The effect of silver nanoparticles in addition to sodium fluoride on remineralization of artificial root dentin caries

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ผลของอนุภาคซิลเว่อร์นาโนร่วมกับฟลูออไรด์ต่อการคืนกลับแร่ธาตุของรอยโรคฟันผุจำลองในเนื้อฟันส่วนรากฟัน

น.ส.พัชรา กองยศสืบ

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พัชรา กองยศสืบ : ผลของอนุภาคซิลเว่อร์นาโนร่วมกับฟลูออไรด์ต่อการคืนกลับแร่ธาตุของรอยโรคฟันผุจำลองในเนื้อฟันส่วนรากฟัน. (The effect of silver nanoparticles in addition to sodium fluoride on remineralization of artificial root dentin caries) อ.ที่ปรึกษาหลัก : อ.ทพญ. ดร.ญาณี ตันติเลิศอนันต์, อ.ที่ปรึกษาร่วม : ผศ. ทพ.ดร.สุชิต พูลทอง

การศึกษานี้เพื่อประเมินประสิทธิภาพของการคืนกลับแร่ธาตุของสารละลายซิลเวอร์นาโนร่วมกับโซเดียมฟลูออไรด์เปรียบเทียบกับสารกระตุ้นการคืนกลับแร่ธาตุชนิดอื่นบนรอยผุจำลองของรากฟัน เป็นการศึกษาทางห้องปฏิบัติการ โดยทำการตัดชิ้นส่วนของรากฟันให้มีขนาด 5x5 ตารางมิลลิเมตร จากฟันที่ถูกถอนของผู้สูงอายุที่มีอายุมากกว่าหรือเท่ากับ 60 ปีขึ้นไป หลังจากนั้นนำชิ้นงานไปสร้างรอยผุจำลองด้วยการแช่ในสารละลายแร่ธาตุ ชิ้นงานทั้งหมดจะถูกแบ่งกลุ่มแบบสุ่มเป็น 5 กลุ่มตามชนิดของสารกระตุ้นการคืนกลับแร่ธาตุ ได้แก่ สารซิลเวอร์ไดอะมีนฟลูออไรด์, สารละลายซิลเวอร์นาโน, สารละลายซิลเวอร์นาโนร่วมกับโซเดียมฟลูออไรด์วานิช, ฟลูออไรด์วานิชและน้ำประปา หลังจากนั้นจะถูกนำเข้าสู่กระบวนการจำลองสภาวะความเป็นกรดด่าง และประเมินผลด้วยการวัดค่าความแข็งผิวระดับจุลภาคแบบนูปด้วยเครื่องทดสอบความแข็งผิวระดับจุลภาค และความลึกรอยโรคด้วยเครื่องไมโครซีที วิเคราะห์ผลทางสถิติโดยเปรียบเทียบค่าเฉลี่ยของค่าความแข็งผิวระดับจุลภาคแต่ละกลุ่มด้วยการวิเคราะห์ความแปรปรวนทางเดียว และทดสอบความแตกต่างระหว่างกลุ่มด้วยการเปรียบเทียบเชิงซ้อนชนิดทูกีย์ที่ระดับนัยสำคัญ0.05 จากการทดลองพบว่าทุกกลุ่มของสารทดสอบมีค่าความแข็งผิวระดับจุลภาคสูงกว่าและค่าความลึกของรอยโรคต่ำกว่ากลุ่มควบคุม ค่าความแข็งผิวระดับจุลภาคแบบนูปและค่าความลึกของรอยโรคในกลุ่มซิลเวอร์นาโนร่วมกับโซเดียมฟลูออไรด์ และกลุ่มโซเดียมฟลูออไรด์ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติและมีประสิทธิภาพที่น้อยกว่าสารซิลเวอร์ไดอะมีนฟลูออไรด์ ภายใต้ข้อจำกัดของการศึกษานี้สรุปได้ว่าการใช้สารละลายซิลเวอร์นาโนกับโซเดียมฟลูออไรด์ให้ผลในการคืนกลับแร่ธาตุบนรอยผุจำลองบนรากฟันได้ด้อยกว่าการใช้สารซิลเวอร์ไดอะมีนฟลูออไรด์

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ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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The aim of this in vitro study was to evaluate the remineralization effect of the silver nanoparticles adjunctively to sodium fluoride varnish compared with other remineralizing agents on artificial root caries. Fifty-five root dentin slices size 5x5 mm were sectioned from extracted human teeth of patients aged 60 years old or more. All specimens were immersed in demineralized solution to create artificial caries. The specimens were randomly allocate into five groups according to the remineralizing agents: silver diamine fluoride, silver nanoparticles solution, silver nanoparticle solution followed by the application of sodium fluoride varnish, sodium fluoride varnish and tap water. After 8 days of pH cycling challenge, the microhardness test and lesion depth evaluation were performed. Data were analyzed using F-test One-way ANOVA following by Tukey's post hoc test and paired T-test. All test groups demonstrated a significantly higher microhardness value and lower lesion depth compared to control group. Despite, no significant difference in lesion depth and microhardness, both silver nanoparticle solution additionally to sodium fluoride varnish and sodium fluoride varnish showed lower efficacy to silver diamine fluoride. Based on the finding of this in vitro study, either silver nanoparticle or sodium fluoride varnish solely remineralize such artificial root caries lesions, yet inferior to SDF.

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Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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Padchara Kongyodsueb

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Background and rationale

The proportion of aging people worldwide increases rapidly compared to any other age groups. The number of aging people (60 years old and above) is projected to double in 2015-2050.[1] Population aging is rising because people are living longer that reflects the success of health promotion policies. This demographic change has had big impact for public health policy to be developed accordingly. Oral health policy therefore focuses on lifelong service of teeth of aging population.

Dental caries has been a major problem that affects people through human lifetime. It can causes pain, discomfort, disfigurement and involves in systemic health problems.[2] Root caries is also a common oral disease that occurs in elderly patients due to increased tooth retention and long term exposed root surfaces.[3] Due to the increase number of older people nowadays, root caries is becoming more significant dental problems. The common location of root caries occurs in supragingival next to cemento-enamel junction and adjacent to the crest of gingiva where dental plaques accumulate especially on proximal surfaces.[4] These surfaces are more susceptible to dental biofilm accumulation and it is difficult to remove these biofilms for elderlies due to limited manual cleaning skill. Similar to coronal caries, the presence of cariogenic biofilms and fermentable carbohydrate are main etiology for the development of root caries.[5] The demineralization process of root caries is more rapid than coronal caries. That is because of their different structures.[6]

The traditional treatment of cavitated root caries includes surgical removal of the carious dental tissue, followed by placement of suitable restorative material. There are many materials that have been used to treat root-surface caries such as amalgam, resin composite, glass ionomer cement and other fluoride releasing materials.[5] Beside, root caries can be arrested by fluoride or

chemical treatment in the same way as enamel caries. Thus, several approaches in minimal invasive dentistry have been proposed to treat root caries lesions. Some chemical agents were showed to prevent new root caries lesion and/or to reverse active lesion into inactive one.[7]

Silver compounds have a long history of their usages in medicine because of their antimicrobial properties. Their mechanism is the release of ionic silver that can cause a structural change in bacterial cell membrane and result in cell death.[8] In dentistry, the first silver compounds were introduced in the 1840s and caries was arrested by silver nitrate ($AgNO_3$) .[9] Although the numerous investigations of silver nitrate medication have been proposed, the limitations of silver nitrate are irritating pulp tissue and discoloring the tooth.[10]

Sodium fluoride is one of most common topical fluoride products that use for preventing caries lesion. Fluoride can react with hydroxyapatite and form fluoroapatite or fluoridated hydroxyapatite. Fluoride based chemotherapeutic agents were developed for preventing and arresting dental caries.[11]

Silver diamine fluoride (SDF) is basically the compound of silver, fluoride, and ammonium. It was introduced as a therapeutic agent in Japan since 1960s and has been used for caries management.[12] Many studies demonstrated that SDF is very effective in arresting dental caries in primary teeth and permanent teeth.[13, 14] However, the dark staining of tooth is the most frequently reported side effect of SDF.[15, 16] Almarwan et al., reported most parents accepted SDF staining on their child's primary teeth rather than permanent teeth as well as staining of their posterior teeth compared to anterior teeth.[17] In order to overcome this side effect of SDF, the use of potassium iodide followed by SDF can reduce the dark staining of teeth however the stain can recover overtime.[18, 19]

The silver nanoparticle is one of the potent antimicrobial agents with low toxicity.[20] Researchers have recently explored the corporation of silver nanoparticles and fluoride to arrest dental caries and promote remineralization.[21] They suggested that silver nanoparticles are effective in arresting dental caries without tooth staining and it can be considered as a suitable alternative to SDF.[22-24] However, these studies focused on primary teeth and coronal tooth structures. Different formulation of silver nanoparticles and fluoride solution also have an impact on the effectiveness of the agents. Thus, the present study is designed to investigate the ability of silver nanoparticles solution coated with sodium fluoride varnish whether they can arrest dental caries in elderly root dentin or not.

Research Question

What are the differences of remineralization effect of silver nanoparticles solution, silver nanoparticles solution in addition to sodium fluoride varnish, sodium fluoride and silver diamine fluoride on elderly root caries?

Research Objective

The objective of this study is to evaluate the remineralization effect of the silver nanoparticles solution in addition to sodium fluoride varnish compared with silver diamine fluoride on elderly artificial root caries lesions.

