THE EFFECT OF NANO-SILVER FLUORIDE IN REMINERALIZATION ON ARTIFICIAL DENTINE CARIES: AN *IN VITRO* STUDY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลของนาโนซิลเวอร์ฟลูออไรด์ต่อการคืนกลับแร่ธาตุบนรอยผุจำลองในชั้นเนื้อฟัน: การศึกษาในห้องปฏิบัติการ

น.ส.พีรญาณ์ พันธุ์เพ็ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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พีรญาณ์ พันธุ์เพ็ง : ผลของนาโนซิลเวอร์ฟลูออไรด์ต่อการคืนกลับแร่ธาตุบนรอยผุจำลองในชั้นเนื้อฟัน: การศึกษาในห้องปฏิบัติการ. (THE EFFECT OF NANO-SILVER FLUORIDE IN REMINERALIZATION ON ARTIFICIAL DENTINE CARIES: AN *IN VITRO* STUDY) อ.ที่ปรึกษาหลัก : ผศ. ทพญ. ดร.ณัฐนันท์ โกวิทวัฒนา, อ.ที่ปรึกษาร่วม : รศ. ทพญ. ดร.พนิดา ธัญญศรีสังข์,ผศ. ดร.พร้อมพงศ์ เพียรพินิจธรรม

การศึกษาในห้องปฏิบัติการนี้มีวัตถุประสงค์เพื่อเปรียบเทียบประสิทธิภาพการคืนกลับแร่ธาตุของสารประกอบนาโนซิลเวอร์ฟลูออไรด์ปริมาณ 400 ppm (NSF400) ซิลเวอร์ไดเอมีนฟลูออไรด์เข้มข้นร้อยละ 38 (38% SDF) และฟลูออไรด์วานิชเข้มข้นร้อยละ 5 (5% NaF) บนรอยผุจำลองในชั้นเนื้อฟันของฟันกรามหรือฟันกรามน้อยแท้ของมนุษย์ โดยนำฟันมาตัดให้เป็นชิ้นของเนื้อฟันจำนวน 120 ชิ้น (ขนาด 3 x 3 x 2 ลูกบาศก์มิลลิเมตร) จากนั้นนำมาสร้างรอยผุจำลองบนชั้นเนื้อฟัน วัดค่าความลึกของรอยผุ (LD) ก่อนการทาสารด้วยเครื่องเอ็กซ์เรย์คอมพิวเตอร์ระดับไมโครเมตร (Micro-CT) (n=15 ชิ้น/กลุ่ม) และวัดค่าความแข็งผิวระดับจุลภาค (SMH) โดยใช้หัวกดชนิดนูป (n=15 ชิ้น/กลุ่ม) สุ่มชิ้นฟันตัวอย่างเข้ากลุ่มทดลอง 4 กลุ่มได้แก่ กลุ่มที่ 1 NSF400 กลุ่มที่ 2 38% SDF กลุ่มที่ 3 5% NaF และกลุ่มที่ 4 น้ำปราศจากไอออน (กลุ่มควบคุม) นำสารทดลองทาลงบนชิ้นฟันตัวอย่าง จากนั้นนำชิ้นฟันมาแช่ในอาหารเลี้ยงเชื้อที่ประกอบด้วยเชื้อ*สเตร็ปโตคอคคัส มิวแทนส์* และเชื้อ*แคนดิดา อัลบิแคนส์* เป็นเวลา 24 ชั่วโมงเพื่อให้เกิดการสร้างไบโอฟิล์ม แล้วจึงนำไปผ่านกระบวนการจำลองสภาวะความเป็นกรดด่างในช่องปากเป็นระยะเวลา 7 วัน ทำการวัดค่าความลึกของรอยผุ และค่าความแข็งผิวระดับจุลภาคภายหลังการทาสารทดลอง นำผลที่ได้มาหาค่าเฉลี่ย และเปรียบเทียบผลก่อนและหลังการทาสารด้วยสถิติ Paired t-test จากนั้นเปรียบเทียบค่าร้อยละการเปลี่ยนแปลงความลึกรอยผุเฉลี่ย (%LD change) และ ค่าเฉลี่ยร้อยละการคืนกลับความแข็งผิวระดับจุลภาค (%SHR) ระหว่างกลุ่มด้วยสถิติ One-way ANOVA ร่วมกับ Tukey's post hoc test โดยกำหนดระดับนัยสำคัญทางสถิติที่ 0.05 ผลการศึกษาพบว่าภายหลังการทาสาร กลุ่มที่ 1 และ 2 มีค่าเฉลี่ย LD ลดลง และกลุ่มที่ 1,2 และ 3 มีค่าเฉลี่ย SMH เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับค่าเฉลี่ยก่อนการทาสาร และเมื่อคำนวณ %LD change ในกลุ่มที่ 1, 2, 3 และ 4 พบว่ามีค่าเท่ากับ -7.80 ± 3.70, -12.69 ± 4.66, 1.66 ± 4.80 และ 8.34 ± 3.62 ตามลำดับ โดยกลุ่มที่ 1 มีค่า %LD change ลดลงมากกว่ากลุ่มที่ 3 และ 4 ($p<0.001$) แต่น้อยกว่ากลุ่มที่ 2 อย่างมีนัยสำคัญทางสถิติ ($p=0.013$) และเมื่อคำนวณ %SHR ในกลุ่มที่ 1, 2, 3 และ 4 พบว่ามีค่าเท่ากับ 7.86 ± 3.15, 10.49 ± 1.82, 1.60 ± 1.52 และ -1.44 ± 0.79 ตามลำดับ โดยกลุ่มที่ 1 มีค่า %SHR มากกว่ากลุ่มที่ 3 และ 4 แต่น้อยกว่ากลุ่มที่ 2 อย่างมีนัยสำคัญทางสถิติ ($p<0.001$) จากผลการศึกษาสรุปได้ว่า สารประกอบนาโนซิลเวอร์ฟลูออไรด์ปริมาณ 400 ppm มีประสิทธิภาพในการคืนกลับแร่ธาตุในรอยผุจำลองในชั้นเนื้อฟันได้ดีกว่าฟลูออไรด์วานิชความเข้มข้นร้อยละ 5 แต่น้อยกว่าซิลเวอร์ไดเอมีนฟลูออไรด์เข้มข้นร้อยละ 38

สาขาวิชา ทันตกรรมสำหรับเด็ก ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Remineralization, Lesion depth, Surface hardness, Silver diamine fluoride, Nano silver fluoride/Silver nanoparticles, Fluoride varnish, Dentine caries, Dual-species biofilm

Peeraya Punpeng : THE EFFECT OF NANO-SILVER FLUORIDE IN REMINERALIZATION ON ARTIFICIAL DENTINE CARIES: AN *IN VITRO* STUDY . Advisor: Asst. Prof. NATTANAN GOVITVATTANA, D.D.S., M.Sc., Ph.D. Co-advisor: Assoc. Prof. PANIDA THANYASRISUNG, D.D.S., Ph.D.,Asst. Prof. Prompong Pienpinijtham, B.Sc., Ph.D.

The aim of this *in vitro* study was to compare the remineralization efficiency among Nano-silver fluoride 400 ppm (NSF400), 38% Silver diamine fluoride (38% SDF) and 5% Sodium fluoride (5% NaF) on artificial dentine caries in permanent molar or premolar teeth. The teeth were sectioned to obtain 120 specimens (3 x 3 x 2 mm^3). After artificial caries were created, the lesion depth (LD) was measured using Micro-computed tomography (Micro-CT) (n=15 specimens/group), and the surface microhardness (SMH) was measured using a Knoop microhardness tester (n=15 specimens/group). Subsequently, all specimens were randomly assigned to four groups: Group 1) NSF400, Group 2) 38% SDF, Group 3) 5% NaF, and Group 4) Deionized water (control group). Experimental treatments were applied. All specimens were immersed in culture media containing *Streptococcus mutans* and *Candida albicans* for 24 hours to create a biofilm and underwent a biofilm pH-cycling for 7 days. The measurements of lesion depth and surface microhardness were conducted after the treatment. The results before and after treatment were compared using the paired t-test. Then, the mean percentage change in lesion depth (%LD change) and the mean percentage of surface microhardness recovery (%SHR) among the groups were compared using One-way ANOVA with Tukey's post hoc test ($p<0.05$). The results showed that after treatment, Groups 1 and 2 had a significant decrease in mean LD, while Groups 1, 2, and 3 exhibited a significant increase in mean SMH compared to their pre-treatment values. The %LD change in Groups 1, 2, 3, and 4 were -7.80 ± 3.70, -12.69 ± 4.66, 1.66 ± 4.80, and 8.34 ± 3.62, respectively. Group 1 exhibited a significant decrease in %LD change compared to groups 3 and 4 ($p<0.001$), but it was significantly lower than group 2 ($p=0.013$). The %SHR of groups 1, 2, 3, and 4 were 7.86 ± 3.15, 10.49 ± 1.82, 1.60 ± 1.52, and -1.44 ± 0.79 respectively. Group 1 exhibited a significantly higher %SHR compared to groups 3 and 4 but it was lower than group 2 ($p<0.001$). In conclusion, the Nano-silver fluoride 5provided remineralization effects on artificial dentine caries better than 5% Sodium fluoride varnish but not as effectively as 38% Silver diamine fluoride.

Field of Study: Pediatric Dentistry Student's Signature .....

Academic Year: 2022 Advisor's Signature .....

Co-advisor's Signature .....

Co-advisor's Signature .....

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Chapter 1

Introduction

Background and significance of the study

Dental caries affecting the primary teeth in preschool children, also known as early childhood caries (ECC), is still a major dental health problem in the developing countries including Thailand.(1) The 8th national oral health survey 2017 of Thailand reported that the prevalence of dental caries in 3- and 5- year-old children is 52.9% and 76.6%, whereas mean decay missing filling tooth (dmft) is 2.8 and 4.5, respectively.(2)

It was reported that untreated dental caries in children can affect the children's quality of life such as eating and sleeping problems.(3) It was found that children with ECC had a poorer oral health-related quality of life than caries-free children. In children with severe dental caries, growth and development can also be affected.(4) The difficulty in accessing dental care services is still a problem in children in the rural area or in low- income families, which can cause diagnosis and treatment delays.(3)

The concept of dental caries management has been changed from removing all the infected tissues and replace with the restorative material to the least invasive method (minimally invasive dentistry) and deferring the time to restore for as long as possible in order to preserve the non-cavitated infected enamel and dentine.(5)

Silver compounds have been used in dentistry since the 1840s.(6) Silver compounds have been shown to be effective agents for preventing and arresting caries in the primary and permanent dentition in vitro, in vivo, and clinical studies.(7) Recently, Silver diamine fluoride (SDF) was approved as a medical device for treating tooth sensitivity and has been used to arrest of dental caries in the primary dentition by the U.S. Food and Drug Administration (FDA) in 2016.(7, 8) Treatment of caries lesions with SDF has been found to be effective with a success rate of 70% in caries arrest in pediatric research.(9) In some studies, SDF has been ranked higher than fluoride varnish, and it is commonly recognized as a low-cost alternative to other topical fluorides.(10)

However, adverse effects of SDF have been reported, including the reversible slightly painful lesions which may disappear within 48 hours, the metallic taste and the black staining of carious tissue due to the oxidation process of the ionic silver in the formulation.(11) Some study found that more than 70 percent of parents with at least one child who had received SDF treatment were dissatisfied with the aesthetics of their upper anterior teeth compared to posterior teeth.(12, 13)

In 2014, Targino AG et al., has introduced the new Nano Silver Fluoride (NSF).(14) This was a new experimental material containing silver nanoparticles and fluoride that combined antimicrobial and preventive properties. The NSF formulation was found to be efficient in inhibiting the growth of *Streptococcus mutans (S. mutans)*, while not staining teeth black.(14) Recent studies found that the effectiveness of NSF in arresting and remineralization in dental caries is controversial. Some study found that NSF has the arresting effect and also remineralization of carious dentine lesion similar to 30% and 38% SDF (15), while some study showed that the effectiveness of NSF in remineralization of artificial enamel caries was less than NaF and SDF significantly. (16) Although the nano-silver fluoride has been shown to have an effect on both enamel and dentine, few studies have been undertaken to investigate its efficacy in carious dentine lesion.

Therefore, the aim of this study was to compare the effectiveness of the Nano-silver fluoride solution, 38% silver diamine fluoride, and 5% sodium fluoride varnish in remineralization of artificial dentine caries.

Research question

Is there a significant difference between the Nano-silver fluoride solution (400 ppm silver and 22,600 ppm fluoride), 38% silver diamine fluoride, and 5% sodium fluoride varnish in remineralization of artificial dentine caries?

Research Objectives

To compare the effectiveness of the Nano-silver fluoride solution (400 ppm silver and 22,600 ppm fluoride), 38% silver diamine fluoride, and 5% sodium fluoride varnish in remineralization of artificial dentine caries.

Research hypothesis

There is no significant difference between the Nano-silver fluoride the Nano-silver fluoride solution (400 ppm silver and 22,600 ppm fluoride), 38% silver diamine fluoride, and 5% sodium fluoride varnish in remineralization of artificial dentine caries.

Study design

Laboratory experimental study

Scope of study

This study is a laboratory experimental study to compare the effectiveness of remineralization of artificial caries in the dentine layer of extracted sound permanent premolars or third molars. After applying the Nano-silver fluoride solution (400 ppm silver and 22,600 ppm fluoride), 38% Silver diamine fluoride, and 5%Sodium fluoride varnish on artificial carious dentine lesions, all the specimens will be subjected to a dual-species microbial pH-cycling model to mimic oral conditions. Remineralization of artificial dentine caries will be evaluated.

Conceptual framework

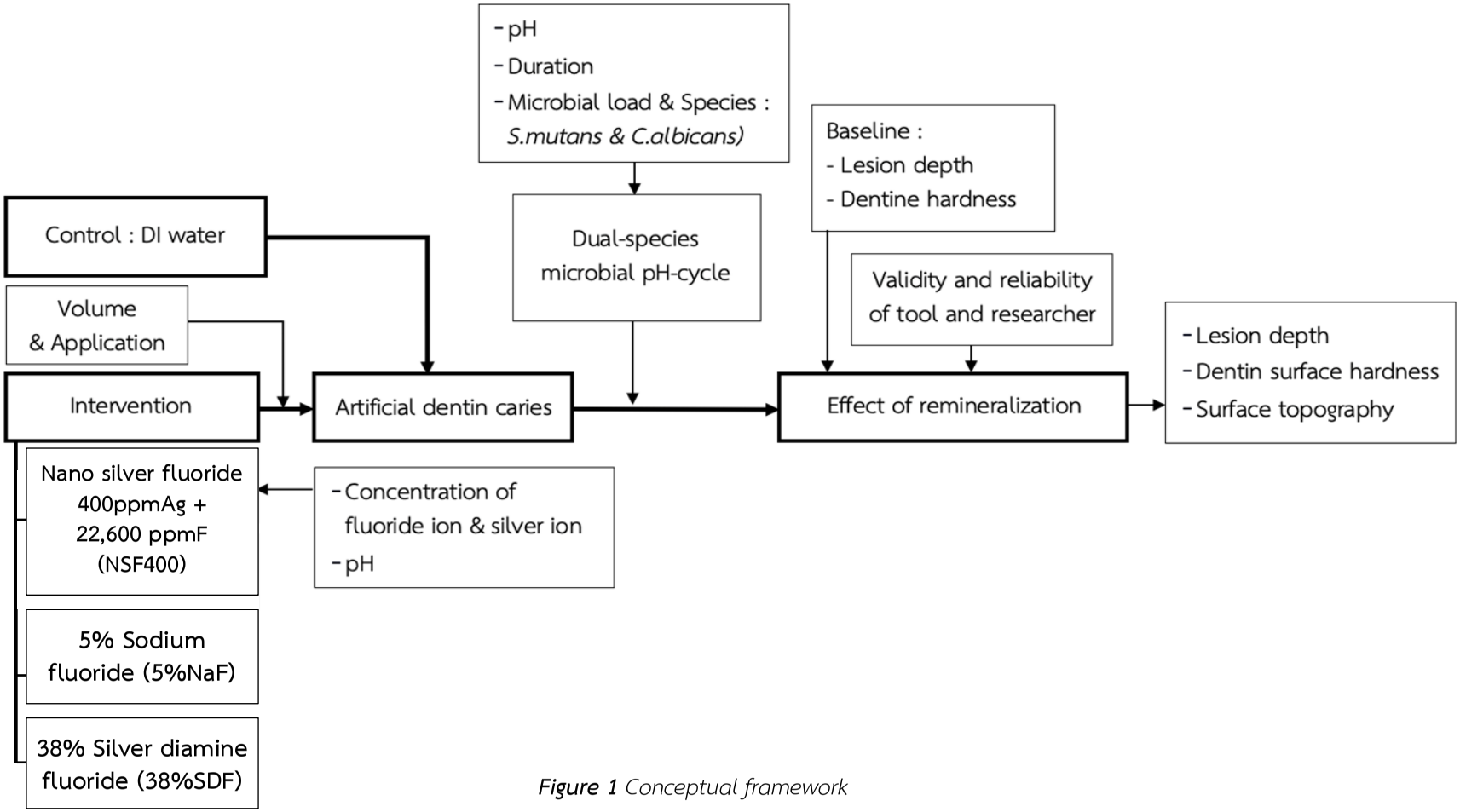

Figure 1 *Conceptual framework*

Limitation of Research

This study is a laboratory study. The dentine carious lesions used in this study was developed in the laboratory settings and underwent a dual-species microbial pH-cycling model simulating oral conditions. Thus, the results of this study may not be applied to the actual carious lesion in the oral cavity.

