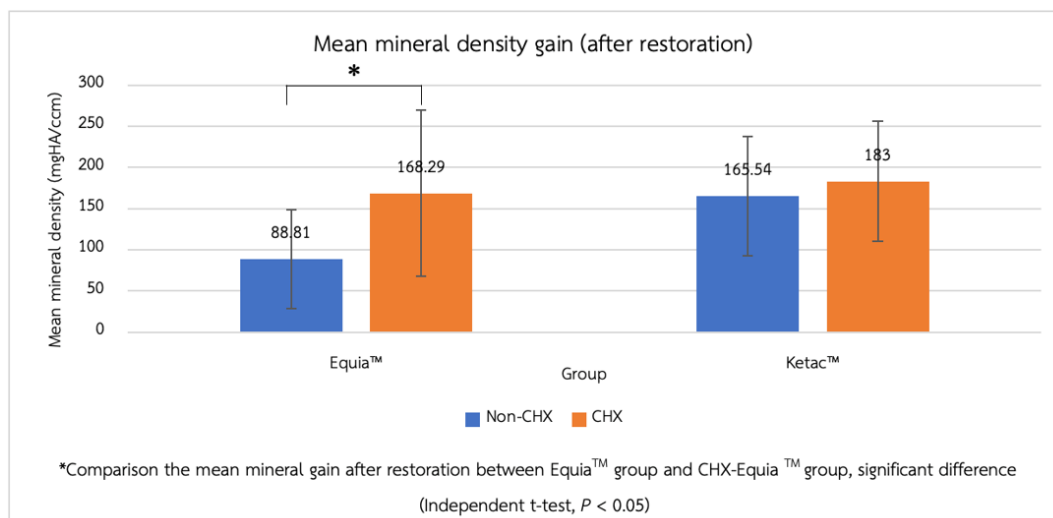
Graph 3. The comparison of mean mineral density gain after restoration between Equia^TM/CHX-Equia^TM group and Ketac^TM/CHX-Ketac^TM group

SEM

Micrographs of the dentin surface in contact with H-GIC restoration were investigated by SEM scanning at 60X and 5000X magnification (figure 10). Adjacent dentin and H-GIC restoration was seen in all groups except CHX-Equia^TM group since the restoration was dislodged during the specimen preparation process (figure 10 A1, B1, C1 and D1.). The area of dentinal tubules in red rectangles were also shown in 5000X magnification.

In the Equia^TM group which dentin conditioner was applied before restoration with H-GIC (Equia Forte^TM), Dentinal tubule orifices were visible (almost superficial minerals from Intertubular dentin removed). Margins of dentinal tubules orifices were rounded. Thin intertubular dentin and small peritubular were detected which indicated the demineralization of superficial dentin (figure 10; A2).

In the CHX-Equia^TM group which 2% CHX was applied for 1 minute followed by the dentin conditioner and restored with H-GIC (Equia Forte^TM), dentinal tubules orifices were sometimes seen. There were different sizes and irregular shapes of dentinal tubule orifices. The peritubular dentin around the orifice was seldomly detected. The Intertubular dentin was thicker than the Equia^TM group. Inside surface of intratubular dentin was rough with some content in the deep part of the dentinal

tubule (ARROW). The SEM image of this group indicated the mild demineralization of dentin on the superficial surface and remineralization inside the dentinal tubule (figure 10; B2).

For the Ketac^TM group, cavity was directly restored with H-GIC (Ketac Universal^TM) without prior treatment. Dentinal tubule orifices were usually visible and rounded. Peritubular dentin was embossed on the dentinal tubule orifice (ARROW). There were several deposits on interdentinal dentin (figure 10; C2).