Type of research

Experimental study

Proposed benefits

This study will clarify the effect of silver nanoparticles solution with sodium fluoride on remineralization of root caries lesions. This result will confirm the effectiveness of silver nanoparticles solution with sodium fluoride in remineralization of root dentine caries. It can be used as an

alternative agent for silver diamine fluoride and further develop the effectiveness remineralizing agent without tooth staining.

Limitation

This experimental design limits to in vitro simulated environment. The result might not be inferred to the real clinical situation. This study investigates the effect of silver nanoparticles solution with sodium fluoride on dentine remineralization compared with silver diamine fluoride, thus the result from this study may not be inferred to other remineralizing agents.

Statistic Hypotheses

- The null hypothesis: There is no significant difference in the remineralization effect on elderly root caries in various remineralization materials.
- The alternative hypothesis: There is significant difference in the remineralization effect on elderly root caries in various remineralization materials.

Conceptual framework

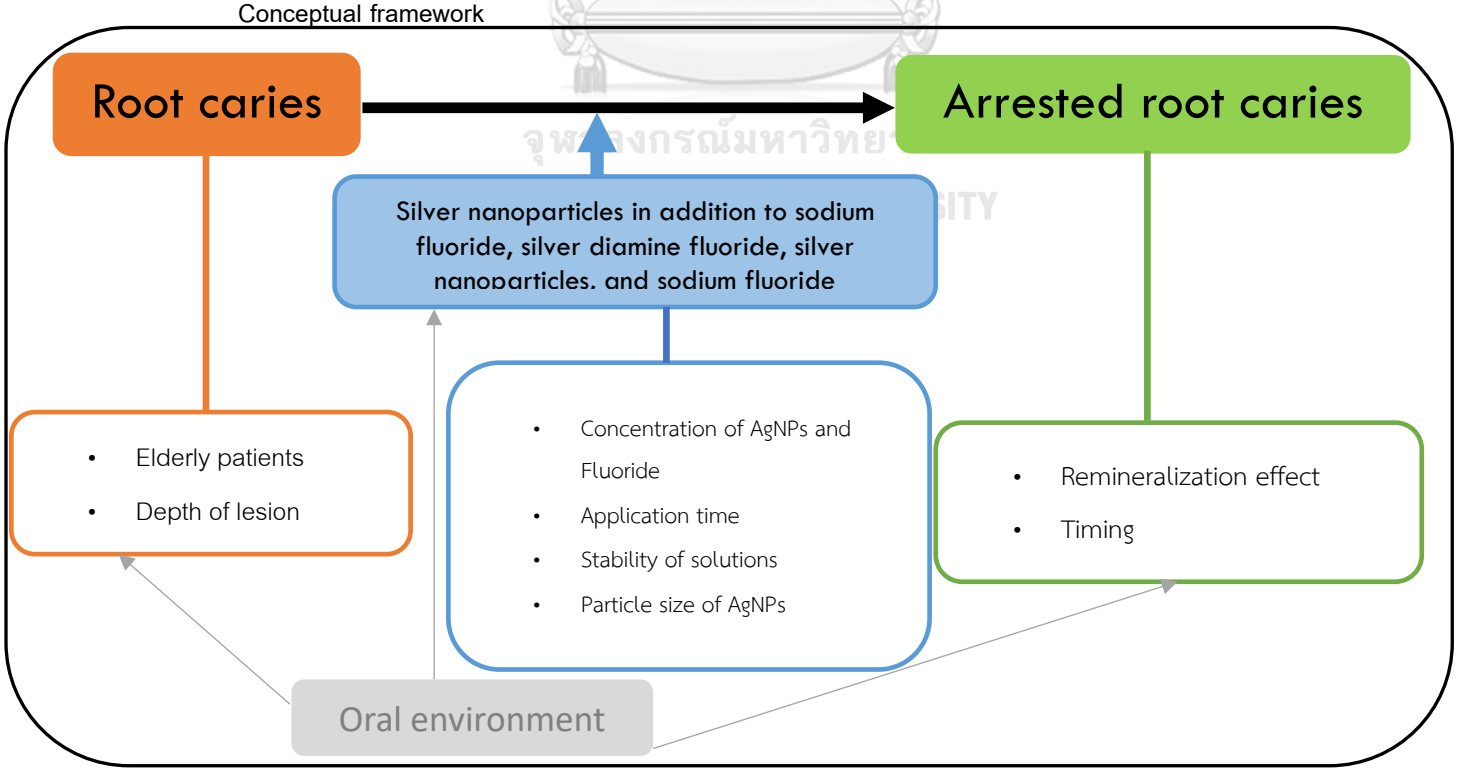

Figure 1 Diagram of conceptual concept

CHAPTER II

Literature review

History of silver

Silver metal has been used as an antimicrobial for a thousand years. In ancient times, silver was used in coin and cutlery because of their corrosion resistance as a noble metal. It was common practice to store water and food in silver vessels or add the silver coin in the vessel for a long journey. Moreover, it has been used for in medical treatment due to its antibacterial effect, lack of bacterial resistance and low toxicity. It was used to prevent infection of burnt skin, treating tetanus, gonorrhea and as a silver suture to close the infection surgical site.[25] In the 19th century, the uses of silver nitrate solution for the therapy of eye infection especially in newborn and several bacterial infections were common. The high solubility of silver nitrate leads to high local silver concentration, which affects bacteria and surrounding tissue. However, the large amount of silver nitrate may cause corneal damage and huge silver oral administration can cause gastrointestinal disturbances.

The use of silver was diminished since antibiotic drugs were discovered.[26] The silver deposition in skin can cause Argyria, that is a mechanism to detoxify silver by sequestering it in the tissue to render it in the form of silver protein complexes or silver sulphide.[27] It develops a bluish-gray discoloration skin but there is no pathologic alteration of the effected organs. The lowest intravenous dose of metallic silver that causes argyria is approximately 0.9 g (6 g of silver arsphenamine).[28] Nowadays, silver compounds such as, silver nitrate and silver sulphadiazine have been used as topical antibacterial agents for burnt skin and chronic ulcer.[29] With many bacteria being resistant against antibiotics, silver has also found in clinical application including cardiac devices, catheters, orthopedic appliances, wound care and bone cement to prevent of infection[30] and even in consumer product such as textiles or sprays to prevent bad odors[31]

In dentistry, silver nitrate was introduced in early 1900s to manage oral ulcers and reduce pain due to aphthous ulcer. Howe's solution or ammoniacal silver nitrate ($AgNH_3NO_3$) has been used as a sterilization agent for disinfecting root canal. Later, it was used to reduce dental caries in primary and permanent dentition.[32] Silver was introduced to combine with fluoride as an anticaries agent. However, the limitation of silver fluoride compound was black staining.[13]

Zhao et al., studied the ability of silver nitrate solution, followed by sodium fluoride varnish to arrest dental caries. They used silver diamine fluoride as positive control and investigated surface morphology, crystal characteristic, carious lesion depth and collagen matrix degradation. They found that the lesion depth of SDF and silver nitrate groups were less than the control group. Dense granular structures of spherical grains were observed in the inter-tubular area in both groups but absent in the control group. The strong peaks were coincident with silver chloride in both sodium fluoride and SDF groups. They concluded the use of $AgNO_3$ solution and sodium fluoride varnish is effective in inhibiting dentine demineralization and dentine collagen degradation.[33]

Double-blind randomized clinical trial compared the effectiveness of the topical semiannual application of a 25% silver nitrate solution followed by a 5% sodium fluoride varnish and 38% silver diamine fluoride solution in arresting caries among preschool children over 18 months.[34] The use of silver nitrate and sodium fluoride varnish was reported to be used as an alternative for managing early childhood caries.

Antibacterial properties of silver

Silver ions (Ag^+) are main cause of antibacterial effect for microorganism whereas metallic silver (Ag or Ag^0) is inert.[35] Antibacterial action of silver relates to extracellular and intracellular

binding properties. There are three mechanisms of silver ions that link to antibacterial effect. Firstly, silver ions can bind with disulphide (S_2^{2-}) in membrane protein allowing membrane permeability. Moreover, the positive charge of silver can bind with negative charge of peptidoglycan in bacterial cell wall resulting in disrupt membrane transportation and leading to destruction of cell wall.[36] Secondly, silver can bind to sulphydryl groups (-SH) which is necessary for enzyme activities. This interaction can inhibit enzyme activities, disrupt metabolic process and cause bacterial cell death.[37] Thirdly, it was reported to inhibit of DNA replication by attaching to guanine which is nucleobase of DNA.