Keywords

Remineralization

Lesion depth

Surface hardness

Silver diamine fluoride

Nano silver fluoride/Silver nanoparticles

Fluoride varnish

Dentine caries

Dual-species biofilm

Definition of Terms

Dentine specimen is the specimen size 3 x 3 x 2 mm^3 (Width x Length x Depth) taken from the sound dentine of an extracted permanent premolar or molar due to dental purposes.

Remineralization is a measurement of mineral changes before and after intervention through oral pH change simulation which was measured by micro-computed tomography (Micro-CT) and surface hardness testing.

A dual-species microbial pH-cycling model is a model using *Streptococcus mutans* and *Candida albicans* to form cariogenic biofilm (demineralization) and artificial saliva are used for remineralization.

Nano silver fluoride is a new experimental material containing silver nanoparticles and fluoride that combined antimicrobial and preventive properties.

Expected Benefits

1. To be an alternative material for arresting caries and promoting the remineralization in dentine carious lesion.
2. To serve as a knowledge foundation for future clinical material development.

Ethical consideration

Permanent premolar or molar teeth used in this study required permission and approval from the patient or the owner of the dental clinic, as well as a review of human research ethic from the Human Ethics Committee of Faculty of Dentistry in Chulalongkorn University (HREC-DCU 2022-041).

Biosafety consideration

Streptococcus mutans and *Candida albicans* used in this study, and unknown pathogens from tooth specimen are risk group 2 infectious pathogens. The researcher applied for approval and was certified by the Institutional Biosafety Committee of Faculty of Dentistry, Chulalongkorn University (DENT CU-IBC 014/2022).

Chapter 2

Literature review

Dental caries

Dental caries is still the most common chronic disease in children and a major cause of tooth loss in adults, especially in developed countries.(17) According to the most recent survey of Global Burden of Disease, untreated caries in permanent teeth was the most common human disease condition worldwide, with untreated caries in primary teeth ranking tenth.(18) Dental caries is now recognized as a complex disease, biofilm-mediated, diet modulated, multifactorial, non-communicable, dynamic disease resulting in net mineral loss of dental hard tissues(19)

Since the role of microbes in the establishment of a carious lesion in 1890 with the chemo-parasitic theory of Miller (20), caries paradigm have evolved to the currently recognized concept, the ecological plaque hypothesis (EPH). Carious lesions are formed when the plaque biofilm microbial flora undergoes a catastrophic ecological change, causing an imbalance in the physiologic equilibrium between tooth mineral and biofilm fluid and shifting the caries balance towards demineralization. Local environmental factors such as frequent dietary sugar exposures or salivary dysfunction, which are key factors that promote an increase in the acidogenic/aciduric component of the oral microbiota, contribute to the development of lesions.(Fig.2) (21) Oral plaque biofilm is a dynamic microbial environment with diverse microbial communities. Highly aciduric bacteria, such as *S. mutans* or lactobacilli, begin to dominate and replace non-MS*, Actinomyces spp*., or *Veillonella spp*., which are the main neutral pH species populating the plaque microflora in the early stages of incipient (non-cavitated) carious lesions when the acidogenic environment is prolonged.(22)

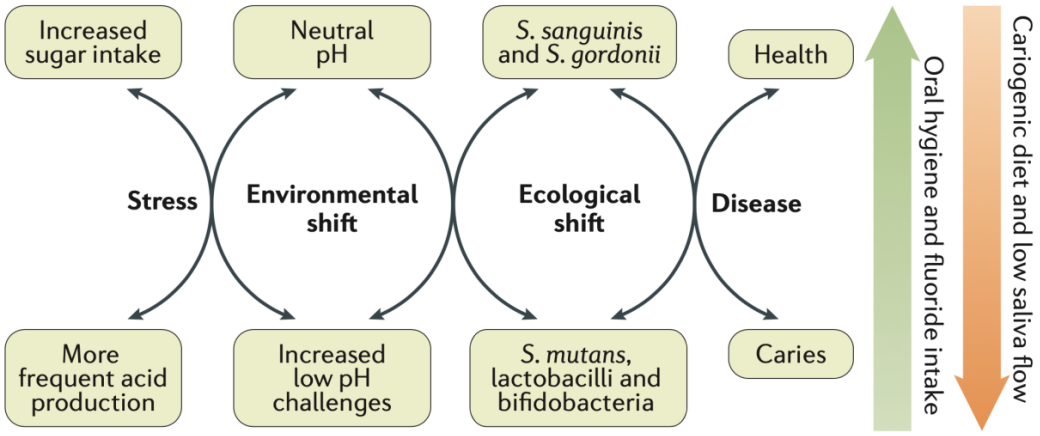

Figure 2 *Ecological plaque hypothesis to explain the etiology of dental caries*(23)

Cariogenic microbiota

Oral biofilms are microbial communities with a complex composition and structure. More than 750 distinct species or phylotypes of bacteria have been found in mature dental plaque, which can differ from one individual to the next or within specific areas of the oral environment.(24) The acquired pellicle on the tooth surface provides binding sites for adherence by early bacterial colonizers such as *Streptococcus sanguinis*, *Streptococcus gordonii, Actinomyces spp.,* or *Veillonella spp.,* leading to dental biofilm formation, and acts as a physical barrier preventing acid diffusion. However, after an unfavorable shift in the resident microbiota's balance caused by changes in the oral environment, such as frequently exposed to fermentable dietary carbohydrates daily, the biofilms experience recurrent low pH circumstances, which favor the development and metabolism of aciduric and acidogenic bacteria like *S. mutans*, Lactobacilli and *Bifidobacterium spp*.(23) *S. mutans* becomes a numerically significant species in cariogenic biofilms due to its ability to generate multiple mechanisms to colonize the tooth surface that increase bacterial adhesion to tooth surfaces, inter-bacterial adhesion, and biofilm formation. *S. mutans* has the ability to transport and metabolize a wide range of carbohydrates into organic acids (acidogenicity), and the ability to growth under environmental stress conditions, particularly low pH (aciduricity).(24, 25) In Thailand, it was also reported that the level of *S. mutans* in dental plaque and saliva in the S-ECC group was significantly higher than those from the caries-free group in preschool children (3–5 years old).(26, 27)

Interestingly, according to the previous studies, in addition to MS infection, the fungus *Candida albicans* has also been found in great numbers in plaque-biofilms from young children with ECC..(28) The children with *C. albicans* colonization had an approximately 5 times higher chance of having ECC than children who are not infected.(29) Furthermore, it is well known that co-species biofilms between *S. mutans* and *C. albicans* have a significant association with ECC patients.(30) A recent study found that the unique interaction of *S. mutans* and *C. albicans* is a symbiotic interaction mediated through the influence of Gtfs exoenzymes. Due to the production of glucan in the presence of sucrose, GtfB binds with exceptional avidity to the surface of *C. albicans* cells, even when they are in hyphal form.(30) When sucrose presenting, it promotes the interactions between *C. albicans* and *S. mutans* by providing a substrate for EPS **α**-glucans production by streptococcal Gtfs that enhances co-adhesion and bacterial–fungal tooth colonization, stimulating cross-kingdom biofilms. This interaction enhances the carriage of the cariogenic pathogen and acid production, while the presence of *C. albicans* increases EPS matrix production (via Gtf induction and fungal-derived EPS) and biofilm aciduricity, resulting in cariogenic conditions on the tooth surface. (Fig.3) (31)

Furthermore, it is also reported that the dual-species *C. albicans* with *S. mutans* biofilm provoked higher surface hardness loss on dentine surface, which represented demineralization, than the *S. mutans* and *C. albicans* biofilm alone.(32)

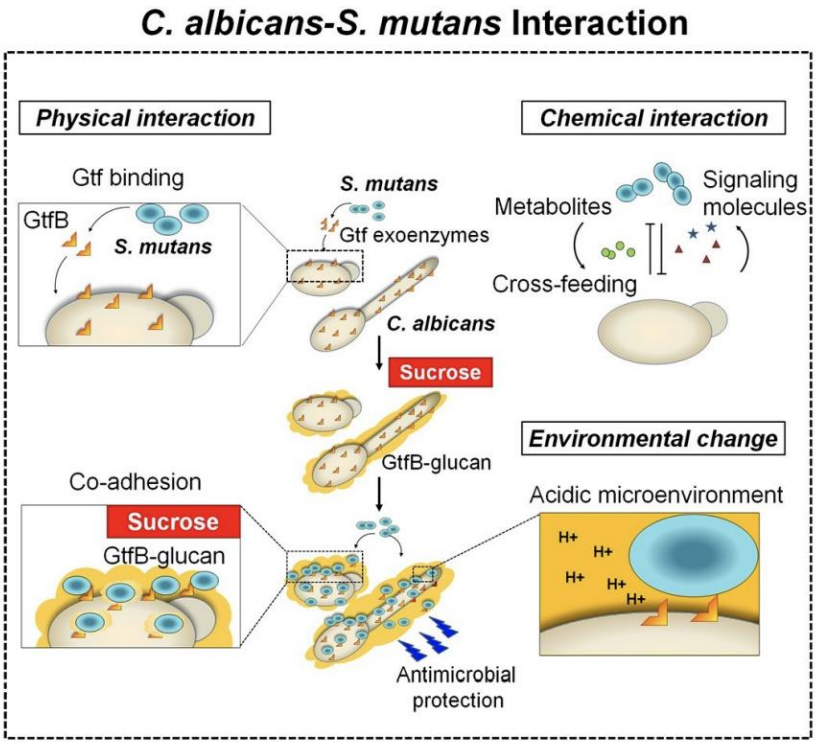

Figure 3 *Pathogenic mechanisms of C. albicans and S. mutans cross-kingdom interactions*(31)

Caries management in dentine lesion

Traditionally, dental caries were managed by removing all demineralized tissue (affected) and bacterially contaminated tissue (infected) and then replacing the cavitated with restoration, but now the new concept of minimal invasive dentistry has been approached.(33) Non-surgical techniques has been included in this approach, or when surgical treatment is indicated, it should be minimally invasive techniques (5) , such as removing or controlling the activity of the biofilm, sealing the tooth surface from the environment, or rebalancing demineralization and remineralization by applying fluoride.(5)

The 8th national oral health survey 2017 of Thailand reported that the prevalence of dental caries in 3- and 5- year-old children is 52.9% and 76.6%, respectively, while the prevalence of untreated dental caries in 3- and 5- year old is 52.0% and 73.8%.(2) There are many children who have not been treated properly, Its effects have been related to dental pain and discomfort, as well as an impairment in oral health-related quality of life.(34) This may be due to a problem with the difficulty of accessing dental care services for children in rural areas or in

low-income families.(3) Therefore, minimal invasive dentistry and prevention of disease play an important role in reducing the progression of the disease and prevention from new lesion developed.(35) There are currently many alternative approaches to remineralization. Fluoride has been widely used as a re-mineralizing agent such as fluoride toothpaste, fluoride mouthwash, fluoride water, fluoride tablet, fluoride gel, and fluoride varnish(36). In particular, recommended to combine silver with fluoride as an anticaries agent for a possible combined effect, including antibacterial agents and promoting remineralization.(37)

Silver nanoparticle in dentistry

The concept of nanotechnology was introduced in 1993 by Robert A. Freitas Jr. and can be applied to various medical fields, including dental treatment.(38) There are various scientific and technological implications, especially in the development of novel materials. Nanoparticles are developed with unique features that make them desirable for materials science and biology.(39) Among several nanoparticles, silver nanoparticles have been one of the most researched fields in recent decades.(40) It is now possible to synthesis silver nanoparticles (AgNPs) with controlled size and morphology probably smaller than 100 nm in diameter, high homogeneity and specific target functions.(41) Due to a large surface-to-volume ratio, silver nanoparticles demonstrate remarkable antimicrobial activity, even at a low concentration.(42) AgNPs, with their desirable characteristic particle, may now be thought of as multifunctional building blocks for dental materials and procedures.(43) AgNPs are used in many fields of dental treatment, such as adding to acrylic resins for fabrication of removable dentures in prosthetic treatment, composite resin for direct restoration, irrigating solution and obturation materials in endodontic treatment, adhesive materials in orthodontic treatment, membrane for guided tissue regeneration in periodontal treatment, titanium coating in dental implant treatment, and also in therapeutic use for caries prevention.(44)

Mechanisms of action of silver nanoparticles

Silver nanoparticles' actions are mostly determined by their nanoscale size, which affects the level of silver ion release and interferes with surface energy. When compared to other antimicrobial agents, silver nanoparticles have strong antibacterial properties due to their large surface area to volume ratio, which provides high interaction with pathogens.(45)

Although the specific mechanism of silver nanoparticles' antibacterial activity has not been fully elucidated, many antibacterial actions of silver nanostructures have been described. Silver nanoparticles can continuously release silver ions, which could be a microbe-killing mechanism.(46) AgNPs have already shown therapeutic efficacy against a variety of pathogens, including bacteria, fungi, and viruses.(47) It consists of the following mechanisms(44) (Fig.4) :

- Silver ions released by silver nanoparticles attach to or pass through the cell wall and cytoplasmic membrane, enhance the permeability and causing cell wall and cytoplasmic membrane disruption.
- After silver ion uptake into cell, silver ions denature ribosomes, inhibiting protein synthesis and deactivate respiratory enzymes in the cytoplasmic membrane, halting ATP production and ultimately cause cell lysis.
- The provocation of cell membrane breakdown and deoxyribonucleic acid (DNA) alteration can be aided by reactive oxygen species. The interaction of silver ions with the sulfur and phosphorus of DNA can result in problems with DNA replication, cell reproduction, and even microorganism death.

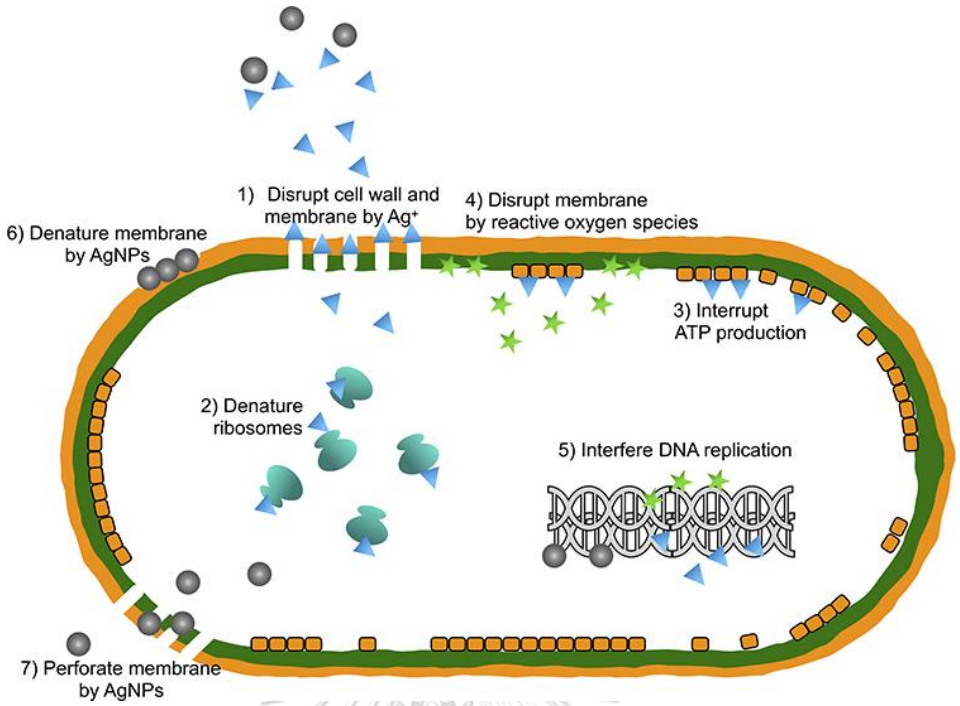

Figure 4 *The antibacterial actions of silver nanoparticles (AgNPs)*(44)

Nano-silver fluoride has been tested against *S. mutans*. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values were 33.54±14.52 and 50.32 μg/ml, respectively, which similar to silver diamine fluoride (SDF).(14) Moreover, nano-silver fluoride was also tested against *C. albicans*, and showed potent antifungal activity with very low MIC of AgNP (0.125 - 0.5 μg/ml) as compared to fluconazole (1-64 μg/ml).(48)

Furthermore, silver particles were shown to inhibit free glycosyltransferases, enzymes that promote glucose and cell adhesion, allowing for the synthesis of glycans and extracellular polysaccharides, thus inhibiting biofilm formation.(47) This was consistent with the study showing that nano-silver fluoride was an effective dental biofilm inhibitor because it reduced the *S. mutans* colony forming unit (CFU) counts, the activity of metabolism, the production of lactic acid, and the expression of the glucosyltransferases gene.(49)

Safety and toxicity of silver nanoparticles

Although the use of silver nanoparticles is very useful in the medical and dental field, knowledge about the effect of human exposure and the possible toxicity of these products is limited. The toxicity of silver nanoparticles directly depends on the

free silver ion released.(50) Some laboratory studies reported the toxicity of AgNPs that can induce oxidative stress and impair mitochondrial function of human neuroblastoma cells.(51) Moreover, the liver and kidneys of rats have been described as the primary organs for silver distribution after oral administration.(52, 53) Another concern is AgNPs' capacity to cross the blood-brain barrier (BBB) via trans-synaptic transport, resulting in final accumulation in the brain when massive AgNPs dosages were intraperitoneally injected in a rat model.(54)

However, in another investigation, silver was found in all organs examined following the oral administration of silver nanoparticles in rats. After 8 weeks of treatment, silver was excreted from most organs in all groups and no observable adverse effect was noted. The excreted Ag in the feces was estimated at about >99% of the intake.(55) Another study found that the AgNPs are biocompatible with human oral cells, such as human gingival fibroblasts and dental pulp stem cells than other silver compounds(56), and NSF had no effect on the membrane of human erythrocytes, regardless of blood type. When comparing absolute cytotoxicity levels, NSF was shown to be less harmful than SDF.(14) Moreover, another study shown that the mammalian cells are most resistant to AgNPs among the tested species.(57) In a clinical trial, there was no toxicity and adverse effects reported from using 5% nano silver fluoride for arresting dental caries in human.(58) Nevertheless, the systemic toxicity of ingested silver nanoparticles has not been reported.