In the CHX-Ketac^TM group: dentin was applied with 2% CHX for 1 minute and restored with H-GIC (Ketac Universal^TM). Dentinal tubules orifices in group 4 had the smallest diameter and were mostly unseen. The size was less than 5 micrometers. Intertubular dentin was the thickest among 4 groups. Many single and cluster particles were scattered on the dentin surface (ARROW). The SEM image showed hypermineralization on the superficial surface of dentin (figure 10; D2).
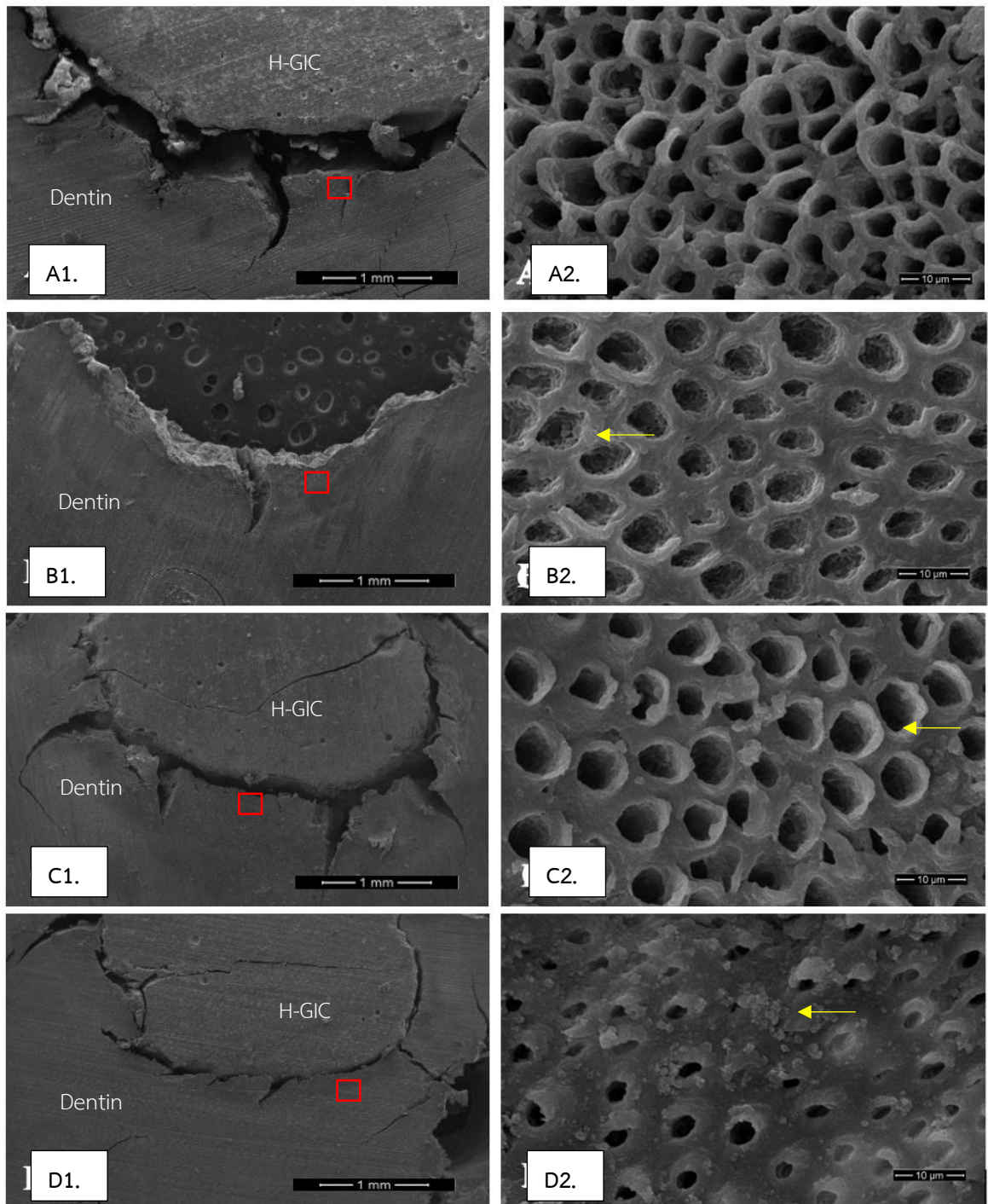
Figure 10. Dentin which contacted H-GIC restoration micrographs of SEM images at 60X magnification (A1.) Equia™ group, (B1.) CHX- Equia™ group, (C1.) Ketac™ group and (D1.) CHX-Ketac™ group and 5000X magnification (A2.) Equia™ group, (B2.) CHX- Equia™ group; the arrow represented inside surface of intratubular dentin, (C2.) Ketac™ group; the arrow represented peritubular dentin and (D2.) CHX-Ketac™ group; the arrow represented particles on the dentin surface

Chapter 5

Discussion

CHX is a well-known antimicrobial agent that is frequently used as a cavity disinfectant to prevent pulpitis caused by bacteria after selective caries removal (77, 94, 95). Several other studies also showed that application of CHX prior to GI restoration helped improve bond strength (29-32). Shear bond strength of H-GIC was superior in CHX treated groups compared with control group which increased success rate in the long term (32). Application of CHX during the bonding procedure also increased the stability of the hybrid layer between composite resin versus dentin and inhibited the reduction in bond strength due to ageing.