Reaction of silver with dental tissue [38]

The mechanism of action between silver compound and dental tissue is still unclear. The chemical reaction of silver compound and the hydroxyapatite (HAp) has been suggested and modified according to the following mechanism:

$$Ca_{10}(PO_4)_6(OH)_2+AgNO_3 \rightarrow Ca(NO_3)2+Ag_3PO_4+Ag_2+H_2O$$

The reaction and subsequent reaction between SDF and HAp present in below assumption:

$$Ca_{10}(PO_4)_6(OH)_2+Ag(NH_3)_2F \rightarrow CaF_2+Ag_3PO_4+NH_4OH$$

$$CaF_2 \rightarrow Ca_2^{+}+2F^{-}$$

$$Ca_{10}(PO_4)_6(OH)_2+2F^{-} \rightarrow Ca_{10}(PO_4)_6F_2+2OH^{-}$$

The formation of CaF_2 and Ag_3PO_4 is considered as a major product of reaction of SDF and tooth structure. As a result, CaF_2 is an important reaction product and acts as pH-regulation slow-release fluoride reservoir during cariogenic challenges. During remineralization, HPO_4^{2-} will adsorb onto the surface of CaF_2 crystal and facilitates it to form fluorapatites (FAp). For Ag_3PO_4, it acts as a

reservoir of phosphate ions facilitating the transformation of CaF_2 and FAp. The CaF_2 can be dissolved and disappeared easily because of its high soluble property. Some study suggested that the therapeutic effect required further consideration because CaF_2 remained for long.[39]

Silver nanoparticles (AgNPs)

Nanoparticles are particles which have size between 1-100 nm. In dentistry, nanoparticle have been used in two purposes including antimicrobial effect and remineralization of tooth.[40] It is known that silver showed broad-spectrum of antimicrobial potential. Following the development of nanotechnology, silver nanoparticles have become increasingly used. Silver nanoparticles are produced by reduction process of soluble silver salts with the decrease of some agents for example citrate, glucose, ethylene glycol or sodium borohydride. The stabilizing compounds are added to prevent aggregation and growth of the newly formed silver nanoparticles.[41]

The morphology and particle size of silver nanoparticles depend on initially form of silver compound, reduction agent additives and method.[42] It is known that the particle morphology is not stable over time. The aggregation of silver nanoparticles may occur after a few hours or a day if the stabilizers are not sufficient. The most common stabilizer is polymer such as poly-N-vinylpyrrolidone(PVS) and substitutes phosphane ligand.[8] Silver nanoparticles can be synthesized in various methods, which include chemical reduction, photochemical reduction, laser irradiation aerosol techniques or by natural biological process.[43]

Silver nanoparticles have an attraction for researcher to use them in dentistry. It shows the properties of antimicrobial, antiviral, and antifungal actions.[44] Their small particle size increases surface area and provides broad spectrum antimicrobial effect on both gram positive and gram

negative organisms and also various drug resistant strains. The antibacterial mechanism of silver nanoparticles is not completely understood. It relies on their ability to penetrate the bacterial cell wall, a large surface area to contact with bacteria, which allows the particles to attach to the cell membrane and easily penetrate into bacteria cell wall. Formation of reactive oxygen species causes changes of cell wall permeability, damage cell membrane, disrupts the DNA replication and inhibits respiratory proteins.[45]

The release of Ag^{+} ions is the crucial mechanism for antibacterial action of AgNPs. The small size of AgNPs provides better antibacterial action than larger AgNPs. In vitro study, AgNPs has been proven to be antimicrobial agent that is effective against cariogenic bacteria such as *Streptococcus mutans*.[46] Bennis et al.,[47] investigated the inhibition of biofilm and antibacterial properties of silver nano-coating on dentine and compared with silver nitrate and chlorhexidine. In the study, microbial growth and cell viability were evaluated by measuring the turbidity, determination of live/dead cell, and lactate production. Silver nanoparticles and silver nitrate showed similar powerful antimicrobial effect (>99.5%) against the oral pathogen, *Streptococcus mutans* and impeding microbial adhesion on dentine surface.

Application of silver nanoparticles in dentistry

Due to the antimicrobial properties against a wide variety of microorganism of AgNPs, they appear to be good alternative chemical agent for prevent infection, treatment of dental caries, controlling plaque-related biofilms, and remineralization of dental caries. AgNPs were added to dental material, such as polymethy methacrylate (PMMA) , adhesive resin, resin composite, orthodontic resin, and implant to prevent cariogenic bacteria and secondary caries.

Oei et al., investigated the effect of AgNPs incorporated in PMMA on mechanical and antimicrobial properties. The light-cure and chemical-cure systems were used to synthesize AgNP-PMMA denture resin. They found that all AgNP-PMMA released Ag^{+} ions for over 28 days and had comparable Durometer-D hardness and modulus to control PMMA. They implied that the addition of AgNPs lead to improvement of mechanical and anti-bacterial properties of PMMA.[48] Shenggui et al., also evaluated the antibacterial effect of polymethyl methacrylate (PMMA)-cellulose nanocrystals (CNCs)-Nag modified resin against *Streptococcus aureus* and *Escherichia Coli*. They concluded that PMMA-CNCs-Nag modified resin exhibited excellent mechanical properties, desirable biocompatibility, and excellent antibacterial activities.[49] The study suggested that AgNPs could effectively be applied against microorganisms with its antimicrobial properties and prevent adhesion of bacteria to surfaces and formation of biofilms.[44]

Melo et al., studied the adding of AgNPs into primer and adhesive at 0.1% by mass and found that AgNPs remarkably improved antimicrobial properties of the material. The improved properties were biofilm metabolic activities, lactic acid production and less colony forming unit count of cariogenic bacteria comparing to the non-containing AgNPs group.[50]

Another experiment conducted the adding of AgNPs into resin composite by das Neves et al. They found the uses of 0.3% and 0.6% AgNPs by weight showed less inhibition of *Streptococcus mutans* and *Lactobacillus acidophilus* than the unmodified resin. Although the modified resin with 0.3% AgNPs could increase compressive strength, the large portion of AgNPs compromised compressive strength and elastic modulus of material.[51] However, some studies showed the incorporation of AgNPs into pit and fissure sealant can reduce tooth demineralization significantly compared to the conventional sealant.[52] Also, some studies showed the adding of AgNPs into

orthodontics adhesive might prevent plaque accumulation and bacterial adhesion. Ahn et al., showed that all groups with the incorporation of AgNPs into composite adhesive reduced bacterial adhesion when compared to the control group without adverse effect on shear bond strength.[53]

The study of Wang et al., demonstrated the good effect of AgNPs-filled hydrogen titanate nanotube layer titanium foil coated on a metallic surface of implant using *in situ* model. The study found that it exhibited high bacteriostatic activity, long term silver ions release capacity and excellent biocompatibility.[54] Moreover, in terms of endodontic treatment, AgNPs were used as an irrigants. The study showed the 50 μg/ml (0.005%) concentration of AgNPs can be considered for intracanal irrigants because of its antibacterial effect on *Enterococcus faecalis* while those above 80 μg/ml concentration may cause cytotoxic.[55] Also, Luna et al., investigated AgNPs of 10 nm in size can used as final irrigation agent. The AgNPs not only showed the effectiveness for inhibit *Enterococcus faecalis* same as 2.25% sodium hypochlorite, but also it presented a good smear layer removal capacity.[56]

As AgNPs have shown to have antibacterial action against gram negative bacteria, it was used for periodontal infection prevention and periodontal regeneration. Rani et al., investigated antibacterial effect of nanosilver-impregnated GTR (guided tissue regeneration) by observation of microbial adherence and penetration of four organisms (*Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*). Results showed the mean bacterial adherence scores and colony forming unit were significantly lower than control group.[57] Lu et al., found the diverse of antibacterial properties of AgNPs depended on size of particles. Five nm in size of AgNPs presented the highest antibacterial activity to anaerobic pathogen.[58]

Silver nanoparticles and remineralization

The mechanism of remineralization process by AgNPs is still unclear. The crystal growth occurs and begins with nucleation around AgNPs which has anchored to a demineralized site. The negatively charged of AgNPs can bind with positively charged demineralized sites, allowing it to act as a seed for potential crystal formation. The stable nanoaggregate could form, causing growth in the presence of calcium and phosphate ions. It was suspected that these crystals remained in a size of nanoscale and contained an AgNPs core.[59] Furthermore, the reduction in the size of AgNPs may increase contact surface area, which is an important condition for the antimicrobial effects of silver and prevent black staining on the teeth.[46]

Nano-silver fluoride (NSF) is a new colloid which contains silver nanoparticles, chitosan and fluoride. It was developed by Targino et al. They evaluated the antimicrobial and cytotoxicity of the compound against *Streptococcus mutans* compared with SDF and chlorhexidine. The NSF was not toxic at any concentration tested with any type of erythrocyte and is less toxic than SDF. This material demonstrated antimicrobial and remineralizing properties and it can be used as a caries arresting agent as same as SDF without staining.[60] However, the cariostatic effect on dental tissue has not been clarified yet, the synergism of the components of their formulation (silver nanoparticles, fluoride and chitosan) may result in the effectiveness in arresting caries.[61]

Sayed et al.,[24] investigated the antibacterial effect and dentine remineralization at collagen level of silver diamine fluoride, nano-silver and nanosilver/potassium fluoride on morphology of dentine collagen and color staining on the bovine root dentin. They found all test groups showed intrafibrillar pattern of mineral deposition and great bacterial effect but only SDF showed alteration in collagen fibril. Regarding nano silver, it showed no color change.

Zhao et al.,[62] investigated the remineralizing and staining effect of sodium fluoride solution with polyethylene glycol-coated silver nanoparticles on artificial dentine caries. They used digital spectrophotometry, x-ray diffraction, micro-computed tomography and spectrophotometry with a hydroxy proline assay. They found that the use of sodium fluoride solution with polyethylene glycol-coated silver nanoparticles could remineralize artificial dentine caries and inhibit collagen degradation without causing tooth staining.

Akyildiz et al.,[63]evaluated the remineralizing effect of nano-silver fluoride formulation on enamel surface using an in vitro remineralization model compared with silver diamine fluoride and sodium fluoride varnish. They found that all remineralizing agents can improve the hardening of demineralized enamel specimens. The effect of sodium fluoride and SDF were similar, but the effect of nano-silver fluoride is lowest.

Teixeira et al.,[21]evaluated the effectiveness of enamel remineralization action of nano-silver fluoride and its antimicrobial action on the acid production and adhesion of *Streptococcus mutans.* The NSF was compared with sodium fluoride (NaF) through the microhardness test, fluorescence spectroscopy and optical coherence tomography (OCT). They found the NaF and NSF presented similar behavior between the remineralization pattern through fluorescence spectroscopy and microhardness. The concentration of NSF was 400 ppm with a particle size of 8.7±3.1 nm. it showed antimicrobial action in relation to its enamel adhesion and its acid production.