Recently, researchers tried to investigate the probable in vivo toxicity dose of using AgNPs. A NOAEL (no observable adverse effect level) of 30 mg/kg dose and a LOAEL (lowest observable adverse effect level) of 125 mg/kg dosage were found in rats after oral administration of AgNPs. Significant dose-dependent changes were found in alkaline phosphatase and cholesterol, indicating that exposure to more than 125 mg/kg of silver nanoparticles may result in slight liver damage.(59) For commercial colloidal silver products (nanoparticle size 5–10 nm, 20 μg/kg/day; or size 25–40 nm, 96 μg/kg/day) the study did not find clinically relevant adverse effects in a 14-day monitored human study.(60)

However, the cytotoxic effects of AgNPs, documented in vitro studies in various cell lines, are determined by various factors such as particle size, shape, coating, dose, route, duration of exposure, and cell type, which the results are inconsistent.(61) Further investigations are required to understand the mechanisms of toxicity and safety for using AgNPs following various modes of exposure to AgNPs.

Application of silver nanoparticles on caries prevention

Since AgNPs have shown therapeutic efficacy against a variety of pathogens, including bacteria, fungi, and viruses.(47) Several studies also have developed silver nanoparticles for the purpose of controlling and preventing dental caries.(46)

Effects of silver nanomaterials on enamel

Currently, *in vitro* studies have shown the use of NSF for preventing and controlling incipient enamel caries. Some studies found that NSF have a greater remineralization efficacy due to their ability to increase surface microhardness on enamel surface in primary teeth.(16, 62-64) For example, Nozari *et al.* found that NSF showed greater remineralization efficacy than NaF varnish and nano-Hydroxyapatite Serum (n-HAP)(62) while three other studies showed similar effects to NaF varnish(63, 64) and one other study found that NSF has a similar effect on limiting enamel demineralization to NaF varnish.(65) In contrast with a study in premolar permanent teeth, NSF showed less remineralization efficacy than NaF varnish and SDF.(16) In another study investigate the mineral content on demineralized enamel permanent teeth by using energy dispersive x-ray spectroscopy (EDS). The result showed that the NSF demonstrated a significant increase in calcium and phosphorus on demineralized enamel surfaces. Moreover, the NSF had potential effectiveness to prevent caries due to inhibiting the formation of *S. mutans* on the enamel surface and preventing a decrease in pH.(64)

Effects of silver nanomaterials on dentine

From the literature review, the AgNPs with NaF can inhibit a demineralizing and promote a re-mineralizing effect on dentine carious lesions, and prevent collagen exposure and degradation in the dentine layer.(66-68) Some in vitro studies reported that artificial dentine caries treated with AgNPs + NaF had a shallow lesion depth and

less mineral loss than deionized water and NaF after biofilm challenge(67), while showing a similar effect when compared with SDF.(68) In contrast, one study found that NSF has a similar effect when compared with SDF and NaF varnish on surface and cross-sectional microhardness test.(69) In terms of the mineral content of demineralized dentine, another study reported that NSF was proven to have the ability to release calcium, phosphate, and fluoride ions and revealed similarities to SDF(70) while other study showed NSF had a lower Ca:P ratio than SDF and NaF.(69) Moreover, the HPO24– : amide I ratio was higher on dentine surfaces treated with AgNPs and NaF than deionized water group which suggests that AgNPs and NaF can decrease dentine demineralization.(67)

Furthermore, the AgNPs with NaF can prevent collagen degradation in the dentine layer. The studies showed that demineralized dentine disc treated with AgNPs with NaF showed smooth surface(67), intact and dense collagen fibers, while the cross-sectional image showed the formation of compact granuliform structures of ball-shaped particles in the inter-tubular region.(68) In addition, one study found that in AgNPs/KF groups showed an intrafibrillar pattern of minerals deposition (biomimetic remineralization) without any effect on collagen fiber morphology.(66)

In term of color staining, there are studies showing that there was no significant black staining on the dentine disc in NSF and AgNP with NaF, compared to the SDF group which has a significant black staining on dentine.(66, 67, 71, 72)

Clinical evidence

Although most studies on caries prevention and anti-cariogenic bacteria are still studied in vitro, controlled clinical trials to assess the efficiency of silver nanoparticles as anticariogenic agents in remineralization have recently been conducted.

In a recent study, controlled clinical trials were conducted to assess the arresting caries effect of the NSF-treated group and the water (control) group in primary teeth. The results showed that 81% of the teeth in the NSF treated group had demonstrated arrested caries, while 0 percent of the teeth in the control group had arrested caries after seven days. Five to twelve months later, the NSF treated group

showed 72.7% and 66.8% of the teeth had demonstrated arrested caries, respectively.(15) Similar to one randomized controlled clinical trial investigated the effectiveness of NSF in preventing and arresting dental caries on primary molars compared to the saline group. This study found that after 7 days of follow up, the high success rate of arresting caries found in NSF was about 78%, compared to 0% in saline group. After 5 and 12 months of follow up, the success rate of arresting caries was 72.91% and 65.21% respectively, compared to the saline group, showed 34% and 28.88%, respectively.(73)

In a similar study, the effectiveness of 0.1 percent 46 nm AgNPs applied to a commercial fluoride varnish on remineralization of white spot lesions on deciduous teeth was examined. The findings showed that teeth treated with fluoride varnish containing silver nanoparticles have greater dental structure than teeth treated with conventional varnish.(74)

Furthermore, A 6-month follow-up double-blind randomized clinical trial compared the arresting caries effects of two different concentrations of NSF on posterior primary teeth with occlusal and proximal dentine lesions. The result found that the NSF 600 solution showed a higher success rate of arresting caries at 72.7%, while the NSF 400 solution was 56.5% for a 6-month evaluation with a single application.(75)

Moreover, Tirupathi *et al* study found that the 5% NSF group had similar clinical efficacy as the 38% SDF group in terms of pain, lesion depth progression and lesion activity but did not stain teeth black during their 12-month follow-up.(58) In term of antibacterial effect that related to caries activity, NSF showed a greater reduction of *S. mutans* bacterial count than SDF on dental biofilm of pre-school children with active carious lesions while not in *Lactobacilli*, and both groups had a percentage of inactive caries with no significant difference after 1 month follow up (NSF was 63.4% while SDF was 64.4%).(76) Similar to other studies found that NSF varnish reduced *S. mutans* level in both saliva and plaque biofilm similar to chlorhexidine varnish after 1 and 3 months follow up (77), another study showed that NSF revealed lower CFU counts on dental biofilm compared to normal saline group after 1 week follow up.(49)

Analytic method of mineral changes

There are various experimental methods used to analyze mineral content changes, surface morphology, and crystal characteristics during dentine demineralization and remineralization, such as micro-computed tomography (Micro-CT), energy-dispersive X-ray spectrometry (EDX), scanning electron microscopy (SEM), transversal microradiography (TMR) and transmission electron microscopy (TEM), atomic absorption spectrometry (AAS), X-ray diffraction (XRD), optical coherence tomography (OCT) and microhardness test.(67, 78, 79) Each measurement method has advantages and limitations. Therefore, when selecting a method to measure mineralization, it is necessary to choose a method that is suitable and accurate.

Analytic of mineral change by micro-computed tomography (Micro-CT)

In the past, Transverse microradiography (TMR) was considered the gold-standard two-dimensional (2D) imaging method for mineral density measurement during demineralization in vitro and in situ. This technique could be used to measure mineral loss and lesion depth (LD), as well as the thickness and degree of mineralization of the surface layer.(80) TMR analysis needs the preparation of transverse thin sections from calcified tissue, the generation of their x-ray absorbance images on photographic plates or film, and the conversion of the optical density to mineral content values using an aluminum step wedge. The thickness of these slices, about 100 um, was not only fragile and a labor-intensive challenge to create, but also limited data to a certain area in the specimen.(81) On the other hand, microfocus X-ray computed tomography (Micro-CT) is a high-resolution three dimensional (3D) X-ray imaging technology that has improved in recent years as computer science has developed. The image analysis methods have been validated to measure TMR lesion parameters such as lesion depth (LD) and mineral loss (ΔZ) based on the CT values representing radiopacity.(82) Therefore, Micro-CT had the ability to assess and measure mineral densities of sound enamel and dentine, carious enamel and dentine, remineralization, and demineralization of teeth.(83) This method using microfocus spot X-ray sources and high-resolution detectors can generate 3D reconstructed images of specimens by rotating projections via many

viewing directions. The images show the regional distribution maps of linear attenuation coefficients, which are influenced by the X-ray source's energy and the atomic composition of the material sample. Because the imaging method is nondestructive and does not require special preparation, the interior characteristics of the same sample may be studied several times, and samples can be used for further biological and mechanical testing after scanning.(84)

Some limitation of using Micro-CT is a significant amount of mineral (or metallic silver) has been gained, and CT images corresponding to those lesions demonstrate radiopacity beyond that of natural tissue, to the point where the lesion has progressed beyond remineralization to a point that can be considered healing. It should also be noted that SDF radiopaque may have a role in the calculation of ΔZ depending on the ROI selection.(85) Due to the benefits of Micro-CT and the limitations of TMR, this study plan uses Micro-CT to evaluate lesion depth of carious dentine.

Microhardness measurement

Microhardness measurement is an indirect technique of following changes in the mineral content of dentine which is a destructive method.(86) This method is easy, quick, and requires only a small area of specimen surface for testing. For measuring the microhardness, the tooth is indented with a diamond indenter at a certain load for a certain period of time. After load removal, diagonals of the indentation were measured with an optical microscope. An increase in the length of the indentation indicates a decrease in the hardness of the tooth, which means demineralization. In contrast, a reduction in indentation length indicates greater hardness of the tooth, which means remineralization of the specimen.(87) There are two type of microhardness measurement following (88):

1. **Surface microhardness testing**

This method uses a diamond indenter to press perpendicularly onto the polished tooth element to obtain surface microhardness. It is easy to measure and can be reproduced. The limitation of this method is that the hardness values are not able to represent the mineral change at the depths below the surface.(88)

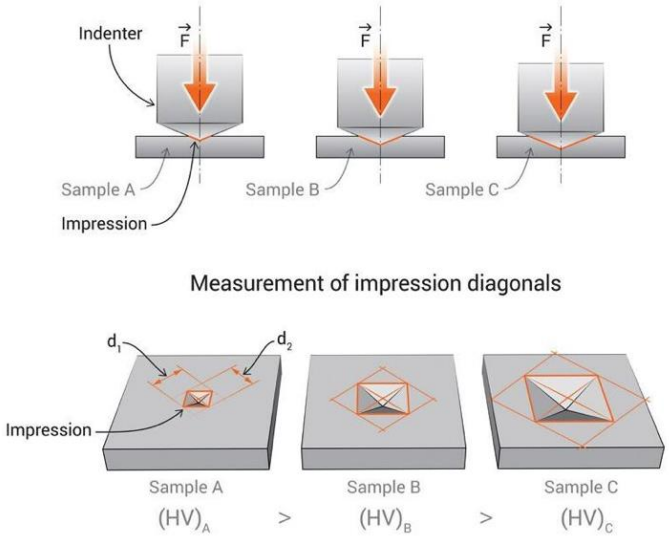

Figure 5 *Surface microhardness testing*(89)

2. Cross-sectional microhardness testing

The sample tooth is cut perpendicular to the surface, then a diamond indenter is pressed parallel to the outer surface of the tooth specimens. This method can measure the microhardness in area below 25 microns from the outer surface.(88) This method is frequently used for the study of mineral change in carious dentine lesions (2, 79, 90-93), and is an alternative to TMR for describing mineral content and hardness profiles.(91) The limitation of this method is that the specimens will be destroyed. As a result, it cannot be replicated.(94)

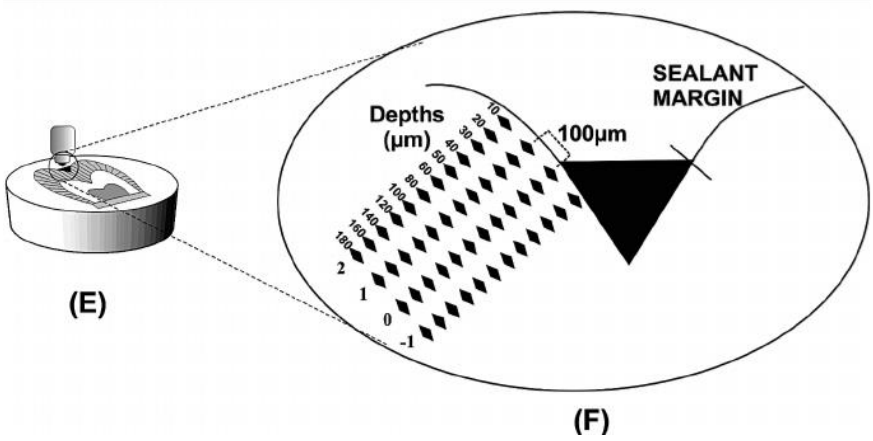

Figure 6 *Cross-sectional microhardness testing*(95)

The commonly used of diamond indenter for measuring microhardness on dentine surface are Vickers and Knoop.

- **Vicker indentation**

The Vickers test is a diamond indenter, which has a square base pyramidal indenter with a 136-degree angle between them (Fig 7). The depth of the indentation is approximately 1/7 times the length of the diagonal, and the diagonals must be measured on both sides and averaged.(88) The hardness number was defined by the ratio between the indentation load and the area of the residual impression. Then the hardness of materials was calculated using these equations (87):

$$H_v = (1.854)\ F/d^2$$

H_V = Vickers Hardness number (kg/mm^2)

F = The indentation load (kg)

d = The diagonal of the indentation (mm)

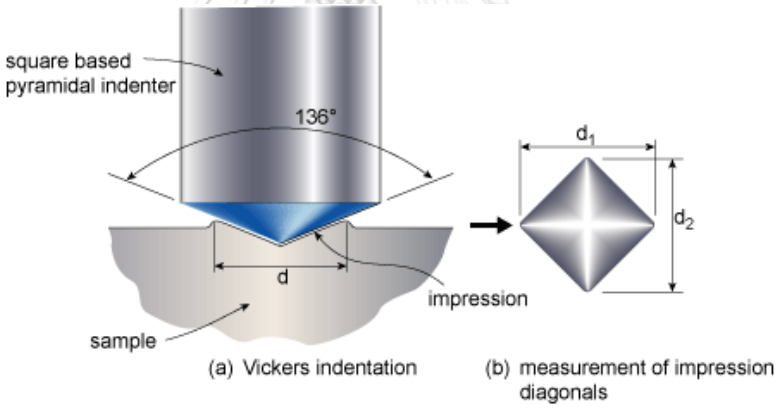

Figure 7 *Vickers hardness test*(96)

- **Knoop indentation**

The Knoop indentation is a pyramid-shaped diamond indenter at an angle of 130 degrees and 172 degree 30'. The indentation has two diagonal lines. The ratio of long to short lines is 7:1. The depth is about one-third of the length of the diagonal (Fig.8). Therefore, the thickness of the object should be at least one-third of the long diagonal. And after pressing, the pressure marks will be restored along the short diagonal line. As a result, the hardness measured does not depend on the flexibility of the specimen being tested.(88) Then the hardness of materials was calculated using these equations:

$H_k = (14.23)\ F/d^2$

H_k = Knoop Hardness number (kg/mm^2)

F = The indentation load (kg)

d = The diagonal of the indentation (mm)

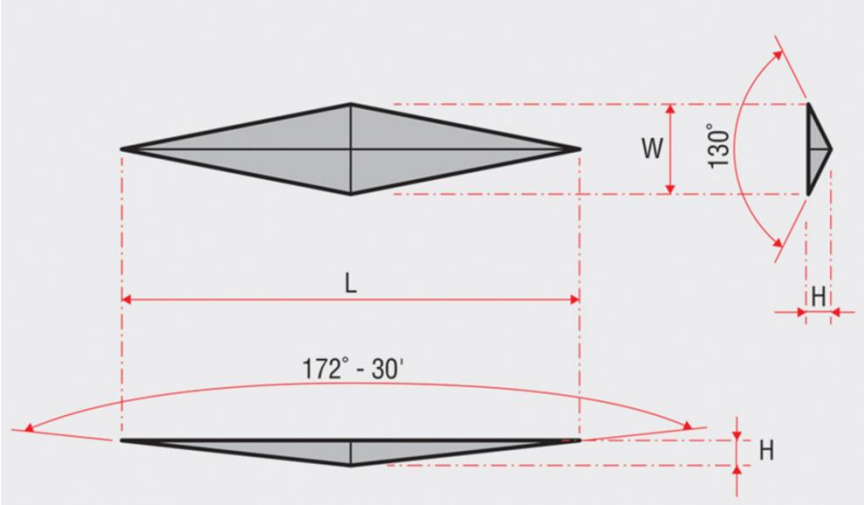

Figure 8 *Knoop hardness test*(97)

The Knoop indentation is suitable for brittle specimens due to its shallow indentation without cracking. In addition, the longer impression is easy to read. From the literature review found that they commonly use a Knoop indenter for measuring microhardness on the dentine surface.(79, 87, 90, 92, 98) Therefore, in this study, we used a Knoop indenter for surface microhardness testing for mineral change in dentine

Chapter 3

Materials and methods

Population and sample

Target population: Dentine carious lesions

Sample population: Sound maxilla or mandibular permanent premolars or molars

Study sample: Dentine slice prepared from sound permanent premolar or molar teeth that fit the inclusion criteria of this study and were created artificial dentine carious lesions in the laboratory.