On the other hand, the study on the effect of CHX on remineralization of dentin is scarce and did not directly measure the mineral content gain. In a study that indirectly examined the remineralization of dentin by the micromorphological appearance using a field emission scanning electron microscope (FE-SEM) found that the application of 0.2% and 2% CHX for 1 minute increased mineral absorption as seen in micrographs and increased elastic modulus compared to the non-treated control (78). To date, our study is the first report on CHX effect in remineralization quantity of demineralized dentin after restoration with H-GIC by using micro-CT analysis.

Our results show that application of CHX disinfectant prior to restoration improved mean mineral density gain in both CHX-Equia and CHX-Ketac groups compared to the non-CHX control. From the previous study, cavity conditioner had partial demineralized effect to dentin and caused microporosities (56). Therefore, CHX might act as a barrier to the dentin surface by neutralizing the dentin surface which is applied by acid conditioner (31).

The effect of CHX on promoting remineralization might be explained via two mechanisms. First, CHX is known to be the inhibitor for two collagen-degrading enzymes present in dentin, matrix metalloproteinases (MMPs) and cysteine cathepsins (96, 97). The MMPs remain inactivated as long as the dentin matrix

structure is mineralized (98). Acid production from cariogenic bacteria or acid etching stimulates dentin degradation triggered by dentin- matrix metalloproteinases and cathepsins activation (91). The exposed collagen network after acid etching is vulnerable to degradation by endogenous metalloproteinases resulting in the destruction of the bonded interface as well (77, 99, 100). CHX has been shown to keep collagen cross-linking of tooth structure from collagen degradation by inhibiting the matrix metalloproteinase (MMP) activity through cation- chelating mechanism (77-79). Accordingly, remineralization of dentin is encouraged from the remaining scaffold collagen fibrils that crystalize minerals (78, 80). Extrafibrillar mineral, intrafibrillar mineral and protein triple helix are organic structures surrounding the collagen fibrils. They create collagen fibrils to elastic behavior and resist to demineralization. Intra- and extrafibrillar mineral also have mechanical recovery themselves into likely normal dentin structure though dentin is partially demineralized. When collagen fibrils are reincorporated with minerals, the remineralization is promoted again (83).

An inhibitory effect of CHX on MMPs tended to be dose dependent and remained active even in low concentration after 6 months of application (79). A metaregression study also found that the effect of CHX might depend on the adhesive system used (101). In resin restoration, the sequential application of phosphoric acid, CHX, and an etch-and-rinse adhesive may be more effective than the self-etching adhesives since CHX acts better on exposed collagen fibrils (101). This observation might also be implied to our results that CHX seems to have better effect on Equia^{TM} which has dentin conditioner step that contain mild polyacrylic acid that can expose collagen fibrils similar to etch-and-rinse adhesive system. Meanwhile, Ketac required no dentin conditioner step therefore collagen fibrils may remain concealed and camouflage the MMP inhibitor effect of CHX, therefore no statistical difference is observed when compared mean mineral density gain between Ketac and CHX-Ketac groups.

Another possible mechanism which CHX might promote remineralization is via electrostatic attraction (78). Interaction between CHX and its target results from a cationic-anionic reaction. The cationic part of CHX molecule can bind to the negative

charge area of the target substrate. Bound CHX on dentin collagen might strongly attract the mineral phosphate via the electrostatic interaction between the protonated amine groups of CHX and the mineral phosphates which promote mineral growth and deposition in demineralized dentin (102, 103).