Silva et al.,[64] studied the remineralization of nano-silver fluoride on deciduous teeth enamel and its antimicrobial action. They divided the specimens into 3 groups; sodium fluoride was used as positive control, nano-silver fluoride was experimental group and deionized water as negative control. The specimens were measured by using microhardness, fluorescence spectroscopy and

optical coherence tomography (OCT). They found that nano-silver fluoride showed similar effect to sodium fluoride on deciduous enamel remineralization.

Nozari A et al.,[65] determined the remineralization ability of sodium fluoride varnish, nano-Hydroxyapatite serum(n-HAP) and nano-silver fluoride on enamel carious lesions in primary anterior teeth. They found that the nano-silver fluoride had the highest surface microhardness values when compared to other groups.

Yin et al.,[66] investigated the remineralizing and staining effects of sodium fluoride with silver nanoparticles on artificial dentine caries using biofilm challenge and remineralization process. They found the mean lesion depth and dentine collagen exposed of 5% sodium fluoride with 4000 ppm silver nanoparticles were lowest when compared to applying silver nanoparticles or sodium fluoride alone. There was no significant interaction effect between silver nanoparticles and sodium fluoride, and they concluded that sodium fluoride solution with silver nanoparticles can remineralize dentine caries without staining. Moreover, they studied the antibacterial activity against *Streptococcus mutans*, cytotoxicity effect and the tooth staining effect of sodium fluoride solution with various concentrations of polyethylene glycol-coated silver nanoparticles (PEG-AgNPs). As a result, a novel anti-caries agent was developed in which the combination of PEG-AgNP and NaF was found to broaden the therapeutic window where cariogenic bacteria can be inhibited without harming human cells.[22]

Santos et al.,[23] conducted a prospective, randomized, double-blinded, clinical control trial on 60 children with mean age of six years old. The examiner used ICDAS criteria for evaluation and diagnosis caries lesions. The tactile measurement was used to evaluate activity of caries lesions both pre- and post-treatment. The test-group was applied with two drops of nano-silver fluoride solution

on 63 teeth and left in contact with the tooth for two minutes while the control group was applied with one drop of nano-silver fluoride solution on 67 teeth. They reported caries arrest of up to 81, 72.7 and 66.7% at 7-days, 5 and 12 months after treatment follow up, respectively. They concluded that the annual application of a nano-silver fluoride solution was effective in hardening and arresting dentine caries in primary teeth. The nano-silver fluoride prevented fraction of caries arrested in the primary teeth showed similar result to SDF.

Tirapati et al.,[67] evaluated the annual application of clinical cariostatic efficacy of 5% Nano-silver incorporated Sodium fluoride varnish (NSSF) and 38% SDF in arresting carious lesion progression on primary molar teeth. They found NSSF presented a success rate of 77% for 12 month follow up period and had the same antibacterial effects as SDF without dark staining of dental tissue.

Nagireddy et al.,[61] investigated the effectiveness of nano-silver fluoride in arresting and preventing caries in 100 deciduous molars from 60 children of 4-9 years old. The nano-silver fluoride solution which was used in this study had a spherical shape with average size of 1.2-3.2 nm. They found that, after 7 days, 5 months and 12 months analysis, the nano-silver fluoride group showed arrest of caries of 78%, 72.91% and 65.21% of teeth, respectively. While the control group (saline) showed no arrest of caries on 7th day of evaluation and after 5 and 12 month showed arrest of caries 34% and 28.88% respectively. Moreover, the single surface carious lesion showed higher caries arrest than multiple surfaces after 5 and 12 months. The present study demonstrated that NSF can be used as an arresting caries agent.

Nerabieah Al et al.,[68] synthesized the nano-silver fluoride using green tea extract (NSF-GTE) in a form of spherical shape with average particle size of 4 nm. This study was a randomized, single-blinded, non-inferiority clinical trial. Sixty-three children with 164 active carious lesions were divided

into two groups, experimental and SDF group. The follow up periods were 21 days, 3 and 6 months. According to the non-inferiority margin 15%, at 6 months, the NSF-GTE showed an arresting rate of 67.9% while SDF exhibited 79.6%. Moreover, single surface lesions and anterior teeth demonstrated a higher arresting rate than multiple surface lesions and posterior teeth. The study concluded that the NSF-GTE possessed the non-inferiority in arresting caries compared with SDF. Multiple studies have proposed their uses in various preparations, showing good results in the treatment of early dental caries.

Root dentin related to aging

Dentin has a hierarchical structure, composed of 70% inorganic material, 20% organic material and 10% water by weight. Most of inorganic materials in dental tissue consist of hydroxyapatite $Ca_{10}(PO4)_6(OH)_2$ while 91% of organic matrix are collagen, especially type I collagen. For dentin, noncollagenous matrix components consist of of phosphoproteins, proteoglycan, gamma-carboxyglutamate-containing proteins (Gla-proteins), acidic glycoprotein, growth factor and lipid.

The morphology of human dentin is the presence of tubules that arrange from 20-30% by volume of intact dentin. The tubules host the major cell process of odontoblasts. The tubules are formed around the odontoblast processes and thus transverse the entire width of the dentin from the DEJ to the pulp. This tapering shape is the result of progressive formation of peritubular dentin, which leads to a continuous decrease in the diameter of the tubules toward the enamel outside. The number and diameter of the tubules are varying with the distance from the pulp, and the mean number and diameter are decreased following the increase distance from the pulp.[69]

Dentin is the major constituent of tooth presented in a crown of tooth as coronal dentin and in a root portion as radicular dentin. Toshiko et al., investigated and compared mineral density of coronal and radicular dentin. They evaluated ten bovine incisor teeth using Micro-CT and found the mineral density of coronal dentin was higher than radicular dentin.[70] According with the study of hardness and Young's modulus of coronal and radicular dentin by using nanoindentation test, they found that the hardness of coronal intertubular dentin and radicular dentin was 0.81±0.05 GPa and 0.55±0.02 GPa respectively. Also, the Young's modulus of coronal dentin was greater than those of radicular dentin.[71] Interestingly, it was found that mineral concentration and crystallite size of root dentine changed with age. However, the change in the mechanical properties of aged root dentin is unclear.

Xu et al.,[72] investigated the mechanical properties of root dentin associated with aging using Nanoindenter, microhardness tester and atomic force microscopy. The characteristic of root dentin was measured by scanning electron microscopy and energy dispersive X-ray spectroscopy. They compared young (17-30 years old) and old (50-80 years old) root dentin and found that aged intertubular dentin had significantly higher nano-hardness, elastic modulus, and calcium-phosphate ratio than young root dentin. Furthermore, the old root dentin showed more mineral salt accumulation in the intertubular dentin because of aging. In addition, Sekimizu et al.,[73] investigated the relationship between hypercalcified dentin and ages by dividing 44 human teeth from 37 patients into 3 groups:10-30 years old, 40-60 years old and 70 years and older. The measurement of mineral density used monochromatic radiation x-ray micro-CT(MR-μCT) and contact microradiography (CMR). The percentage of hypercalcified dentin in the group of 40-60 years old was significantly higher than the group of 10-30 years old but there was no significant difference with the group of

over 70 years old. However, this study might require more sample sizes to obtain more reliable result.

Demineralization-remineralization models

A number of demineralization-remineralization models have been used for cariology studies. Caries lesions generated in animal or human studies can greatly mimic the natural caries formation process. A balance between *in vitro* and *in vivo* studies have been proposed as *in situ* studies. The *in situ* models are useful as the role of a large-scale, randomized controlled clinical trial(RCT) in caries studies.[74] It provided essential data for the physical-chemical characteristics of the oral environment. However, the characteristic of natural caries is difficult to control for any confounding factors in both *in vivo* and *in situ* studies. Therefore, the *in vitro* studies are mostly used to investigate the demineralization-remineralization process in caries research. The *in vitro* study can generate controllable oral environment according to the purpose of the investigation with least time and cost. However, it might not be inferred to the real clinical situation.[75] Chemical and microbiological models are used to perform artificial caries like lesion in the *in vitro* studies.

Chemical models can be divided into simple mineralization models and pH cycling models. For simple mineralization models, they use low pH solution to generate the demineralization. The pH value ranged from 4.4 to 5.0 can produce subsurface caries lesions. Mild acid can create a demineralized surfaces like a natural caries lesion. Lactic acid and acetic acid as organic acids have been used to create demineralized lesions in most experimental designs. The demineralization process will stop after mineral saturation is reached. The amount and viscosity of the gel applied on the top of the substrate will affect the time of mineral saturation. Besides, acidified gel such as carboxymethycellulose-based lactic acid gel and ethylenediaminetetraacetic (EDTA) acid gel are

widely used because they are simple and can generate lesions that are histologically similar to natural enamel caries evaluated by light polarized microscopy.[76] For the carboxymethylcellulose-based lactic acid gel, the consistency of the gel provides a diffusion barrier on the surface of substrate resulting in a slow demineralization process. Moreover, it may combine with calcium and reduce the demineralizing activity of calcium.[75]

The pH-cycling models are laboratory models based on a pattern that challenged the neutral pH environment and was periodically interrupted by an acid environment. Acid exposure in pH cycling protocol represents demineralized periods and neutral pH environment infers to remineralized periods. This model mimics the dynamic of mineral loss and gain that involves in caries formation.[77] The pH values of acid ranged from 4.4-5.5 are used as demineralized solution depending on different substrates and demineralized acid whereas pH value of 7 is used for remineralized solution. The duration of pH cycling varies from several days to months. The pH-cycling models often used for the study of dentin caries.[78] Other advantages of this model are the high level of scientific control and the lower variability to *in vitro* models. With this kind of experiment, small sample size can also be used.