Criteria of study sample

Inclusion criteria

Sound permanent premolar or molar teeth without dentine carious lesion, crack line, restorations or abnormalities of enamel and dentine.

Exclusion criteria

Permanent premolar or molar teeth that are unable to prepare dental specimens of the required size.

Sample size calculation

Sample size and power calculations were performed using G*Power program version 3.1.9.4 with F test: one way ANOVA. The sample size was determined based on a micro-computed tomography (Micro-CT) result of Zhao *et al.*(67, 68) comparing lesion depth of artificial dentine caries specimens after Nano silver particles (400 ppm silver) solution with 2.5% NaF solution (11,310 ppm fluoride) and 12% Silver diamine fluoride (80,000 ppm silver and 14,150 ppm fluoride) application. However, the aim of this study is to compare the effectiveness of different concentrations of nano-silver fluoride solution, 38% silver diamine fluoride, and 5% sodium fluoride varnish in the remineralization of artificial dentin caries, which has not been studied before. As a result, it is thought to increase the effect size to 0.48 as suggested by the statistics G*program to be a large effect size. After the specimen calculation with an alpha value of 0.05 and a power of 0.8, the total sample size was 52 specimens with the actual power of 0.81. Finally, 60 specimens (15 specimens/group) were used in the Micro-CT study, which compensated for 10% laboratory errors (Fig.9).

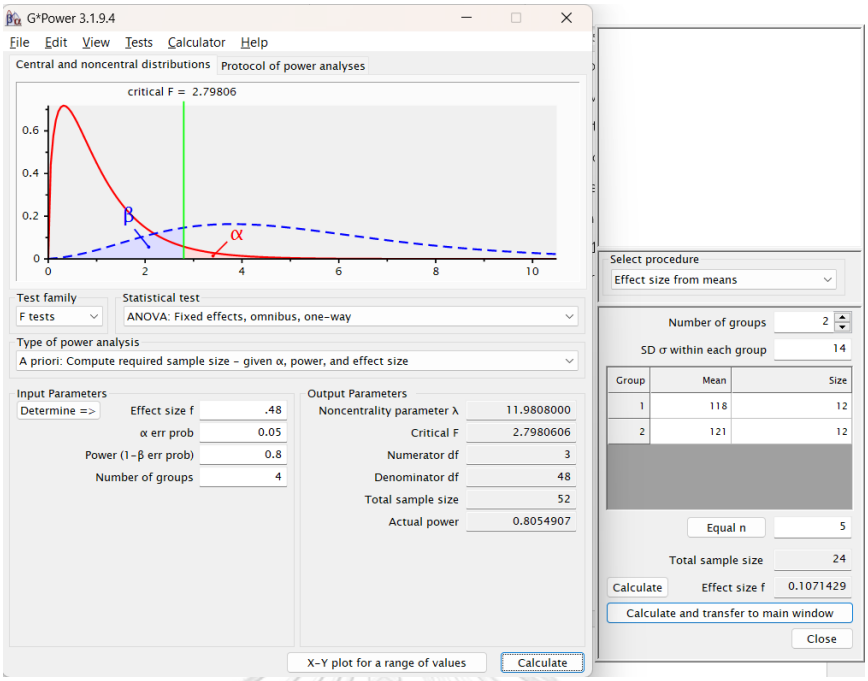

Figure 9 *Calculation of sample size by using G*Power program version from mean caries lesion depth*

For surface microhardness test, the sample size was determined based on the study of J. Cai *et al*. (79) that compared the surface dentine hardness using Knoop hardness indentation between SDF/KI and DI water (a control group). After the specimen calculation with an alpha value of 0.05 and a power of 0.8, the total sample size was 12 specimens with effect size was 1.12.

Since DI water has no effect on the hardness and SDF was used for resembling effect of NSF, whereas our study aims to compare the remineralizing agents (which are known to have an effect on the hardness) including the nano-silver fluoride solutions, 38% silver diamine fluoride, and 5% NaF-varnish. As a result of this, as well as the limitation on the number of specimens that can be handled, the effect size was adjusted to 0.48 (reduced to 57%). The sample size was determined with an alpha value of 0.05 and a power of 0.8. As a result, the total sample size was 30 specimens with the actual power of 0.81. Finally, 60 specimens (15 specimens/group) were used in the hardness study, which compensated for 10% laboratory errors (Fig.10).

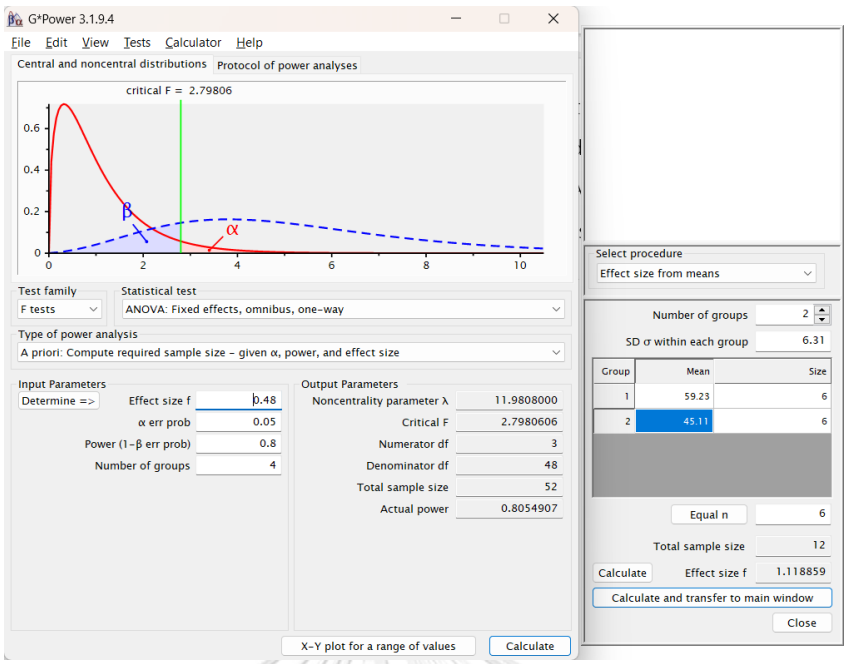

Figure 10 *Calculation of sample size by using G*Power program version from mean surface hardness*

Materials and instruments

Instruments

1. Analytical Balance (Satorius, BP110S, Germany)
2. Hot plate stirrer (Fermo®-Geratetechnik, Germany)
3. Forma™ SteriCycle™ CO_2 Incubators (Thermo Scientific, USA)
4. Laminar airflow biosafety cabinet (NU-440,Nuaire, USA)
5. GENESYS™ 20 Visible Spectrophotometer (Thermo Scientific, USA)
6. pH meter (HORIBA LAQUAtwin pH-11 Compact pH meter)
7. Fluoride meter (Thermo Orion, VERSA STAR, USA)
8. GENESYSTM 20 Visible Spectrophotometer (Thermo Scientific, USA)
9. Low speed cutting machine (ISOMET 1000™, Buehler, USA)
10. Polishing machine (MINITECH 233, PRESI, France)
11. micro-fine 800, 1000, 1200-grit sanding paper
12. Micro Computerized Tomography (Micro-CT) (SKYSCAN1272, Bruker, Germany)
13. Microhardness tester machine (FM-810, FUTURE-TECH, Japan)
14. Scanning electron microscope (Quanta 250, FEI, USA)

15. Inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima 7300 DV, Perkin Elmer, USA)
16. Autoclave (Tuttnauer, USA)
17. Plasma sterilization machine (STERRAD 100NX, ASP, USA),
18. Refrigerator Temperature Monitoring (Canon Ball manufacturing, Thailand)
19. Shaker incubator (Stuart Scientific Ltd.,UK)
20. Vortex mixer (VX-200, Labnet, India)
21. High speed refrigerated microcentrifuge (MX-305, TOMY, Japan)
22. Measuring cylinder
23. Funnel
24. Plastic beaker
25. Stirring rod
26. Test tube
27. Plastic centrifuge tubes
28. Automated pipette with tip (10, 200 and 1000 µl)
29. Petri dish
30. Inoculating loop
31. Cuvette
32. Glass bead 3 mm.
33. 24-well plate (Thermo Fisher Scientific, China)
34. Silicone mold block
35. Hot melt adhesive with gun
36. Surgical blade no.15

Material

1. Silver nanoparticle (Prime nanotechnology Co.,ltd)
2. Sodium fluoride salt (NaF)
3. 38% Silver diamine fluoride (Saforide®: Toyo Seiyaku Kesei Co. Ltd)
4. 5% Sodium fluoride (5%NaF) (Duraphat®: Colgate®, Canton, MA)
5. *Streptococcus mutans* UA159
6. *Candida albicans* SC5314

7. Brain Heart Infusion Agar (HiMedia, India)
8. Brain Heart Infusion broth (BHI) (HiMedia, India)
9. Tryptone with yeast extract powder (HiMedia, India)
10. Sucrose Uniar® (Ajx Finechem, Australia)
11. D-glucose andydrous® (Fisher Scientific, England)
12. Phosphate Buffered Saline (PBS)
13. Demineralization solution containing 2.2 mM KH_2PO_4, 2.2 mM $CaCl_2$ and 50 mM acetate (Prepared by the Department of Pharmacy Faculty of Dentistry Chulalongkorn University)
14. Artificial saliva without fluoride (Prepared by the Department of Pharmacy Faculty of Dentistry Chulalongkorn University) containing:
    1. Potassium Chloride; KCl 130 mM
    2. Calcium Chloride; $CaCl_2$) 1.5 mM
    3. Potassium Dihydrogen Phosphate; KH_2PO_4 0.9 mM
    4. 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES) buffer 20 mM
    5. Potassium Hydroxide 1mM
11. Deionized water
13. Clear epoxy resin
14. Double sided tape
15. Microbrush applicators with 1 mm. diameter
16. Mixing well
17. Nailed coat (Revlon, New York, USA)

Interventions

1. Nano-silver fluoride (Ag^+ 400 ppm + F^- 22,600 ppm, NSF400) (Prime nanotechnology Co.,ltd)
2. 38% Silver diamine fluoride (Saforide®: Toyo Seiyaku Kesei Co. Ltd)
3. 5% Sodium fluoride (5%NaF) (Duraphat®: Colgate®, Canton, MA)
4. Deionized water (DI water) (The Office of Research Affairs, Faculty of Dentistry, Chulalongkorn University)

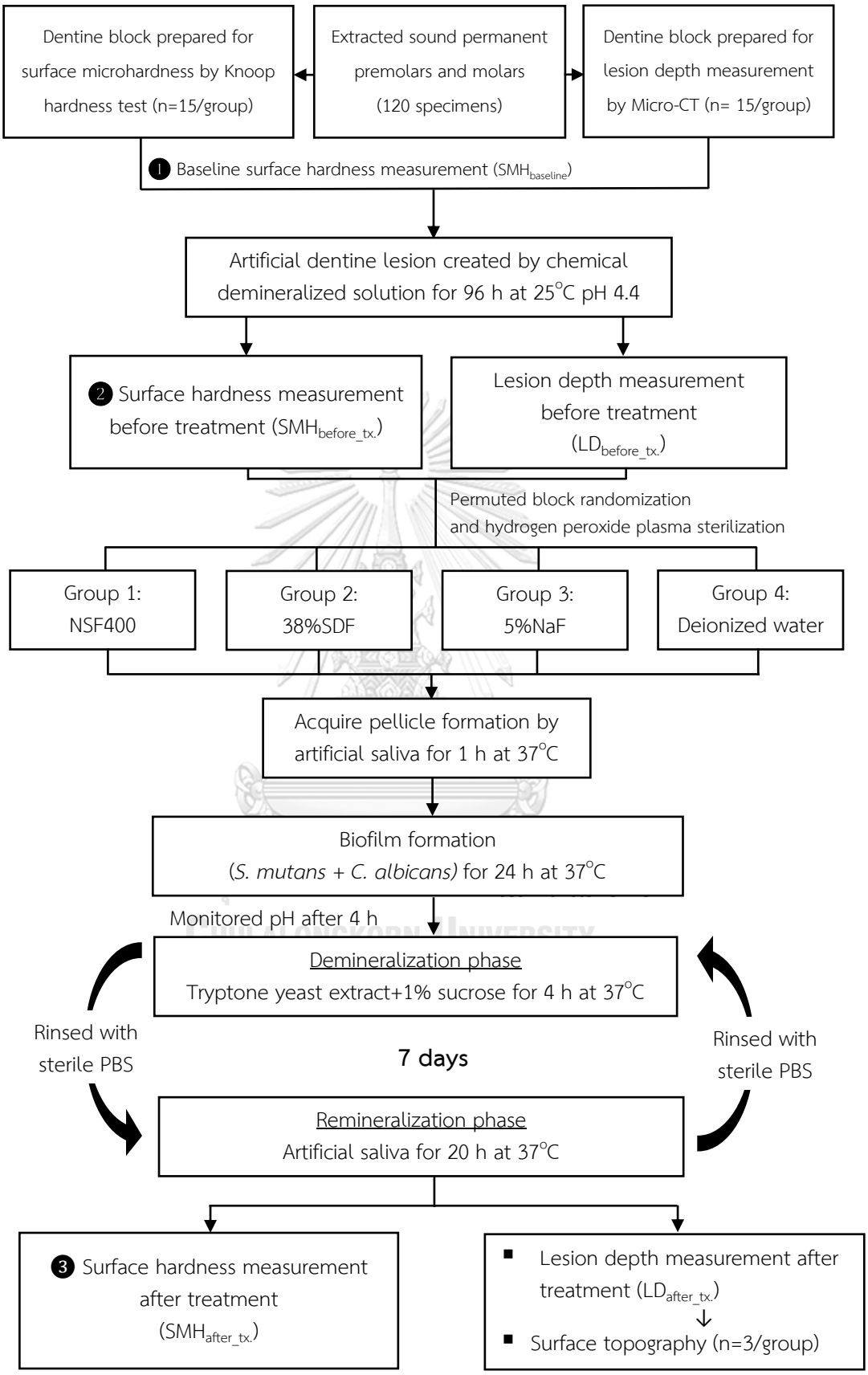

Figure 11 *Flow chart of the experimental design*

Research methodology

This study was divided into six main steps, which showed as follows:

1. Nano-silver fluoride preparation and characterization

1.1 Preparation of Nano-silver fluoride

The synthesis of Nano-silver fluoride is adapted from Yin *et al*.(72) and the synthesis of silver nanoparticles was manufactured by Prime nanotechnology Co., Ltd., which contained Ag^{+} and tannic as a stabilizer. The average particle size is 6 $\pm$ 4 nm. with silver concentration 10,000 ppm. NSF solution was prepared freshly before use with the steps as follows:

1.1.1. The silver nanoparticles solution was diluted with deionized water to concentration 400 ppm silver. The color changed from dark yellow colloid to light yellow and keep in plastic centrifuge tube.

1.1.2. The sodium fluoride 250 mg (22,600 ppm; 5% NaF) was added into 5 ml of silver nanoparticles solution at room temperature and mixed by vortex mixer to form Nano-silver fluoride solution.