Since the KetacTM and CHX-KetacTM group received no dentin conditioner step, demineralized effect on dentin from mild acid conditioner did not occur in these groups. The collagen fibrils were undegraded and maintained for remineralization as observed in the micrographs of KetacTM and CHX-KetacTM group. The Ketac[TM] group, dentinal tubule orifices were usually visible and rounded. Peritubular dentin was embossed on the dentinal tubule orifice. Among 4 groups, CHX-Ketac[TM] group exhibited the thickest Intertubular dentin with smallest diameter of the dentinal tubule indicating the mineral deposition around the collagen fibrils (78). These results from SEM were corresponding to micro-CT analysis that the mean mineral gain after restoration is highest in the CHX-Ketac[TM] group. In Equia[TM] group, thin intertubular dentin and small peritubular were detected which indicated the demineralization of superficial dentin. CHX-Equia[TM] group showed different sizes and irregular shape of dentinal tubule orifices which peritubular dentin around the orifice was seldomly detected. Interestingly, the intertubular dentin was thicker than of Equia[TM] group. Inside surface of intratubular dentin was rough with some content in the deep part of the dentinal tubule. The SEM image of this group indicated the mild demineralization of dentin on the superficial surface and remineralization inside the dentinal tubule. From the SEM results, applying CHX as a cavity disinfectant significantly maintained the dentin around the tubules. There were thick Intertubular dentin and particles scattering on the dentin surface among the CHX treated group. Our study was in accordance with previous studies that dense granular deposition of nanoparticles was shown after application of CHX (78, 104). When small particles deposited around the collagen fibrils, the Intertubular dentin appeared to become thicker. As well as collagen structure was not destroyed, the remineralizing agent could effectively deposit to collagen (78).

In this study, we chose to analyze the remineralization in actual dentin carious lesions in order to mimic the clinical scenario as possible. However, naturally

occurred cavities were different in size, shape and baseline mineral content which may influence the remineralization quantity at the H-GIC-dentin interface. For instance, a large multi-surface cavity which contains plenty of H-GIC contact area might exhibit more mineral gain compare to small cavity. Furthermore, the chemical pH cycling model to imitate the oral environment (93)seems to have little effect in our study. This might due to the appearance of the cavities which most of them are class I deep cavity. The bottom of these cavities are unreachable by pH cycling solution, therefore significant statistical different was not observed in any group after pH cycling.

Forty samples (10 samples each) were scanned with the micro-CT scanning machine at baseline, after restoration and after the pH cycling process. The micro-CT contrast value setting would be performed at -1,000 for lower threshold and +550 for upper threshold on micro-CT 3D program for 3D construction. These values were derived from the lowest mean values between 2 examiners identified the difference contrast excluding restoration of 40 samples. The contrast setting for the analysis in each sample was determined from the difference in density between H-GIC and dentin. The contrast of H-GIC and dentin after restoration was easier to identify than dentin alone in the baseline samples. Therefore, the H-GIC restored samples were analyzed before the baseline samples.

Accordingly, our study corresponded with the previous study which investigated the remineralization of dentin through elastic modulus (78). Their study showed the elastic modulus of the CHX treated group on the demineralized dentin block was more than the non-CHX group. Besides, the higher concentration of CHX, the more elastic modulus was found. Therefore, the application of CHX on demineralized dentin is effective in promoting the remineralization of deep residual caries.

Considering that using CHX could be beneficial to ART method due to the antimicrobial property and promoting remineralization, but the effect of CHX on GI bond ability is unclear. Recent studies showed that there was no significant difference in bond ability of H-GIC after application of CHX (31-33). Given that new H-GIC is currently developing, it will be beneficial to see how CHX affects the bond

ability and stability. Moreover, the clinical study on the survival rate of ART treated teeth when using CHX with H-GIC restoration is of particular interest in order to improve the clinical success of ART in the future.

Conclusion

According to our findings, the group that used 2% CHX as cavity disinfectant with H-GIC restoration had higher mean mineral density gain than the group with H-GIC restoration alone. When dentin was demineralized, CHX helped remineralization by maintaining the collagen fibrils and mineral phosphates attraction. Consequently, 2% CHX enhances the remineralization of the adjacent dentin of the H-GIC restoration.

Appendix

Artificial saliva

Preparation from Department of Biochemistry, faculty of dentistry, Chulalongkorn university

Ingredients

1. Potassium chloride   0.75 grams
2. Magnesium chloride   0.07 grams
3. Calcium chloride   0.199 grams
4. di-Potassium hydrogen phosphate   0.965 grams
5. Potassium dihydrogen phosphate   0.439 grams
6. Sodium carboxymethyl cellulose   6 grams
7. Sorbital 70%   36 grams
8. Sodium benzoate   2.4 grams

Volume summary 1000 ml.

Sterilization with autoclave at temperature 121 Celsius degrees, pressure 15 pounds for 15 minutes

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