Microbiological models are another methods that aim to simulate caries process using bacterial strains or mixed cultures in the same way as acid procedure in culture process. This model reproduces some characteristics of natural dentin caries. *Streptococcus mutans* was commonly used as a single species within a pure culture system. Besides, the bacteria were used in cariogenic consortia biofilms which are *Streptococcus mutans, lactobacillus, bifidobacteria,* or *actinomyces.* Biofilm closed system models have been used in recent studies. The culture medium is provided in a sealed container and the growth condition will change considerably with the consumption of the

nutrients and accumulation of metabolic products. So, the physiology and biology may be not comparable to the natural biofilm. The advantages of closed system model include simple, repeatable, controllable, and inexpensive.[75]

Marquezan et al., compared the ability of chemical and microbiological methods in dentine caries induction. They found all methods of demineralization showed less microhardness value than sound dentine at 50 μm depth. Acidified gel provided higher microhardness value than natural caries lesion, except at the depth of 10 μm. The pH cycling method provided hardness values similar to natural caries affected dentin until 40 μm of depth, and lower than sound dentin until 200 μm of depth. The microbiological method provided lower microhardness value than natural caries lesions at depth below 200 μm.[76]

In the present study, the demineralization-remineralization model will be used as a pH-cycling model. This is because the model simulates the pH associated with the natural dentin-caries process. The model also possesses dynamic variation in mineral saturation, simplicity, low cost, and efficiency.

Laboratory studies related to remineralization

The measurement of change in mineral content in caries studies has been determined by a variety of methods such as chemical analysis, microhardness, polarized light microscopy, transverse microradiograph, microprobe analysis and Micro-computed tomography(Micro-CT).[79]

Transverse microradiography (TMR) has been used as a standardized quantitative method in studies on de/remineralization of teeth for decades. The principle of this method is the measurement of absorption of monochromatic x-rays by a tooth section, compared with absorption by a simultaneously exposed standard. The result of measurement is a curve of mineral content as a

function of z-direction in the tissue. However, the major problem for this method is destructive and time consuming. The measurement requires a preparation of flat section at a 100 μm in thickness.[80] Moreover, the air drying of dentine lesions can result in a characteristic surface shrinkage. As a result, it is difficult to determine the original location of dentine lesion.

Polarized light microscope (PLM) also has been widely used in hard tissue studies especially as a qualitative method. The principle of this method is an unpolarized beam of light hits the material, it splits into two plane-polarized rays. This is referred to as the ordinary and extraordinary rays, they vibrate at right angles to each other. For those rays, the material exhibits different refractive indices, these are revealed as differences of intensity under crossed polars. This method usually uses to distinguish areas of varying intensity. The measurement procedure in PLM is much more laborious than TMR, but the cost of PLM is less expensive.[80]

Micro-CT was developed in 1980s and used in studies on mineral density or structure of bone or others mineral tissues. It is a non-invasive and non-destructive method that can be used to illustrate morphological characteristics of substrate in detail without destruction and does not need a sample preparation.[81] Micro-CT operation using microfocal spot x-ray sources and high resolution detectors, allows projections to rotate through the sample in multiple viewing directions. Later it can be reconstructed as images of the sample with volumetric pixel (voxels) in the range of 5-50 μm.[82] Micro-CT can be used for measuring and evaluating both the internal and external structure of tooth. It provides reproducible data in all three dimensions. The information after scanned can be created in any plane and the data can be presented both in two-dimensional and three-dimensional images.

The Micro-CT is widely used in dentistry. It can be used to determine biomaterials, root canal morphology, craniofacial skeleton structure, micro finite element modeling, dental tissue

engineering, mineral density of hard tissue, microleakage around filling and dental implants.[83] The limitations of Micro-CT are longer time of scanning, large storage data, large image volume and limiting size of sample. These limitation restricts some analysis in dental research area.[84] Micro-CT cannot be used in a daily clinical setting due to the high radiation level of exposure.[85]

Microhardness testing is an indentation technique that uses a diamond indenter of special shape. The indenter is pressed onto the test material under a designed load and then left on the specimen for a given time. The size of resultant indentation is measured under optical microscope, and the hardness is evaluated as a mean stress pressed by the indenter.[79] There are two types of indentations used in dental research. One is a Vickers indenter, which produces a square indentation and the other is a Knoop indenter which produces oblong indentation. The Knoop indenter is recommended for a brittle and/or anisotropic material where a shallow depth of impression is required.[86]

Scanning electron microscope (SEM) is one of electron microscopy that can obtain a three-dimensional-like images of the surfaces in the magnification ranged from 10-10,000x. The basic components of SEM included the lens system, the electron gun, the electron collector, the visual and photorecording cathode ray tubes, and the associated electronics. The concept of SEM was the analysis of the area irradiated with a finely focused electron beam. The irradiated area may be swept in a raster across the surface of the specimen to form images. The signals as a result of the interaction of the electron beam and the sample include secondary electrons, backscattered electrons, characteristic x-rays, and other photons of various energies. The SEM can be used to analyze a selected point location of sample and the three-dimensional appearance of the images is

developed from the large depth of field of the scanning electron microscope as well as the shadow relief effect of the secondary and backscattered electron contrast.[87-89]

Energy Dispersive X-ray Spectrometer (EDS) is an instrument that can obtain the elemental analysis or chemical characterization of the samples. It is based on the interaction of some source of x-ray excitation and a sample. The system of EDS includes four primary components which are the excitative source, the x-ray detector, the pulse processor, and the analyzer. These x-ray detectors commonly used the lithium drifted silicon (Si(Li)) solid-state detector. When the electron beam hits the sample, it excites an electron in an inner shell and causes its ejection to generate the formation of an electron hole in the electronic structure of the element. An electron from the outer higher-energy shell then fills the hole, and the difference in energy between higher energy shell will be released in the form of x-ray. The energy of x-ray is characterized from the difference in energy between the two shells, and from the atomic structures of the element which they are emitted. As a result, a spectrum that displays the peaks will be correlated to the elemental composition of the investigated sample.[87, 88]

Although polarizing microscopy and transverse microradiography are the standard methods in the measurement of tooth properties, they are invasive, require a thin section, and the procedure is difficult and time consuming. Thus, Micro-CT and microhardness are the methods that are more popular in dental research to qualify and quantify the remineralization potential. Moreover, Micro-CT is a non-destructive method, and it can represent the lesion depth in 3D image. The microhardness will be used in this study together with micro-CT as indirect measurement of remineralization. Besides, the SEM-EDS will be used to characterize remineralized surfaces and perform chemical analysis.

CHAPTER III

Research methodology

Sample Description

Sample size was calculated by using the data from our pilot study. The sample size was generated using G*Power 3.1 (Kiel university, Kiel, Germany) by selecting F-test family for one-way ANOVA with effect size f = 1.51, power β = 80%, and α = 5%. The total sample size calculated is at least 15, 3 samples for each group. However, to avoid discrepancy, the samples of this study will be increase to 11 samples per group which will be a total 55.