1.1.3. The Nano-silver fluoride solution was kept in a plastic centrifuge tube and stored at 4°C until used.

1.2 Characterization of Nano-silver fluoride solution.

1.2.1 pH measurement

pH measurement was performed by using pH meter (HORIBA LAQUAtwin Compact, Japan) The calibration of a pH meter was performed before testing. pH measurement in each solution was repeated three times, the average was calculated

1.2.2 Silver ion and fluoride ion concentration

The Nano-silver fluoride samples were diluted 100 times and digested the sample in 10% nitric acid and analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) before measuring concentration of silver and fluoride free ion. The calibration of a fluoride meter (Thermo Orion, VERSA STAR®, USA) and heavy mineral spectrometer (Optima 7300DV, PerkinElmer®, USA) is performed before testing. The measurement of silver and fluoride ion concentration in each solution was repeated three times, the average was calculated.

2. Preparation of dentine specimens

2.1 Extracted permanent premolar and molar human teeth which met the inclusion criteria were collected with patient consent. Teeth were stored in normal saline and sterilized with 0.1% thymol for 15 minutes before tooth section. Teeth were cleaned to remove debris, gingival calculus, and washed with distilled water.

2.2 The enamel layer was cut to the junction of enamel and dentine (DEJ) then dentine slices with a thickness of 2 mm are prepared. (Fig.12a) The surface of dentine slices was polished with micro-fine 800, 1,000 and 1,200 grit sanding paper. The polished dentine slices were examined using a stereomicroscope to exclude samples with cracks or other defects.

2.3 Each dentine slice was cut into 3 x 3 x 2 mm^3 (width x length x depth) dentine block. All blocks were covered with acid-resistant nail varnish, except for the experimental window of 2 x 2 mm^2 (yellow area) on the occlusal surface, (blue area, Fig. 12b).

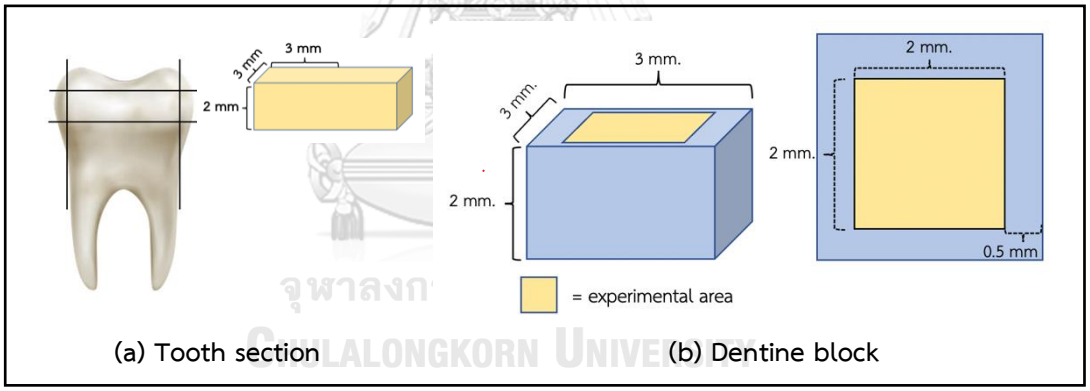

***Figure 12 (a-b)**: Preparation of dentine specimen block*

2.4 Dentine blocks were prepared for Micro-CT and surface dentine microhardness evaluation as follows:

2.4.1 Micro-CT (lesion depth) evaluation: Each dentine block (n=60) was placed on epoxy resin mold with the experimental window raised about 2 mm from the resin surface and parallel to the floor. The first reference point was marked on resin mold and second reference point was marked on dentine block. (Fig.13a)

2.4.2 Surface dentine microhardness evaluation**:** Each dentine block (n=60) was embedded in an acrylic resin mold, with the plane parallel to the mold's surface (Fig.13b).

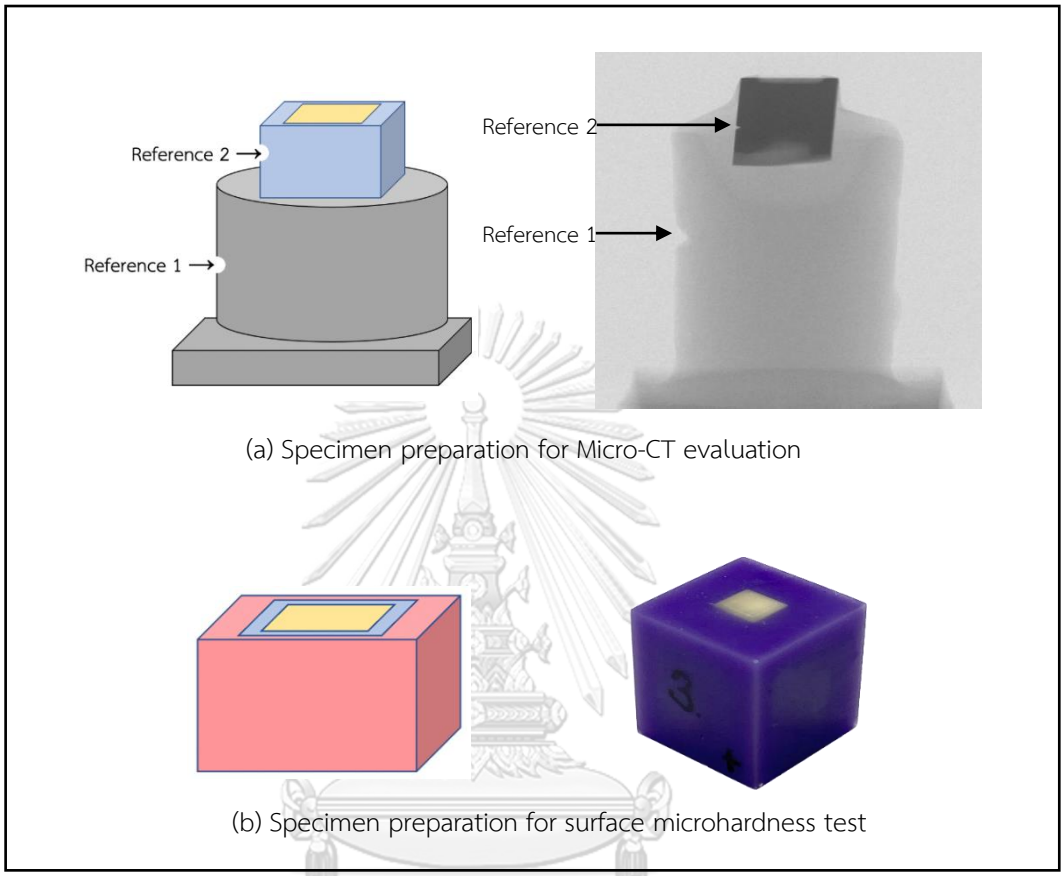

Figure 13 (a, b) *Specimen preparation for Micro-CT and surface microhardness test*

2.4.2.1 The baseline surface hardness (❶ $SMH_{baseline}$): The measurement of baseline surface hardness was performed before artificial dentine caries was created. Five indentations were performed on each specimen at the center with approximately 150 μm apart as shown in Fig.14 The dentine block selected for the study must have an average surface hardness of at least 50-70 KHN.(98)

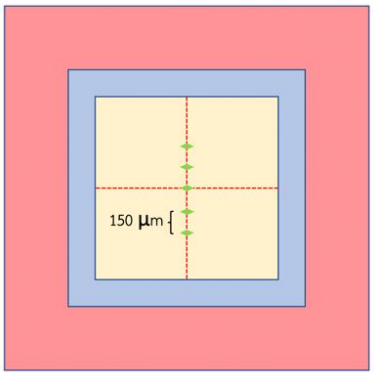

Figure 14 *An indentation track for measuring baseline surface hardness*

3. Artificial dentine caries formation

All specimens were placed into 24-well plate and immersed in a demineralization solution containing 2.2 mM KH_2PO_4, 2.2 mM $CaCl_2$, and 50 mM acetate at pH 4.4 in 25°C for 96 h to develop artificial dentine caries lesions which were 180-280 µm in depth. The demineralization solution was refreshed every 24 h. After lesion developed, the specimen was rinsed thoroughly with deionized water.(99)

4. Experimental treatment

4.1 Randomized allocation

The specimens for each evaluation (Micro-CT and surface dentine microhardness) were randomly allocated using a permuted block randomization (Blocks of 4) as described below:

1. Micro-CT (lesion depth) evaluation: After the artificial dentine caries formation, the lesion depth of all specimens (n=60) was measured ($LD_{before\_tx.}$), sorted in ascending order, and divided into 15 sets (4 specimens/set). The excel application was used to assign each specimen with randomly generated numbers. The specimen with the lowest random number was in group 1, while the specimen with the highest random number was in group 4.

2. Surface dentine microhardness evaluation: After the artificial dentine caries formation, the surface microhardness of all specimens (n=60) was measured (❷ SMH_{before_tx}, Fig.15). The percentage change of surface microhardness was calcuated as following.

$$\text{The percentage change of surface microhardness} = \frac{(SMH_{before\_tx.} - SMH_{baseline}) \times 100}{SMH_{baseline}}$$

The percentage change of surface microhardness was then sorted in ascending order and divided into 15 sets. The excel application was used to assign each specimen with randomly generated numbers. The specimen with the lowest random number was in group 1, while the specimen with the highest random number was in group 4.

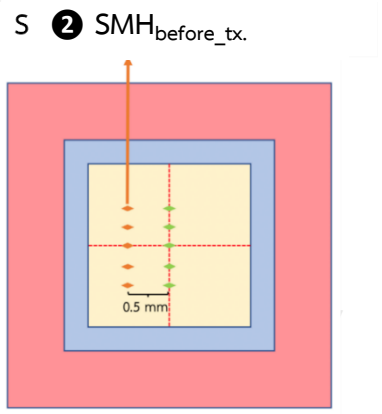

Figure 15 *An indentation track to measuring Knoop surface microhardness before treatment.*

4.2 Experimental treatment

After randomization, all specimens were sterilized with hydrogen peroxide gas plasma sterilization (STERRAD 100NX, ASP, USA), then immersed into sterile artificial saliva (0.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl and 20 mM HEPES and then adjusted to pH 7.0 with 1 mM KOH) for 1 hour to rehydration of dentine specimen.(100) All specimens then received the treatment as detailed below:

Group 1: Nano-silver fluoride (Ag+ 400 ppm + F- 22,600 ppm, NSF400): For each specimen, 5 µl of NSF solution was applied by using a micro brush to the dentine surface with an agitation technique for one minute, and leaved the solution for one minute. Then, rinsed with 1 ml PBS solution to remove excessive solution.

Group 2: 38% Silver diamine fluoride (38%SDF): For each specimen, 5 µl of 38%SDF solution was applied by using a micro brush to the dentine surface with an agitation technique for one minute, and leaved the solution for one minute. Then, rinsed with 1 ml PBS solution to remove excessive solution.

Group 3: 5% Sodium fluoride (5%NaF): A thin layer of fluoride varnish was applied on specimens by using micro brush for one minute and stored in artificial saliva for 24 hours. After that, the varnish was removed from the surface with a scalpel blade without scratching the dentine surface. The specimens were then cleaned with an ultrasonic cleaner for 2 minutes.

Group 4: Deionized water (Negative control, DI water): For each specimen, 5 µl of deionized water was applied with a micro brush to the dentine surface for one minute. and leaved the solution for one minute. Then, rinsed with 1 ml PBS solution to remove excessive solution.

5. Dual-species microbial pH-cycling method

5.1 Microbial strain and culture method

S. mutans UA159 and *C. albicans* SC5314 were inoculated in brain heart infusion (BHI) agar plates and incubated at 37°C with 5% CO_2 for overnight. After that, colonies of *S. mutans* were inoculated into 10 ml of BHI broth (pH = 7.4 ± 0.2) while *C. albicans* were inoculated into 10 ml of tryptone yeast extract (TYE) containing 1% of glucose (w/v) broth (pH = 6.5 ± 0.2) and incubated at 37°C, 5% CO_2 for overnight (approximately 16 hours). These starter cultures were adjusted to OD_{600nm} = 0.1 in 15 ml of the same medium used in the previous step and incubated at 37°C with 5% CO_2 until they reached the mid-log growth phase (OD_{600nm} ≈ 0.56 ± 0.02) of *S. mutans* and

C. albicans. Then, the log-phase cultures were centrifuged (120x100g, 15 min, 25°C, MX-305, TOMY) and the cell pellets were resuspended and diluted 100 times in tryptone yeast extracted (TYE) broth containing 1% sucrose (w/v) (101). Each diluted culture was mixed in a 1:1 ratio to achieve a concentration of *S. mutans* ≈ 1.95 x 10^6 CFU/mL and *C. albicans* ≈ 1.46 × 10^4 CFU/mL (CFU: colony forming units), which is the proportion of microorganisms found in saliva samples of children with ECC.(30) These mixed-cultures were used for dual- species biofilm formation.

5.2 Dual-species biofilm formation

The process for forming dual-species biofilm formation was modified from the study of C. I. V. Lobo *et al.*(101) The specimens were placed vertically in 24-well plates containing 2 ml of sterile artificial saliva and incubated at 37°C for 1 hour to form the acquired pellicle. In each well of the 24-well plates, 2 ml of the dual-species culture were added. To form mature biofilm, the specimens were incubated in the dual-species culture for 24 hours at 37°C with 5% CO_2.

5.3 Demineralization-remineralization cycle

The demineralization-remineralization cycle was modified from Z. Yu *et al.* study. (102) To simulate the demineralization process, the biofilm-formed specimens were incubated in 2 ml of TYE+1% sucrose (w/v) broth for 4 hours at 37°C with 5% CO_2. The pH of culture was measured before incubated (pH = 6.54 ± 0.03). After 4 hours of incubation, the pH of culture was 5.96±0.15, which is below the critical pH for dentine (pH = 6.2-6.5).(103) After washing with 2 ml of sterile PBS, the specimens were incubated for 20 hours (pH after 20-h incubation = 6.82 ± 0.04) at 37°C with 5% CO_2 in 2 ml of sterile artificial saliva (pH ≈ 7.00) The specimens went through this demineralization-remineralization cycle for a total of 7 days (Fig.11). After that all specimens were washed with PBS and biofilm were removed by using ultrasonic cleaner (Elma S30H, Germany) with 37 kHz for 5 minutes.(102) Then, the blocks were sterilized with hydrogen peroxide gas plasma sterilization before further analysis.

6. Analytic method

6.1 Lesion depth evaluation by Micro-CT

The lesion depth was measured by microcomputed tomography (Micro-CT) (SKYSCAN1272, Bruker, Germany). For bone mineral density (BMD) calibration, a series of Bruker-micro-CT BMD calibration phantoms were scanned, which included calcium hydroxyapatites (CaHA) of 0.25 and 0.75 g/cm^3 disks.(104) The X-ray source was set at a voltage of 100 kV and a current of 80 μA with 0.6 degree of rotation step. A 1-mm aluminum filter was used to cut off the softest X-rays and set the reference line as a reference point on the dentine specimens. The images were set to 9 μm with a resolution of 1,024 x 1,024 pixels per projection.(68) The total integration time is 14 minutes/specimen about 200-300 slides. NRecon reconstruction software (SkyScan Bruker, Germany) was used to reconstruct the scanning images for misalignment compensation and ring artifacts reduction. After reconstruction, DataViewer software (SkyScan Bruker, Germany) was used to view, and ten cross-sectional images were simple randomly selected to evaluate the lesion depth. For lesion depth evaluation, quantitative line profiles were evaluated across the lesions perpendicularly from the imagination line as a reference (superficial sound dentine surface) and traversing into a depth of 2 mm. to define grey value of dentine. The lesion depth was measured using image analysis software (Image J; National Institutes of Health, USA) Three parallel lines at the difference point (mid-lesion, ½ of the left half, and ½ of the right half) were measured for mean lesion depth/specimen (Fig.16).(105) The grey value curve at each tooth depth was determined via the "Plot profile" feature. The lesion depth was measured from the reference line to sound dentine, while mean gray values of sound dentine were determined via "Measure" feature (Fig.17). Then, 95% of the sound dentine gray value was considered as the bottom of the lesion depth (Fig.18).(106)

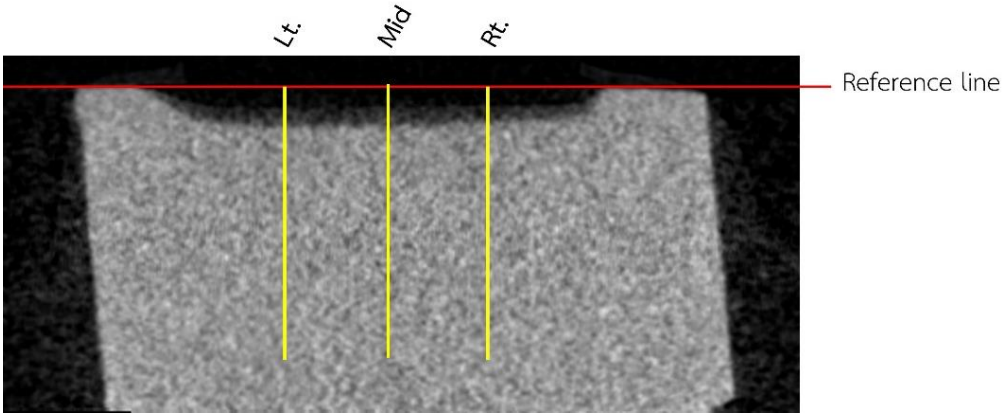

Figure 16 *Lesion depth measurement point*

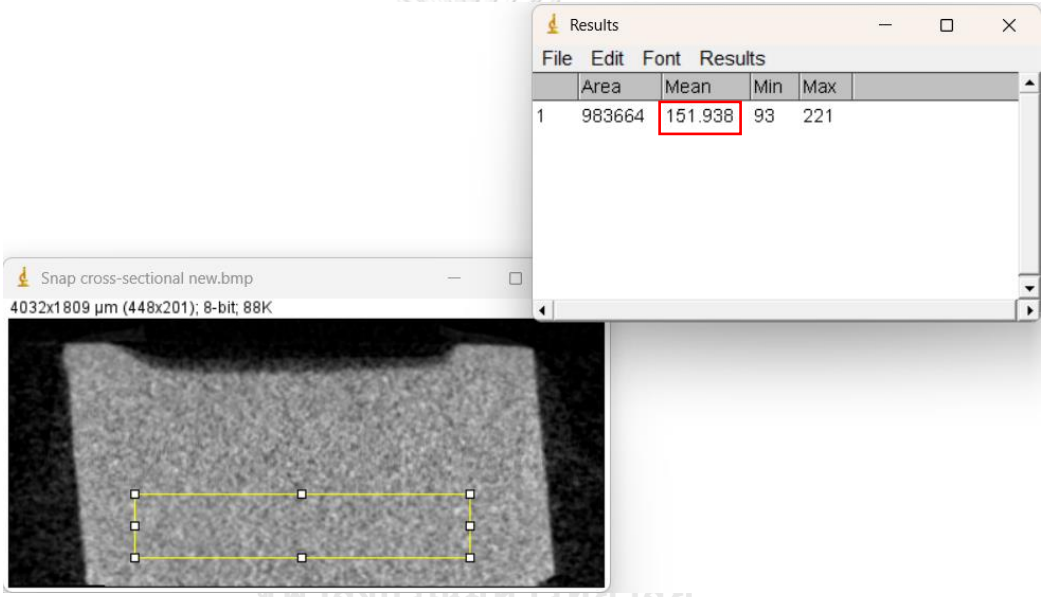

Figure 17 *Grey value of sound dentine measurement*

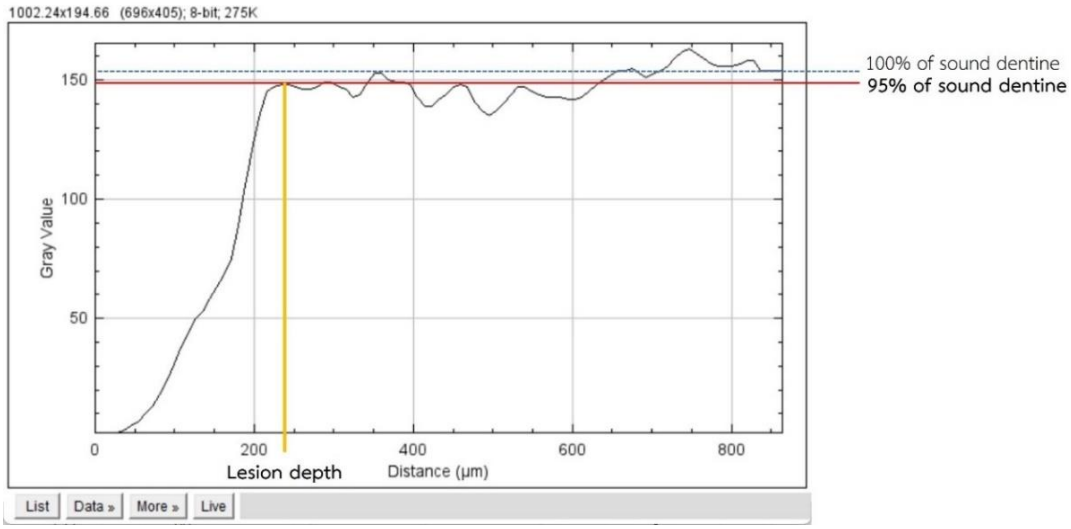

Figure 18 *Grey value curve through the depth of the lesion (µm) of the specimen*(107)

The means lesion depth for each specimen was calculated by averaging the depths of all 10 slides. The mean lesion depth at two different time, before treatment ($LD_{before\_tx.}$) and after treatment ($LD_{after\_tx.}$) were compared as the percentage change of lesion depth (%LD) as following (108):

$$\text{The percentage change of lesion depth} = \frac{(\text{Lesion depth}_{before\_tx} - \text{Lesion depth}_{after\_tx.}) \times 100}{\text{Lesion depth}_{before\_tx.}}$$

6.2 Surface microhardness by Knoop hardness test

The Knoop indentation was used with a load of 10 grams (98 μN) for 10 seconds dwell time. For the surface microhardness after treatment (the mean value of surface microhardness after the application of the treatment and subsequent microbial-pH cycling, ❸ $SMH_{after\_tx.}$), five indentations parallel tracks approximately 0.5 mm apart from the center track to the right edge, and the distance between each point is 150 μm. (Fig.19).

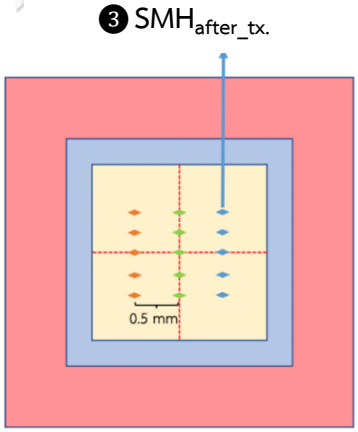

Figure 19 *An indentation track to measuring Knoop surface microhardness test after biofilm pH-cycling.*

The mean Knoop microhardness value at the three different time points (baseline, before treatment, and after treatment) were compared as the percentage surface hardness recovery (%SHR) then were calculated as following(108):

$$\text{The percentage surface hardness recovery (\%SHR)} = \frac{❸\,SMH_{after\_tx.} - ❷SMH_{before\_tx.} \times 100}{❶SMH_{baseline} - ❷SMH_{before\_tx.}}$$

6.3 Surface topography by Scanning Electron Microscopy (SEM)

After assessing the lesion depth of caries after treatment, three of fifteen Micro-CT specimens from each group were randomly selected for surface topography. The dentine specimen was removed from the block and fixed in a 2.5% glutaraldehyde solution at 4°C for 8 hours and dehydrated in a series of ethanol (30%, 50%, 70%, 90% and 100% of ethanol). The blocks were then critical-point dried, sputtered with a thin layer of gold and attached to the aluminum stubs. The surface was scanned by SEM with the magnification of 1,000X, 5,000x and 20,000X. The most representative image of dentine surface was captured.(67, 68)

Statistical analysis

1. Statistical Analysis was performed using SPSS Statistics – Version 28.0 computer software (IBM Corporation, Armonk, NY, USA). The level of statistical significance for all tests is 0.05.
2. Quantitative data was represented as means ± standard deviation. The normal distribution and assumptions of homogeneity of variance were checked by Shapiro-Wilks test and Levene's test, respectively.
3. The characteristics of the Nano silver fluoride solution including pH, Ag^{+} ion and F^{-} ion concentration was reported with mean and standard deviation.
4. The mean baseline surface microhardness, the mean lesion depth and the mean surface microhardness before treatment, the percentage change of lesion depth (%LD), and the percentage of surface hardness recovery (%SHR) were compared among groups using one-way ANOVA with Tukey's post hoc and Dunnett's post hoc multiple comparisons, respectively.
5. The mean lesion depth and surface microhardness between before treatment and after treatment in each group was compared using paired T-test.

Chapter 4

Results

Characterization of Nano-silver fluoride

In this study, Nano-silver fluoride was locally synthesized, and the silver nanoparticle solution was manufactured by Prime Nanotechnology Co., Ltd. The solution contained Ag+ and tannic acid as a stabilizer. The average particle size was 6±4 nm. Upon mixing the silver nanoparticle solution (400 ppm silver) with the sodium fluoride solution (22,600 ppm; 5% NaF), the initial yellow color immediately changed to brown. Measurements of pH, Ag+, and F- were taken to determine the characteristics of the Nano-silver fluoride solution one day after mixing. The results showed that the pH of the Nano-silver fluoride was 7.04 ± 0.03. Free silver ion and free fluoride ion concentrations are 432.49 ± 3.32 ppm and 23,423 ± 430.67 ppm, respectively.

Lesion depth evaluation by micro-CT scanning

The mean lesion depth was measured before and after treatment. The results showed that there was no statistically different of mean lesion depth before treatment between groups ($p=0.884$) as shown in Table 1. However, it was found that, after treatment, the mean lesion depth of Nano-silver fluoride group and 38% silver diamine fluoride group significantly decreased when compared with before treatment ($p<0.001$), while 5% Sodium fluoride group showed no significant difference ($p=0.380$). Additionally, it was shown that the mean lesion depth of the DI water group (negative control) increased statistically significantly ($p<0.001$) (Table 1).

The percentage change of lesion depth (%LD change) was also calculated. Nano-silver fluoride group and 38% Silver diamine fluoride group exhibited a negative %LD change, indicating a regression of the carious lesions. In contrast, the 5% Sodium fluoride group and the DI water group (negative control) showed positive values, indicating a progression of the carious lesions (Table 1).

When comparing the differences of the percentage change of lesion depth (%LD change) between groups, it was demonstrated that Nano-silver fluoride group, 38% silver diamine fluoride group, and 5% Sodium fluoride group had statistically significant differences compared to DI water group. Furthermore, there was a statistically

significant difference among the Nano-silver fluoride group, the 38% silver diamine fluoride group, and the 5% Sodium fluoride group (p<0.001). The 38% silver diamine fluoride group showed a statistically higher %LD change regression than the Nano-silver fluoride group, while the Nano-silver fluoride group showed a statistically higher %LD change regression than the 5% Sodium fluoride group. (Table 1).

Table 1 *The mean lesion depth before and after treatment* (mean ± standard deviation)

Experimental group (n = 15)	LD before treatment (μm)	LD after treatment (μm)	%LD_change
	Mean (SD)	Mean (SD)	Mean (SD)
NSF400	232.33 (22.79)[a]	213.71 (16.72)*	-7.80 (3.70)[a]
38%SDF	230.70 (26.26)[a]	200.84 (19.42)*	-12.69 (4.66)[b]
5%NaF	225.68 (25.48)[a]	229.08 (24.92)	1.66 (4.80)[c]
DI water	229.87 (19.09)[a]	245.54 (20.32)*	8.34 (3.62)[d]

Difference superscript letter indicated statistically significant differences between group at p-value < 0.05 (One-way ANOVA with Tukey's test).

Asterisk (*) indicated statistically significant within group at p-value < 0.05 (Paired T-test).

Surface microhardness change by Knoop hardness test

The results showed that there was no statistically significant difference between groups in mean baseline surface microhardness (❶) and mean surface microhardness before treatment (❷) (p=0.371 and p=0.899, respectively) (Table 2).

Furthermore, it was found that after treatment (❸), the Nano-silver fluoride group, 38% Silver diamine fluoride group, and 5% Sodium fluoride group showed a statistically significant increase in surface microhardness (p<0.001, p<0.001, and p=0.001, respectively) when compared with before treatment. Additionally, the DI water group showed a statistically significant decrease compared to before the treatment (p<0.001). (Table 2).

The percentage of surface hardness recovery (%SHR) was also calculated. The results showed that there were significant differences between groups. The Nano-silver fluoride group, the 38% silver diamine fluoride group, and the 5% Sodium

fluoride group had significantly higher %SHR compared to DI water group. Additionally, the 38% silver diamine fluoride group showed a statistically higher %SHR than Nano-silver fluoride group (p=0.023), while Nano-silver fluoride group showed a statistically higher %SHR than 5% Sodium fluoride group (p<0.001). (Table 2).

Table 2 *The surface microhardness at baseline, before treatment, after treatment, and the percentage of surface hardness recovery.* (mean ± standard deviation)

Experimental group (n = 15)	❶ SMH baseline (KHN)	❷ SMH before treatment (KHN)	❸ SMH after treatment (KHN)	%SHR
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
NSF400	58.32 (4.48)[a]	5.67 (1.67)[a]	9.50 (2.52)*	7.86 (2.62)[a]
38%SDF	56.14 (3.16)[a]	5.60 (1.31)[a]	10.89 (1.51)*	10.49 (1.82)[b]
5%NaF	58.48 (4.11)[a]	5.88 (1.60)[a]	6.75 (0.40)*	1.60 (1.52)[c]
DI water	57.58 (4.12)[a]	5.97 (1.54)[a]	5.24 (1.38)*	-1.44 (0.79)[d]

Difference superscript letter indicated statistically significant differences between group at p-value < 0.05 (One-way ANOVA with Dunnett's test).

Asterisk (*) indicated statistically significant within group at p-value < 0.05 (Paired T-test).

Surface topography by Scanning Electron Microscopy (SEM)

An observation under SEM image represented the dentine surface from each group. The dentine surface treated with Nano-silver fluoride (Group 1) exhibited an intact and smooth intertubular area, with less exposed collagen and no mineral precipitation in the dentinal tubules (Fig. 20A-C). In contrast, the dentine surface treated with 38% Silver diamine fluoride (Group 2) showed an intact intertubular area with mineral precipitation on the dentine surface and within the dentinal tubules, as well as some collagen fiber exposure (Fig. 20D-F). The 5% Sodium fluoride (Group 3) exhibited a dense but rough inter-tubular surface with more exposed collagen fibers (Fig. 20G-I). The DI water (Group 4: negative control) showed the roughest inter-tubular surface with a large amount of collagen fiber exposure and widespread dispersion (Fig. 20J-L).

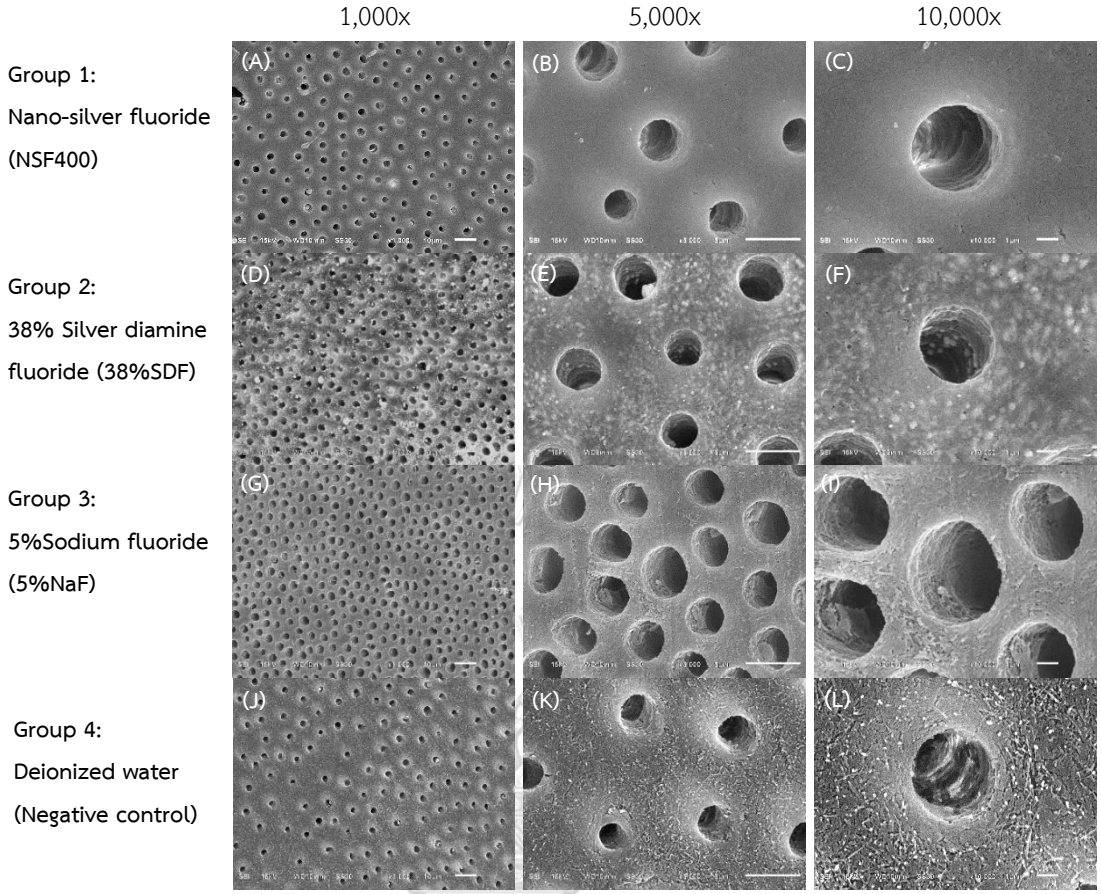

Figure 20 *representative scanning electron microscope images of the dentine surface after the entire experiment*

Chapter 5

Discussion and conclusion

This study investigated the remineralization effects of the Nano-silver fluoride solution (400 ppm silver and 22,600 ppm fluoride) compared to 38% Silver diamine fluoride and 5% Sodium fluoride varnish on artificial dentine carious lesion. The results indicated that Nano-silver fluoride (NSF400) exhibited lower remineralizing effect than 38% Silver diamine fluoride, while demonstrating a higher remineralizing effect compared to 5% NaF varnish in artificial dentine caries. This was determined through measurements of lesion depth, surface microhardness change, and surface topography using SEM scanning.