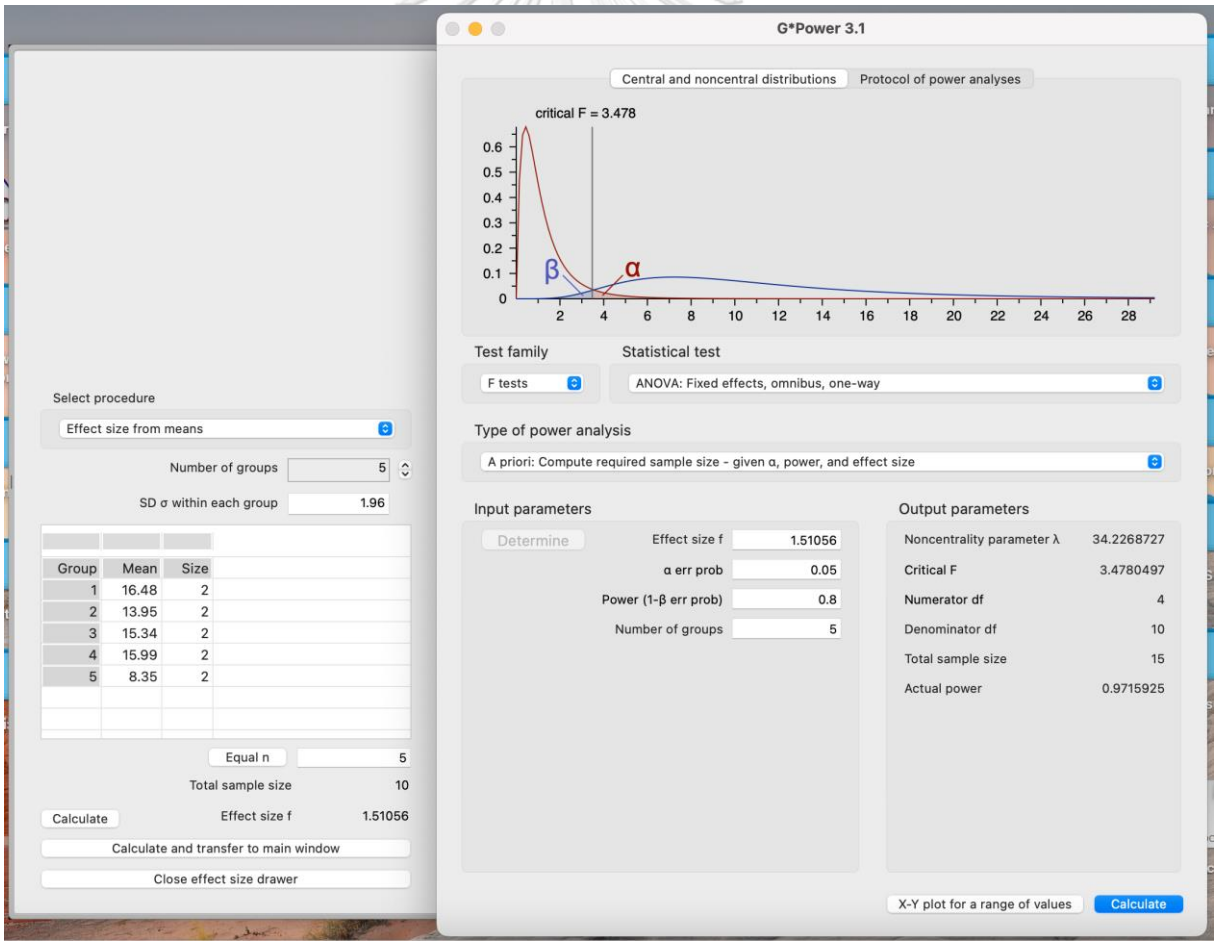

*Figure 2 Demonstrates sample size calculation by G*Power program for microhardness measurement.*

Materials

1. Fifty-five extracted human teeth (more than 60 years of age)

2. Thymol solution (0.1%) (M DENT, Mahidol university, Bangkok, Thailand)

3. Silver nanoparticles solution (10 nm particles size 0.02 mg/mL in aqueous buffer) (Sigma-Aldrich, Darmstadt, Germany)

4. Silver diamine fluoride solution (38%) (Topamine™ Dental life, Ringwood, Australia)

5. Sodium fluoride varnish (5%) (Duraphat®, Colgate-Palmolive Ltd, Guildford, UK)

6. Demineralizing solution (Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

7. Remineralizing solution (Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

8. Artificial saliva (Pharmacology Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

9. Cylindrical diamond bur (Jota, Rüthi, Switzerland)

10. Silicon carbide papers (600, 1000 and 1200- grit) (TOA Co., Ltd., Bangkok, Thailand)

11. Acid-resistant nail varnish (Revlon Professional®, New York, USA)

12. Deionized water (Dental Material Research and Development Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

13. Epoxy resin (Rungart®, Bangkok, Thailand)

14. Micro-brushes (Micro applicator – regular, Premium Plus International Ltd., Hong Kong, China)

15. Ethanol (Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)

16. Adhesive tape (3M Scotch Blue™ Painter's Tape, Minnesota, USA)

Equipment

1. Stereomicroscope (ML 9300®, Meiji Techno Co., Ltd., Saitama, Japan)

2. Slow speed cutting machine (IsoMet® 1000, Buchler Ltd., Illinois, USA)

3. Automatic polishing machine (Nano 2000, Pace technologies Inc., Arizona, USA)

4. Incubator (Contherm 160M, Contherm Scientifie Ltd., Wellington, New Zealand)

5. Ultrasonic cleanser (5210, Heidolph, Illinois, USA)

6. Micro-CT (μCT 35, Scanco Medical, Bassersdorf, Switzerland)

7. Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer (JSM-IT300 InTouchScope™ Scanning Electron Microscope, JEOL Inc., Massachusetts, USA)

8. Microhardness tester (FM810, Future-Tech Corp., Kanagawa, Japan)

9. Digital Vernier Caliper (Mitutoyo Corp., Kanagawa, Japan)

10. Automated sputter-coater (JFC-1200 Fine Coater JEOL, USA)

Sample preparation

The study was approved by the Ethics Committee of Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2021-082). The inclusion criteria were permanent teeth from patients aged 60 years old or more, free from caries, crack, or sclerotic dentin on root surfaces. Teeth with root caries, root fracture or sclerotic dentin were excluded from this experiment.

Fifty-five extracted teeth were washed thoroughly under running water and all blood and adherent tissues were removed. The teeth were collected every week and stored in a 0.1% Thymol solution (M DENT, Mahidol university, Bangkok, Thailand) at 4°C at least for one week and not longer than two months after extraction. The teeth were inspected under a stereomicroscope for visibly observable cracks or other defects.

For the method, the first step was separating the crown and root at the Cemento-enamel Junction (CEJ) (Figure 3a) using a low-speed saw with a diamond blade (IsoMet® 1000, Buchler Ltd., Illinois, USA) under water cooling. Next, the root dentin slices were cut to obtain a cross-sectional area of root dentin size 5x5 mm^2 at the mesial or distal aspect of the root. The top border of root dentin slices was on the level of CEJ in width of 5 mm. and the length of 5 mm was parallel to the tooth axis and cut the root dentin slice perpendicularly to the tooth axis. The thickness of root dentin slices was less than 1.5 mm from the root canal wall to the external surface (Figure 3b). The desired root dentin thickness was measured using a digital vernier caliper (Mitutoyo Corp., Kanagawa, Japan)

Then, the root dentin slices were embedded in epoxy resin blocks which are 1.5 cm in diameter and 1.5 cm in height (one slice for one block). The experimental surface was applied with adhesive tape (3M Scotch Blue™ Painter's Tape, Minnesota, USA) and faced the experimental area

down into the silicone mold. Next, the epoxy resin (Rungart®, Bangkok, Thailand) was poured into the silicone mold and take about 24 hours to cure. (Figure 4)

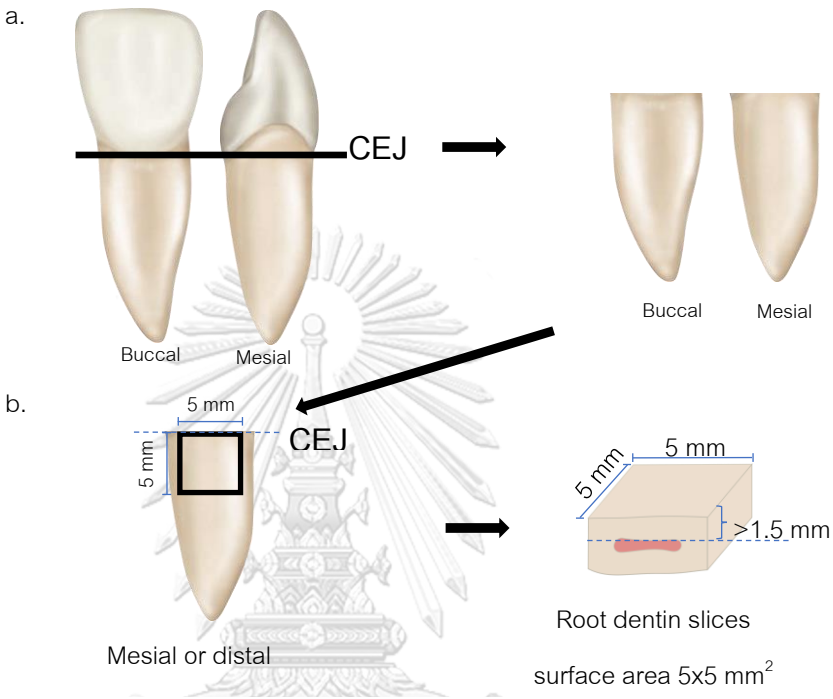

Figure 3 Diagrammatic presentation of sectioned root dentin slices

a.) The tooth was separated crown and root at CEJ

b.) The root fragments of square shape size 5x5 mm^2 at mesial or distal aspect were cut to obtain the root dentine slices and the thickness of root dentin slices was not less than 1.5 mm from the root canal wall to the external surface.

After the curing of epoxy resin block, the adhesive tape was removed. The embedded surfaces of specimens were polished with 600, 1000, and 1200 grit silicon carbide papers and water to obtain a flat, smooth surface and parallel to the top plane of the epoxy resin block. Finally, the specimens were polished with flannel disk and aluminum oxide powder 0.05 μm in particle size using an automatic polishing machine (Nano 2000, Pace technologies Inc., Arizona, USA) under running

water for 60 seconds at the speed of 200 rounds per minute to obtain the glossy surface. Any surface debris were removed by ultrasonic cleaning (Ultrasonic cleanser 5210, HEIDOLPH, Germany) for 15 minutes in deionized water after the polishing process.

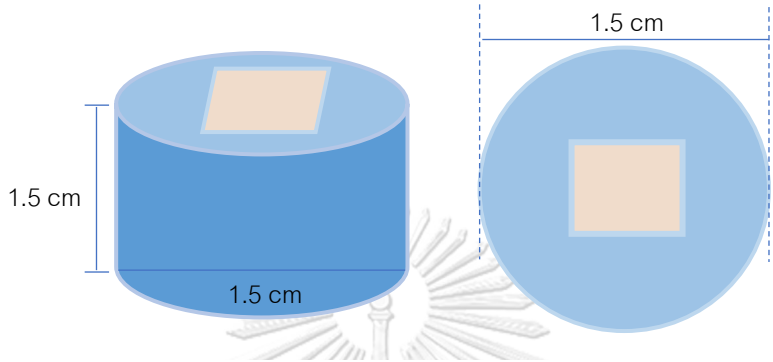

Figure 4 Diagrammatic presentation of root dentin slice embedded in Epoxy resin block.