The results of this study was consistent with the studies showing that, after the treatment, Nano-silver fluoride exhibited a greater percentage of surface hardness recovery (%SHR) (62) and decrease in lesion depth (67) compared to 5% sodium fluoride varnish. This may be attributed to the presence of the combination of silver ions and also fluoride of Nano-silver fluoride. Silver nanoparticles exhibit a potent antimicrobial effect, while fluoride contributes to the reduction in demineralization and increase in remineralization. However, some study had shown that there was no significant difference in surface microhardness recovery of artificial dentine carious lesions after treatment with Nano-silver fluoride, 35% Silver diamine fluoride, and 5% sodium fluoride varnish (69), which is inconsistent with the results of this study. This might be due to the different methods that were employed. Previous study conducted a chemical pH cycling to simulate the environment in the oral cavity, without the presence of microbial factor, which might underestimate the efficacy of Nano-silver fluoride and silver diamine fluoride that have antimicrobial effects. Moreover, without removal of 5% sodium fluoride varnish before entering pH cycling, also might overestimate the remimneralizing effect of fluoride varnish. Similarly, no significant difference in lesion depth of artificial dentine caries was observed after treatment with AgNPs+2.5% sodium fluoride and 12% silver diamine fluoride(68), which is inconsistent with the results of this study. However, it should be noted that the concentration of Nano-silver fluoride and silver diamine fluoride used in the

previous study significantly differed from that of this study, which may pose challenges in comparing the experimental results.

Nano-silver fluoride substance contains silver nanoparticles and has the ability to release a significant amount of silver ions. In this study, silver nanoparticles with a size 6±4 nm were employed in conjunction with sodium fluoride at concentration of 22,600 ppm. The synthesis of silver nanoparticles can yield a wide range of sizes, depending on the synthesis method employed. The size of the synthesized silver nanoparticles has implications for their ability to effectively eradicate microbial organisms. It was shown that smaller particle sizes demonstrating greater antimicrobial efficacy.(109) It was also reported that particle size bigger than 50 nm can prevent biofilm penetration. (110)

Silver nanoparticles exhibit a more potent effect against both planktonic and colonized microbes and are capable of eradicating bacteria even at low concentrations, while also reducing the kinetics and viability of the biofilm.(111) Although the specific mechanism of silver nanoparticles' antibacterial activity has not been fully elucidated, several antibacterial actions of silver nanoparticles have been described. Silver nanoparticles have demonstrated therapeutic efficacy against various pathogens, including bacteria, fungi, and viruses (47). For *Streptococcus mutans,* the silver nanoparticle has shown the capability to enter the bacterial cell walls and induce subsequent modifications in the composition and arrangement of the cell membrane. Once silver ions enter the cell, they can denature ribosomes, inhibit protein synthesis, and deactivate respiratory enzymes in the cytoplasmic membrane, which leads to the cessation of ATP production and ultimately cell lysis. Furthermore, the interaction between silver ions and sulfur and phosphorus in DNA can result in issues with DNA replication, cell reproduction, and even the death of microorganisms.(44) Moreover, silver ions can effectively inhibit the adhesion of bacteria to tooth surfaces and the formation of biofilms through the inhibition of the enzyme glucosyltransferase.(112) For *Candida albicans,* the antifungal effectiveness of silver nanoparticles is associated with the permeabilization of the cell membrane. Silver nanoparticles can penetrate the cell wall during the exponential growth phase of fungal cells, as increased porosity of the cell wall allows molecules with low

molecular weight to pass through, which may be attributed to the interactions with negatively charged components on the fungal surface, such as phosphatidyl serine and phosphatidyl inositol, leads to destabilize the membrane barrier. In addition to increasing cell membrane permeability, high ROS destroys a number of substances, including nucleic acids, proteins, and lipids that lead to cell death. Furthermore, the disturbance of the cell cycle caused by silver nanoparticles suggests an impact on the S and G2/M phases.(113) It has been found in some study that silver ions reduced lactic acid production in the biofilm, which decreased the demineralization process on the tooth structure.(111)

Furthermore, the precipitation of silver phosphate or silver chloride, the reaction between silver ions and hydroxyapatite, which produce silver phosphate (Ag_3PO_4) and calcium fluoride (CaF_2) as the ended products. Subsequently, the silver compounds can react with chloride present in the environment, resulting in the formation of silver chloride (AgCl), which exhibits lower solubility in comparison to silver phosphate. The formation of silver chloride contributes to an increase in dentine hardness (114), which leads to the enhancement of surface microhardness recovery of artificial dentine caries treated with Nano-silver fluoride.

The enhanced remineralization effect could also be attributed to the high concentration of fluoride (more than 10,000 ppm). In this study, sodium fluoride at concentration of 22,600 ppm was incorporated with the Nano-silver particles. It was reported that fluoride ions reacted with the calcium on the dentine surface, which was an inorganic component of dentine and formed temporary CaF_2, which served as a reservoir for fluoride ions. When the biofilm had a low acidic environment, the fluoride ions were released from CaF_2 and substituted hydroxyl ions in hydroxyapatite in the dentine matrix, transforming it into fluorapatite, which has lower solubility compared to hydroxyapatite. Consequently, the hard tissue of the teeth exhibited enhanced resistance to acid and was less prone to demineralization.(115) However, despite having a high concentration of fluoride but lacking silver ions, the 5% sodium fluoride varnish group exhibits lower antimicrobial properties, resulting in lower efficacy to inhibit demineralization when compared to the group treated with Nano-silver fluoride (NSF).

Moreover, in this study, SEM images revealed that the Nano-silver fluoride and 38% Silver diamine fluoride groups exhibited the most intact and smooth inter-tubular area with less exposed collagen fiber. Additionally, only the 38% Silver diamine fluoride group showed mineral precipitation in the dentinal tubule, while the 5% Sodium fluoride group exhibited a rougher inter-tubular surface with more exposed collagen fibers. The DI water group showed the roughest inter-tubular surface with a large amount of collagen fiber exposure. This finding was consistent with the earlier study reported that the higher mineral loss was observed in the DI water group, the inter-tubular mineral content was significantly dissolved, leading to a roughened surface(67) Furthermore, the substantial dissolution of minerals resulted in an increased exposure of collagen fibers.(67) Both silver ions and fluoride ions has been shown to have a capability to protect collagen degradation. Silver ions has been demonstrated to has a capability to protect against the degradation of collagen fibers by inhibiting collagenase enzymes, such as matrix metalloproteinases and cysteine cathepsins. Fluoride also has been shown to inhibit matrix metalloproteinases, as well as the activities of cathepsin B and K.(114) Type-1 collagen is a major component of the organic matrix in dentine. The presence of intact collagen fibers acts as a scaffold that facilitates the accumulation of minerals and inhibits the diffusion of calcium and phosphate, thus preventing further demineralization.(114)

This study has several strengths. It is one of the few studies to look into the remineralization potential of Nano-silver fluoride in dentine carious lesions. The majority of studies have examined the ability of Nano-silver fluoride in enamel carious lesions. Silver ions has been shown to be more effective in treating dentine caries. The higher content of proteins, carbonates, and phosphates in dentinal tissue provides more reactive sites for silver ions. In contrast, these compounds are scarce in enamel tissue.(116) Additionally, the small size of silver nanoparticles allows for better penetration into the porous structure of dentinal tubules and their adhesion to the tubule walls, resulting in superior efficacy compared to the non-porous structure of enamel.(117)

In order to mimic the cariogenic conditions encountered in the oral cavity and due to the predominant antimicrobial effect of Nano-silver fluoride, a dual-species microbial pH-cycling models (102) was conducted in this study. The microbial pH-cycling models are more likely to be resemble the conditions in the oral cavity compared to the use of chemical pH-cycling, as they incorporate microbiological factors, such as the breakdown of dentine's organic matrix by enzymes derived from bacteria and the metabolism of sugars. In this study, dual-species biofilms of *Streptococcus mutans* and *Candida albicans* were used to mimic the conditions in the oral cavity. In recent studies, It has been demonstrated that the combination of *Streptococcus mutans* and *Candida albicans* was found in ECC (Early Childhood Caries) patients(30) The presence of *Candida albicans*, in combination with *Streptococcus mutans*, led to the synergistic enhancement in biofilm virulence.(30) Additionally, it was also reported that the dual-species of *Streptococcus mutans* and *Candida albicans* biofilm provoked higher surface hardness loss on dentine surface, which represented demineralization than in the *Streptococcus mutans* or *Candida albicans* biofilm alone.(32) In this cycling, the demineralization process was conducted for 4 hours using a sugar-containing medium to facilitate microbial metabolism and increased acid production. To confirm the acidic conditions necessary for dentine mineral loss, After 4 hours of incubation, the pH of culture medium was measured to be lower than the critical pH for dentine (pH=6.2-6.5).(103)

However, the limitations of this study were that it was conducted in a laboratory setting, which might not simulate the actual oral conditions, and cannot be directly extrapolated to clinical outcomes inpatients. The dentine carious lesions used in this study also was artificially created by immersing the specimens in a demineralization solution, resulting in the development of carious lesions that resemble affected dentine rather than natural or infected dentine due to their lack of mineral content or a collagen network.(98) Moreover, Nano-silver fluoride solutions used in this study was freshly prepared every time before using. Therefore, the stability of the substance has not been studied. Further study should investigate the stability and shelf-life of the substance to determine its efficacy. Additionally, there are variations of the silver nanoparticle used in each study, including particle size, concentration,

pH, optical properties, and dielectric constant, which might have the potential to induce diverse effects, both in terms of mechanical and physicochemical properties, on demineralized dentine lesions. Hence, further investigations should be conducted in the future.

Nano-silver fluoride is an economical and non-invasive agent that demonstrates an enhanced safety profile in both children and adults.(118) The application of NSF effectively inhibits demineralization and promotes the remineralization of dentin carious lesions, without causing black staining on the teeth. This contributes to improve the aesthetic acceptance among patients and parents. Moreover, Nano-silver fluoride can be suitable not only for young children in the control of dental caries but also for adult patients, particularly those with root caries. Nevertheless, further development of Nano-silver fluoride is necessary to optimize its efficacy, ensure long-term stability, and facilitate easy clinical application.

Conclusion

Nano-silver fluoride provided remineralization effects on artificial dentine caries, demonstrating superior efficacy compared to 5% Sodium fluoride varnish but not as effective as 38% Silver diamine fluoride

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Appendix

Appendix A

Study protocol and consent form approval

No. 043/2022

Study Protocol and Consent Form Approval
Certificate of Exemption

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and patient/participant information sheet dated and/or amended as follows in compliance with the **ICH/GCP with exemption**

Study Title	:	The effect of nano-silver fluoride in remineralization on artificial dentine caries: An *in vitro* study
Study Code	:	HREC-DCU **2022 - 041**
Study Center	:	Chulalongkorn University
Principle Investigator	:	Ms. Peeraya Punpeng
Protocol Date	:	April 22, 2022
Date of Approval	:	May 6, 2022
Date of Expiration	:	May 5, 2024

(Assistant Professor Dr. Kanokporn Bhalang)
Chairman of Ethics Committee

(Professor Dr. Thanaphum Osathanon)
Associate Dean for Research

*A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached (upon requested). This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Approval is granted subject to the following conditions: (see back of the approval)

Appendix B

Biosafety certificate

99-FM-34-15 ฉบับที่ 01 วันที่บังคับใช้ 15 พ.ย. 2564 หน้าที่ 1/1
CU-IBC10

Faculty of Dentistry
Chulalongkorn University
Institutional Biosafety Committee

Certificate of Approval

Approval No.	: DENT CU-IBC 014/2022
Project title	: THE EFFECT OF SILVER COMPOUNDS IN REMINERALIZATION ON ARTIFICIAL DENTINE CARIES: AN *IN VITRO STUDY*
Subproject title	: THE EFFECT OF NANO-SILVER FLUORIDE IN REMINERALIZATION ON ARTIFICIAL DENTINE CARIES: AN *IN VITRO STUDY*
Principal investigator of the project	: Assistant Professor Nattanan Govitvattana, DDS, Ph.D.
Principal Investigator of the subproject	: Assistant Professor Nattanan Govitvattana, DDS, Ph.D. Associate Professor Panida Thanyasrisung, DDS, Ph.D.
Affiliation	: Department of Pediatric Dentistry
Risk group	:

Pathogen ☐ Risk group 1 ☑ Risk group 2 ☐ Risk group 3 ☐ Risk group 4

Animal toxin ☐ Risk group 1 ☐ Risk group 2 ☐ Risk group 3

Other..... Risk group/LD_{50}.....

Biocontainment level :

☐ Biosafety level 1 ☑ Biosafety level 2 ☐ Biosafety level 2 enhanced ☐ Biosafety level 3 ☐ Biosafety level 4

This project has been reviewed and approved by CU-IBC in accordance with the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016).

The official signing to certify that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016).

The approval is subjected to assurance given in the levels of risk in pathogens and animal toxins list in the Risk Group of Pathogen (2018) and Animal Toxin (2019) published by Department of Medical Sciences (Ministry of Public Health), the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology BIOTEC (2016) and may be required for future investigations and reviews.

If there are any changes in information, please notify CU-IBC.

Effective date: July 08, 2022 **Expiration date:** June 30, 2023

Signature .....
(Assistant Professor Lertrit Sarinnaphakorn, DDS, M.Sc. Dent, Ph.D.)
DENT CU-IBC Chair

Appendix C

Informed consent form for tooth donation.

79-FM-32-10 วันที่บังคับใช้ 6 ก.ค. 2564 หน้าที่ 1/1

เอกสารยินยอมมอบ ฟัน/เนื้อเยื่อ/ภาพรังสี/ข้อมูล/หรือสิ่งอื่นๆ เพื่อใช้ในการทำวิจัย

การวิจัยเรื่อง ผลของนาโนซิลเวอร์ฟลูออไรด์ต่อการคืนกลับแร่ธาตุบนรอยผุจำลองในชั้นเนื้อฟัน: การศึกษาในห้องปฏิบัติการ

ผู้วิจัยหลัก ทันตแพทย์หญิงพีรญาณ์ พันธุ์เพ็ง

ก่อนที่จะลงนามในเอกสารยินยอมนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย รวมถึงประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว ข้าพเจ้าขอมอบ

- ☑ ฟันที่ได้รับความยินยอมจากผู้ป่วยให้ถอนออกเนื่องจากเหตุผลทางการแพทย์
- ☐ เนื้อเยื่อที่ได้รับความยินยอมจากผู้ป่วยให้ตัดออกเนื่องจากเหตุผลทางการแพทย์
- ☐ ภาพรังสี
- ☐ ข้อมูล (โปรดระบุ) .....
- ☐ สิ่งอื่นๆ (โปรดระบุ) .....

จำนวนเท่าที่ผู้วิจัยขอมา ที่อยู่ในความดูแล/ครอบครองของข้าพเจ้า เพื่อนำไปใช้ในการวิจัยดังกล่าว

ลงนาม.....ผู้ยินยอม

(ทันตแพทย์อนันต์ พีระนันท์รังษี)
หัวหน้ากลุ่มงานทันตกรรม โรงพยาบาลพิบูลมังสาหาร
วันที่ 7 เมษายน 2565

ที่อยู่ที่สามารถติดต่อได้
กลุ่มงานทันตกรรม โรงพยาบาลพิบูลมังสาหาร
20/6 เทศบาล 2 ตำบลพิบูล อำเภอพิบูลมังสาหาร อุบลราชธานี 34110

เริ่มใช้ กรกฎาคม 2564 เอกสารถูกจัดทำครั้งที่ 1 วันที่จัดทำ 7 เมษายน 2565

Appendix D

Statistical analysis

The normality test of mean lesion depth before and after treatment, and the percentage lesion depth change. (Shapiro-Wilk test)

Tests of Normality

	1=NSF400, 2=SDF, 3=5% NaF, 4=DI	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
LD_before	1	.136	15	.200*	.976	15	.934
	2	.189	15	.153	.882	15	.051
	3	.179	15	.200*	.933	15	.300
	4	.145	15	.200*	.949	15	.504
LD_after	1	.146	15	.200*	.978	15	.953
	2	.163	15	.200*	.923	15	.210
	3	.127	15	.200*	.956	15	.623
	4	.118	15	.200*	.954	15	.588
PercentLD_change	1	.266	15	.006	.892	15	.072
	2	.164	15	.200*	.972	15	.890
	3	.180	15	.200*	.920	15	.195
	4	.132	15	.200*	.960	15	.701

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

The normality test of surface microhardness baseline, before and after treatment, and the percentage of surface hardness recovery. (Shapiro-Wilk test)

Tests of Normality

	1=NSF400, 2=SDF, 3=5% NaF, 4=DI	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
KHN_baseline	1	.129	15	.200*	.944	15	.434
	2	.198	15	.119	.915	15	.160
	3	.156	15	.200*	.904	15	.111
	4	.183	15	.188	.948	15	.497
KHN_before	1	.141	15	.200*	.969	15	.845
	2	.161	15	.200*	.901	15	.100
	3	.151	15	.200*	.941	15	.402
	4	.161	15	.200*	.965	15	.786
KHN_after	1	.111	15	.200*	.953	15	.576
	2	.117	15	.200*	.972	15	.882
	3	.195	15	.130	.899	15	.091
	4	.169	15	.200*	.953	15	.581
SHR	1	.152	15	.200*	.906	15	.119
	2	.144	15	.200*	.944	15	.434
	3	.138	15	.200*	.964	15	.767
	4	.154	15	.200*	.901	15	.099

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

The comparison of the mean lesion depth before treatment in each group (One-way ANOVA with Tukey's post hoc test)

Descriptives

LD_before

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	15	232.3309	22.79048	5.88448	219.7099	244.9518	188.10	274.87
2	15	230.7039	26.26330	6.78116	216.1598	245.2481	200.10	286.43
3	15	225.6794	25.48251	6.57956	211.5677	239.7912	184.07	263.83
4	15	229.8653	19.09333	4.92988	219.2918	240.4389	202.20	272.40
Total	60	229.6449	23.10065	2.98228	223.6774	235.6124	184.07	286.43

1=NSF400 group, 2=38%SDF group, 3=5%NaF group, 4=DI (Control) group

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
LD_before	Based on Mean	.799	3	56	.500
	Based on Median	.531	3	56	.663
	Based on Median and with adjusted df	.531	3	46.838	.663
	Based on trimmed mean	.762	3	56	.520

ANOVA

LD_before

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	361.645	3	120.548	.217	.884
Within Groups	31123.130	56	555.770		
Total	31484.775	59			

The comparison of the mean lesion depth before and after treatment in each group (Paired t-test)

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	NSF400_LD_bf	232.3309	15	22.79048	5.88448
	NSF400_LD_af	213.7067	15	16.71507	4.31581
Pair 2	SDF_LD_bf	230.7039	15	26.26330	6.78116
	SDF_LD_af	200.8444	15	19.41884	5.01392
Pair 3	NaF_bf	225.6794	15	25.48251	6.57956
	NaF_af	229.0844	15	24.91881	6.43401
Pair 4	DI_bf	229.8653	15	19.09333	4.92988
	DI_af	245.5356	15	20.32096	5.24685

Paired Samples Test

		Paired Differences					t	df	Significance	
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			One-Sided p	Two-Sided p
Pair 1	NSF400_LD_bf - NSF400_LD_af	18.62422	10.44211	2.69614	12.84158	24.40687	6.908	14	<.001	<.001
Pair 2	SDF_LD_bf - SDF_LD_af	29.85950	13.17935	3.40289	22.56102	37.15798	8.775	14	<.001	<.001
Pair 3	NaF_bf - NaF_af	-3.40501	10.98806	2.83710	-9.49000	2.67997	-1.200	14	.125	.250
Pair 4	DI_bf - DI_af	-15.67022	10.86635	2.80568	-21.68781	-9.65264	-5.585	14	<.001	<.001

The comparison of the differences in the percentage change of lesion depth among all experimental groups (One-way ANOVA with Tukey's post hoc test)

Descriptives

PercentLD_change

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	15	-7.7977	3.70420	.95642	-9.8490	-5.7463	-16.29	-2.01
2	15	-12.6940	4.66338	1.20408	-15.2764	-10.1115	-21.34	-3.30
3	15	1.6599	4.80039	1.23945	-.9984	4.3183	-4.87	9.03
4	15	8.3387	3.62140	.93504	6.3332	10.3442	3.02	16.32
Total	60	-2.6232	9.20880	1.18885	-5.0021	-.2444	-21.34	16.32

1=NSF400 group, 2=38%SDF group, 3=5%NaF group, 4=DI (Control) group

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
PercentLD_change	Based on Mean	1.189	3	56	.322
	Based on Median	.981	3	56	.408
	Based on Median and with adjusted df	.981	3	52.936	.409
	Based on trimmed mean	1.223	3	56	.310

ANOVA

PercentLD_change

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4000.549	3	1333.516	74.471	<.001
Within Groups	1002.771	56	17.907		
Total	5003.320	59			

Multiple Comparisons

Dependent Variable: PercentLD_change

	(I) 1=NSF400, 2=SDF, 3=5%NaF, 4=DI	(J) 1=NSF400, 2=SDF, 3=5%NaF, 4=DI	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1	2	4.89630*	1.54517	.013	.8049	8.9877
		3	-9.45759*	1.54517	<.001	-13.5490	-5.3662
		4	-16.13634*	1.54517	<.001	-20.2278	-12.0449
	2	1	-4.89630*	1.54517	.013	-8.9877	-.8049
		3	-14.35389*	1.54517	<.001	-18.4453	-10.2625
		4	-21.03264*	1.54517	<.001	-25.1241	-16.9412
	3	1	9.45759*	1.54517	<.001	5.3662	13.5490
		2	14.35389*	1.54517	<.001	10.2625	18.4453
		4	-6.67875*	1.54517	<.001	-10.7702	-2.5873
	4	1	16.13634*	1.54517	<.001	12.0449	20.2278
		2	21.03264*	1.54517	<.001	16.9412	25.1241
		3	6.67875*	1.54517	<.001	2.5873	10.7702
Dunnett T3	1	2	4.89630*	1.53771	.021	.5478	9.2448
		3	-9.45759*	1.56556	<.001	-13.8888	-5.0263
		4	-16.13634*	1.33755	<.001	-19.9055	-12.3672
	2	1	-4.89630*	1.53771	.021	-9.2448	-.5478
		3	-14.35389*	1.72802	<.001	-19.2235	-9.4843
		4	-21.03264*	1.52450	<.001	-25.3468	-16.7185
	3	1	9.45759*	1.56556	<.001	5.0263	13.8888
		2	14.35389*	1.72802	<.001	9.4843	19.2235
		4	-6.67875*	1.55260	.001	-11.0767	-2.2808
	4	1	16.13634*	1.33755	<.001	12.3672	19.9055
		2	21.03264*	1.52450	<.001	16.7185	25.3468
		3	6.67875*	1.55260	.001	2.2808	11.0767

*. The mean difference is significant at the 0.05 level.

The comparison of the mean surface microhardness before and after treatment in each group (Paired t-test)

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	NSF400_HN_bf	5.6679	15	1.66661	.43032
	NSF400_HN_af	9.4988	15	2.51859	.65030
Pair 2	SDF_HN_bf	5.6015	15	1.30572	.33713
	SDF_HN_af	10.8907	15	1.51205	.39041
Pair 3	NaF_HN_bf	5.8792	15	1.60294	.41388
	NaF_HN_af	6.7445	15	1.51460	.39107
Pair 4	DI_HN_bf	5.9745	15	1.53763	.39702
	DI_HN_af	5.2448	15	1.38377	.35729

Paired Samples Test

		Paired Differences					t	df	Significance	
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			One-Sided p	Two-Sided p
Pair 1	NSF400_HN_bf - NSF400_HN_af	-3.83093	1.42084	.36686	-4.61777	-3.04410	-10.442	14	<.001	<.001
Pair 2	SDF_HN_bf - SDF_HN_af	-5.28920	.92766	.23952	-5.80292	-4.77548	-22.082	14	<.001	<.001
Pair 3	NaF_HN_bf - NaF_HN_af	-.86533	.81779	.21115	-1.31821	-.41245	-4.098	14	<.001	.001
Pair 4	DI_HN_bf - DI_HN_af	.72973	.39426	.10180	.51140	.94807	7.168	14	<.001	<.001

The comparison of the differences in percentage surface hardness recovery (%SHR) among all experimental groups (One-way ANOVA with Dunnett's post hoc test)

Descriptives

SHR

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	15	7.8666	2.62663	.67819	6.4120	9.3212	4.86	12.63
2	15	10.4874	1.81775	.46934	9.4807	11.4940	7.32	12.95
3	15	1.5966	1.52182	.39293	.7539	2.4394	-1.51	3.95
4	15	-1.4415	.78595	.20293	-1.8767	-1.0063	-2.57	-.43
Total	60	4.6273	5.12000	.66099	3.3046	5.9499	-2.57	12.95

1=NSF400 group, 2=5%NaF group, 3=DI (Control) group

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
SHR	Based on Mean	6.744	3	56	<.001
	Based on Median	5.023	3	56	.004
	Based on Median and with adjusted df	5.023	3	34.050	.005
	Based on trimmed mean	6.592	3	56	<.001

ANOVA

SHR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1362.732	3	454.244	138.309	<.001
Within Groups	183.919	56	3.284		
Total	1546.651	59			

Multiple Comparisons

Dependent Variable: SHR

	(I) 1=NSF400, 2=SDF, 3=5%NaF, 4=DI	(J) 1=NSF400, 2=SDF, 3=5%NaF, 4=DI	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1	2	-2.62078*	.66174	.001	-4.3730	-.8686
		3	6.26996*	.66174	<.001	4.5177	8.0222
		4	9.30809*	.66174	<.001	7.5559	11.0603
	2	1	2.62078*	.66174	.001	.8686	4.3730
		3	8.89075*	.66174	<.001	7.1385	10.6430
		4	11.92887*	.66174	<.001	10.1767	13.6811
	3	1	-6.26996*	.66174	<.001	-8.0222	-4.5177
		2	-8.89075*	.66174	<.001	-10.6430	-7.1385
		4	3.03813*	.66174	<.001	1.2859	4.7903
	4	1	-9.30809*	.66174	<.001	-11.0603	-7.5559
		2	-11.92887*	.66174	<.001	-13.6811	-10.1767
		3	-3.03813*	.66174	<.001	-4.7903	-1.2859
Dunnett T3	1	2	-2.62078*	.82476	.023	-4.9650	-.2766
		3	6.26996*	.78380	<.001	4.0230	8.5169
		4	9.30809*	.70790	<.001	7.2136	11.4025
	2	1	2.62078*	.82476	.023	.2766	4.9650
		3	8.89075*	.61211	<.001	7.1622	10.6193
		4	11.92887*	.51133	<.001	10.4401	13.4176
	3	1	-6.26996*	.78380	<.001	-8.5169	-4.0230
		2	-8.89075*	.61211	<.001	-10.6193	-7.1622
		4	3.03813*	.44224	<.001	1.7626	4.3137
	4	1	-9.30809*	.70790	<.001	-11.4025	-7.2136
		2	-11.92887*	.51133	<.001	-13.4176	-10.4401
		3	-3.03813*	.44224	<.001	-4.3137	-1.7626

*. The mean difference is significant at the 0.05 level.

Appendix E

The mean lesion depth of Nano-silver fluoride group (n=15)

Group	Before_tx (μm)	After_tx (μm)	%LD_change
1_(NSF400)	225.63	210.90	-6.53
1	264.83	221.70	-16.29
1	259.33	221.70	-14.51
1	230.27	213.00	-7.50
1	274.87	245.40	-10.72
1	219.77	198.60	-9.63
1	207.43	192.00	-7.44
1	235.33	218.40	-7.20
1	240.00	226.20	-5.75
1	228.60	215.70	-5.64
1	209.10	204.90	-2.01
1	246.90	236.10	-4.37
1	188.10	177.70	-5.53
1	225.00	210.00	-6.67
1	229.80	213.30	-7.18

The mean surface microhardness test of Nano-silver fluoride group (n=15)

Group	Baseline (KHN)	Before_tx (KHN)	After_tx (KHN)	%SHR
1_(NSF400)	66.06	5.26	9.19	6.45
1	54.46	7.00	11.48	9.44
1	51.59	6.96	9.34	5.33
1	52.66	4.22	10.34	12.63
1	64.96	6.97	13.66	11.53
1	53.98	8.52	13.87	11.77
1	54.97	7.83	12.15	9.15
1	58.93	5.40	8.00	4.86
1	57.61	6.43	10.05	7.08
1	57.65	4.20	7.12	5.48
1	58.26	3.57	6.40	5.17
1	59.83	6.33	10.61	8.01
1	57.91	2.71	7.68	8.99
1	61.02	5.41	9.35	7.09
1	64.89	4.20	7.24	5.01

The mean lesion depth of 38% Silver diamine fluoride group (n=15)

Group	Before_tx (μm)	After_tx (μm)	%LD_change
2_(38%SDF)	224.77	192.07	-14.55
2	278.96	234.00	-16.12
2	259.73	204.30	-21.34
2	214.13	188.70	-11.88
2	286.43	243.30	-15.06
2	219.43	189.90	-13.46
2	205.77	179.70	-12.67
2	238.83	218.70	-8.43
2	200.10	174.60	-12.74
2	211.50	186.30	-11.91
2	220.90	213.60	-3.30
2	208.80	188.40	-9.77
2	215.70	202.50	-6.12
2	232.80	201.60	-13.40
2	242.70	195.00	-19.65

The mean surface microhardness test of 38% Silver diamine fluoride group (n=15)

Group	Baseline (KHN)	Before_tx (KHN)	After_tx (KHN)	%SHR
2_(38%SDF)	53.76	4.23	9.77	11.19
2	54.03	6.68	12.74	12.80
2	57.63	5.04	10.27	9.94
2	55.76	6.03	9.67	7.32
2	60.36	5.21	11.85	12.04
2	54.80	4.22	10.77	12.95
2	55.22	7.53	12.76	10.98
2	53.91	7.67	11.76	8.84
2	52.65	4.92	9.00	8.56
2	62.50	4.37	10.52	10.58
2	56.68	3.99	8.35	8.26
2	61.54	4.35	9.43	8.89
2	51.75	6.59	12.11	12.21
2	55.66	7.37	13.44	12.56
2	55.90	5.82	10.92	10.18

The mean lesion depth of 5% sodium fluoride group (n=15)

Group	Before_tx (μm)	After_tx (μm)	%LD_change
3_(5%NaF)	215.35	230.13	6.86
3	263.83	263.43	-.15
3	187.00	191.70	2.51
3	245.24	239.70	-2.26
3	207.20	197.10	-4.87
3	255.87	245.40	-4.09
3	249.60	239.40	-4.09
3	222.97	220.50	-1.11
3	184.07	191.70	4.15
3	208.67	220.20	5.53
3	211.80	226.20	6.80
3	252.90	273.90	8.30
3	225.90	246.30	9.03
3	248.10	244.20	-1.57
3	206.70	206.40	-.15

The mean surface microhardness test of 5% sodium fluoride group (n=15)

Group	Baseline (KHN)	Before_tx (KHN)	After_tx (KHN)	%SHR
3_(5%NaF)	58.41	6.13	7.11	1.87
3	56.96	7.73	8.12	.78
3	55.12	7.06	7.34	.58
3	64.54	4.89	6.55	2.79
3	53.15	6.74	8.52	3.83
3	62.41	7.07	7.27	.37
3	52.62	7.43	8.19	1.68
3	52.67	8.33	7.66	-1.51
3	62.36	3.82	6.13	3.95
3	60.95	5.05	6.15	1.96
3	53.97	4.23	4.98	1.52
3	59.64	6.65	7.35	1.32
3	62.28	6.03	8.03	3.56
3	62.39	3.76	4.63	1.50
3	59.66	3.28	3.13	-.26

The mean lesion depth of DI water (Control) group (n=15)

Group	Before_tx (μm)	After_tx (μm)	%LDchange
4_(DI)	226.39	257.10	13.57
4	211.93	234.20	10.51
4	246.43	272.93	10.75
4	240.33	264.60	10.10
4	228.50	240.60	5.30
4	232.67	252.90	8.70
4	211.40	230.70	9.13
4	245.70	260.10	5.86
4	245.70	241.20	6.10
4	227.33	221.70	7.88
4	205.50	233.10	16.32
4	213.00	220.50	3.52
4	202.20	219.30	8.46
4	238.50	245.70	3.02
4	272.40	288.40	5.87

The mean surface microhardness test of DI water (Control) group (n=15)

Group	Baseline (KHN)	Before_tx (KHN)	After_tx (KHN)	%SHR
4_(DI)	65.67	4.58	4.13	-.74
4	52.79	6.68	6.06	-1.34
4	60.69	4.97	4.56	-.73
4	51.17	6.98	6.29	-1.57
4	54.62	9.38	8.49	-1.98
4	54.52	6.47	5.24	-2.56
4	55.74	7.94	6.84	-2.30
4	56.00	7.55	6.52	-2.13
4	59.51	5.47	4.90	-1.06
4	61.83	5.91	4.48	-2.57
4	61.14	4.94	3.72	-2.17
4	61.47	3.48	3.23	-.43
4	53.68	5.28	5.01	-.56
4	59.69	5.47	5.20	-.49
4	55.24	4.51	4.01	-.99

VITA

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