Baseline microhardness test (KHN_0)

To avoid dehydration, the surface of specimen was wiped with a cotton pad to get a dry surface and microhardness testing was finished within ten minutes for each specimen. The microhardness measurement was conducted with a Knoop Hardness Tester (FM810, Future-Tech Corp., Kanagawa, Japan) under a load of 25 g applied for 15 seconds.[90] Five different indentations were indented on the surface area with a spaced 0.5 mm from each other and the first indentation was positioned at the dentin surface 1mm from left and upper border (Figure 6a). The diagonal length of each indentation was measured by a built-in scaled micrometer and calculation was automatically done by the Knoop Hardness tester to obtain the Knoop Hardness number for each indentation. The specimens were stored in an incubator at 37 °C with 100% relative humidity during the non-experimental period.

Fifty-five specimens were assigned to five group with stratified random sampling. The specimens were arranged in ascending order according to the Knoop hardness value from minimum value to maximum value. The specimens which minimum value from the first to the 25th were

randomly divided into five groups based on experimental treatment (Group 1-5). Then, the 26th specimen to 50th specimen were randomly divided into five groups again.

Development of artificial dentin caries

The specimen's surface area size 1.5x5 mm^2 were coated with acid-resistant nail varnish (Revlon Professional®, New York, USA) to act as a baseline lesion. Each specimen was individually immersed in 10 mL of the demineralization solution (1.5 m*M* $CaCl_2$, 0.9 m*M* KH_2PO_4, 5 m*M* NaN_3, and 50 m*M* acetic acid buffer, pH = 5.0) at 37 °C for 48 hours to create artificial caries lesions.[91] The specimens were washed for 30 seconds with deionized water after lesions developed.

Knoop microhardness assessment after artificial lesion formation (KHN_1)

After artificial caries lesions developed, the microhardness measurement of the surface was carried out. The five different indentations were indented at the location of 2.5 mm from the left border and 0.75 mm from the upper border. (Figure 6b) The loads utilized and all conditions are the same as the initial one.

Micro-CT assessment after artificial lesion formation (L_1)

Micro-computed tomography was used to scan the tooth surface blocks to measure the lesion depth after demineralization. The specimens were put in the holder with sponge stabilization and then the holder was placed in the micro-CT machine. The voltage and current of the x-ray source were 70kV and 100 μA, respectively. The highest spatial scanning resolution was 9 μm. The specimens were filtered for radiation using aluminum foil with 0.5 mm thickness. The position of each specimen within the micro-CT machine for the pre-treatment scan was stored in a file that could be loaded in a subsequent scan to return it to the same position. The specimens were stored in an incubator at 37 °C with 100% relative humidity during the non-experimental period.

Experimental treatment

All specimens were assigned to five groups. In group 1, a topical application of 38% silver diamine solution (Topamine™ Dental life, Ringwood, Australia) were applied on the root surface according to the manufacturer's recommendation. In group 2, the specimens were applied with a topical application of AgNPs solution (Sigma-Aldrich, Darmstadt, Germany) . In group 3, the specimens were applied with a topical application of AgNPs solution (Sigma-Aldrich, Darmstadt, Germany) and followed by the application of a thin layer of 5% NaF varnish (Duraphat®, Colgate-Palmolive Ltd, Guildford, UK). In group 4, the specimens were applied with a topical application of 5% NaF varnish (Duraphat®, Colgate-Palmolive Ltd, Guildford, UK) with a thin layer. In group 5, the specimens were applied with tap water (negative control). Micro-brushes (Micro applicator – regular, Premium Plus International Ltd., Hong Kong, China) were used to apply all solutions to the specimens' surfaces.[33] The solution in all groups were left on the surfaces of the specimens for three minutes before they will experience further treatment. The treatment agents of all groups were received one-off. Before the pH cycling procedure, the specimens were kept at room temperature for 30 minutes. The commercial name, composition, and method of use of materials in this study are listed in Table 1.

Table 1 Composition and method of use of materials used in present study.

Materials	Compositions	Manufacturer	Application
Silver nanoparticle solution	10 nm particle size 0.02 mg/mL in aqueous buffer	Sigma-Aldrich, Darmstadt, Germany	Apply AgNP solution with regular size of micro-brush, air dry for 3 minutes, one application per one sample[24]
38% Silver diamine fluoride solution	Fluoride = 44,880 ppm, Ag^{+}=24-27%, Ammonia, pH 11-12	Topamine™ Dental life, Ringwood, Australia	Apply SDF with regular size of micro-brush and rub for 1 minutes, air dry for 3 minutes, one application per one sample[24]
5% Sodium fluoride varnish	Fluoride = 22,600 ppm, neutral pH	Duraphat®, Colgate-Palmolive Ltd, Guildford, UK	Apply sodium fluoride varnish as a thin layer using regular size of micro-brush, remove the excess varnish with another micro-brush, left for 3 minutes[33]

pH cycling

All treated specimens were experimented using a pH cycling model[92] through demineralization solution (50 mM acetate; 2.25 mM $CaCl_2.H_2O$; 1.35 mM KH_2PO_4; 130mM KCl; pH 5.0; 30 minutes) and remineralization solution (20 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES); 2.25 mM $CaCl_2.H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl, pH 7.0; 10 minutes) for eight days. All solutions were freshly made daily prior to use. Six cycles per twelve hours were performed each day for eight days. (Fig.5) The specimens were kept in the neutral buffer overnight.

The compositions of the solutions used in pH cycling are listed in Table 2.[92]

Table 2 Composition of the demineralizing and remineralizing solutions used in pH cycling.

Solution	Composition
Demineralizing solution	50 mM acetate; 2.25 mM $CaCl_2.H_2O$; 1.35 mM KH_2PO_4; 130mM KCl; pH 5.0
Remineralizing solution	20 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES); 2.25 mM $CaCl_2.H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl, pH 7.0

Knoop microhardness assessment after pH cycling challenge (KHN_2)

Following the pH cycling process, the specimens were put in an ultrasonic cleaner for 15 minutes. The surface of specimens was wiped with a cotton pad to get a dry surface and the microhardness testing will be finished within ten minutes for each specimen. The post-treatment surface microhardness test was conducted at 3.5 mm from the left border and 1 mm from the upper border (Figure 6c) under the same conditions (process, load, time, and machine).

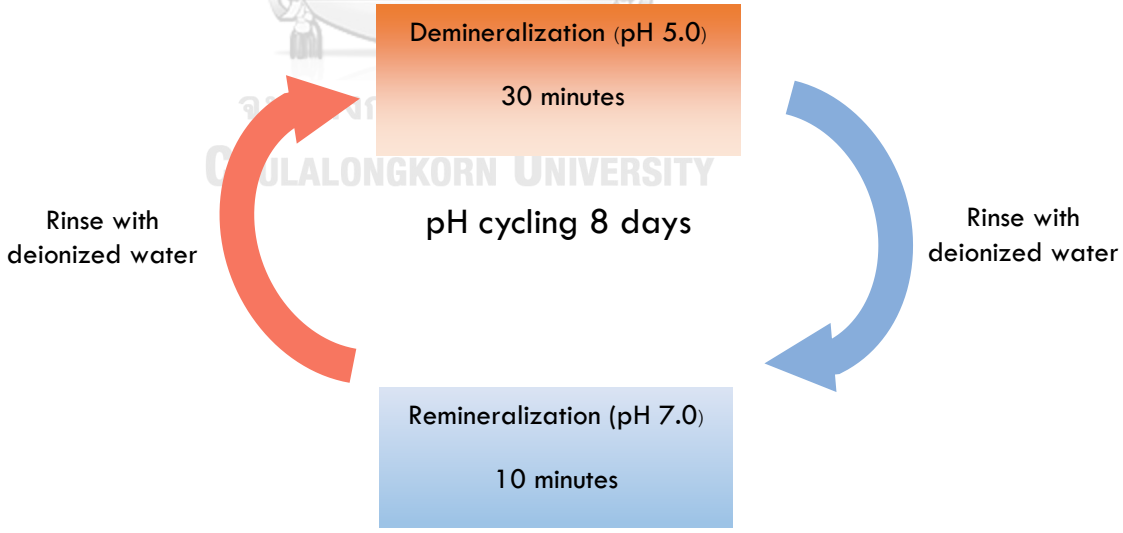

Figure 5 Diagram of the pH cycling model.

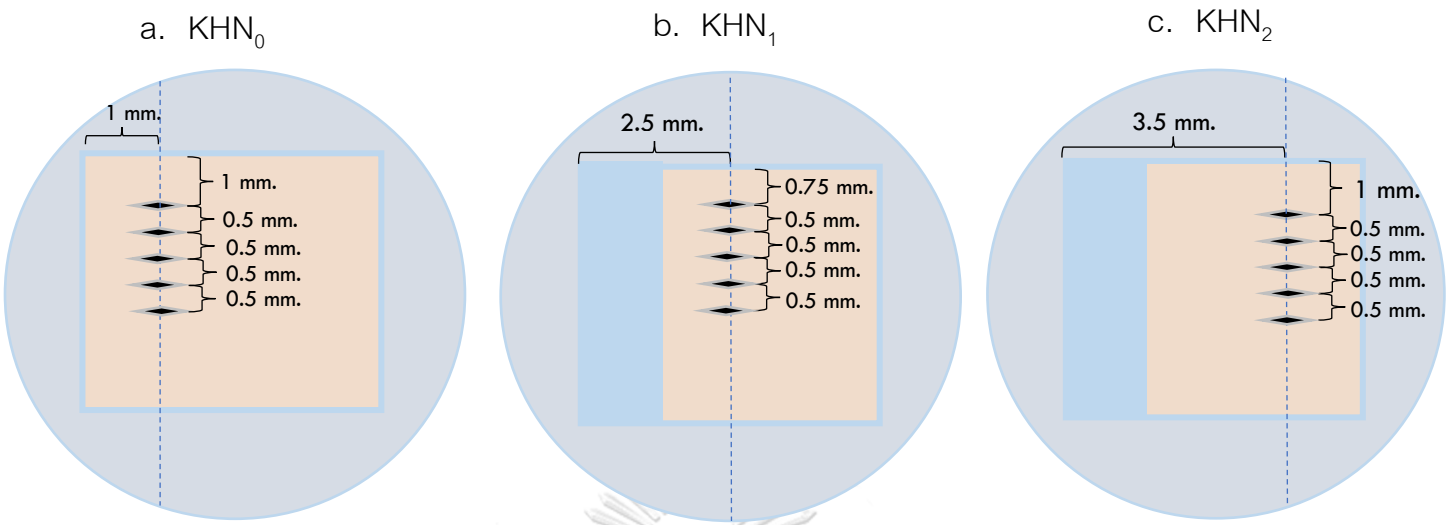

Figure 6 Knoop microhardness was determined in five different vertical indentations

a.) Baseline Knoop microhardness (KHN_0) was determined at 1 mm from the left border and 1 mm from upper border, b.) Knoop microhardness after artificial lesion formation (KHN_1) was determined at 2.5 mm from the left border and 0.75 mm from upper border, c.) Knoop microhardness after immersion in each tested solution under pH cycling model (KHN_2) was determined in other five locations at 3.5 mm from the left border and 1 mm from upper border.

Micro-CT assessment after pH cycling challenge (L_2)[33, 66]

Micro-computed tomography was used to scan the tooth surface blocks to measure the lesion depth after pH cycling. The same scanning parameters as those for the pre-treatment scans were used to ensure consistency in the greyscale values. The Pre-treatment specimens' position was loaded to ensure that each specimen was scanned in the same position to minimize misalignment of the two image sets. The scanning results were reconstructed using software (μCT35, Scanco Medical, Bassersdorf, Switzerland) and process the reconstructed three-dimensional images at the precipitation and lesion areas. From the reconstructed images of each specimen, cross-sectional images were located. From these lesion images, 10 images were randomly selected. The lesion depth of different groups was measured using image analysis

software (Image J; National Institutes of Health, Bethesda, MD, USA) , with internal control as a reference line. The lesion depth was measured by the paired T-test of pre-treatment lesion depth and post-treatment lesion depth.

Scanning electron microscopy and energy-dispersive x-ray spectroscopy (EDS) analysis[66]

Scanning electron microscopy (SEM) was used to examine the specimens' surface morphology. Four specimens from each group were dehydrated in a series using ethanol solution (Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand) (70% for 10 minutes, 95% for 10 minutes and 100% for 20 minutes) then mounted on aluminum stubs, dried in a desiccator, and finally sputter-coated with gold coating. The surfaces were evaluated at a magnification of 1,000X, 5,000X and 10,000X at an acceleration voltage of 15 kV.

Four specimens for each group were used to characterize the chemical composition by EDS analysis. The precipitation surface was quantified the carbon, fluoride, nitrogen, and silver at 15 kV of accelerating voltage and 50 nA of beam current.

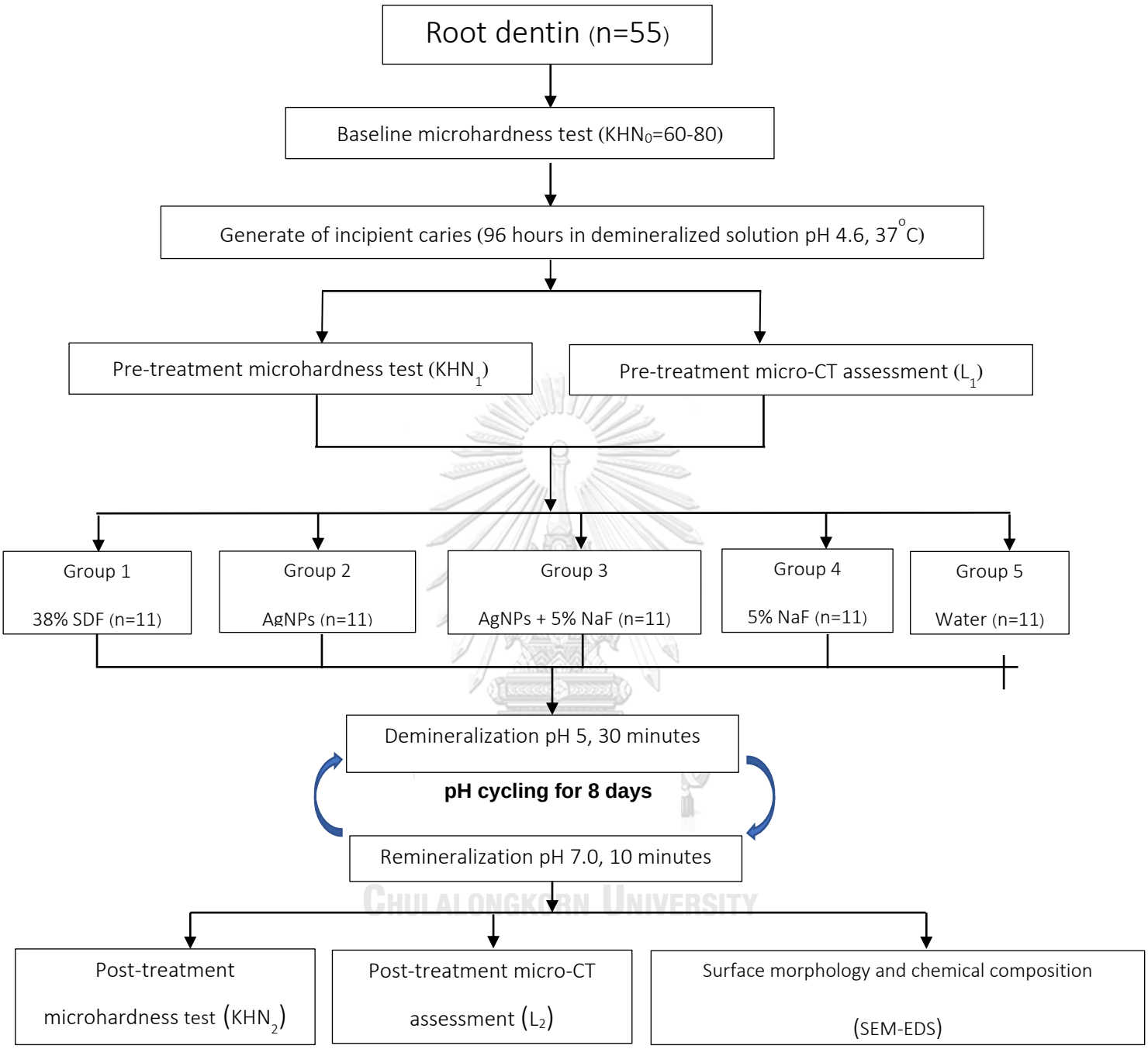

Figure 7: Diagram of the study design

Statistical analysis

The characteristics of crystals and minerals compositions were evaluated by SEM and EDS and not subjected to statistical analyses.

The intragroup mean difference of surface microhardness and lesion depth were analyzed using paired T-test. Data was analyzed using statistical analysis software (SPSS 26.0; SPSS Inc, Chicago, USA.). A normal distribution is tested by a Kolmogorov-Smirnov test. One-way ANOVA with Tukey's post hoc test were used to analyze the intergroup mean difference of lesion depth and surface microhardness. The level of statistical significance for all tests is 0.05.

Ethical consideration

Ethical consideration was approved by the research ethics review committee of the Faculty of Dentistry, Chulalongkorn University for research involving human research participants. (HREC-DCU 2021-082).

CHAPTER IV

Results

At baseline, KHN_0 values of all samples ranged from 60.32 to 72.66 (mean 64.99±2.94) and no statistically significant differences were seen between groups (p=0.863). After generating artificial caries, KHN_1 values ranging from 10.11 to 18.11 (mean 14.45±2.00), were significantly lower than baseline Knoop hardness values. However, KHN_1 values were not significantly different between groups (p=0.761). The highest KHN_2 value were observed in SDF group followed by AgNPs+NaF group, NaF group, AgNPs group and control group (Table 3). SDF group showed statistically significant difference in all group. However, AgNPs group, AgNPs+NaF group and NaF group were not different statistically. Pairwise comparison of KHN_1 and KHN_2 revealed significantly different statistically between group AgNPs, NaF and control.

Table 3 : Mean of Knoop microhardness measurement in 5 experimental groups.

Baseline Knoop microhardness (KHN_0), Knoop microhardness after artificial lesion formation (KHN_1), and Knoop microhardness after pH cycling challenge (KHN_2).

Groups	KHN_0	KHN_1	KHN_2
SDF	65.17±3.98 [a, A]	14.71±2.01 [b, B]	15.29±2.22 [c, B]
AgNPs	64.34±3.49 [a, A]	14.13±2.53 [b, B]	11.21±2.31 [d, C]
AgNPs+NaF	65.58±2.49 [a, A]	14.31±2.15 [b, B]	12.48±1.68 [d, B]
NaF	65.28±2.98 [a, A]	15.05±1.11 [b, B]	12.20±1.45 [d, C]
Control	64.99±1.61 [a, A]	14.45±2.01 [b, B]	8.50±1.43 [e, C]

Different lowercase letters in each column presents statistically significant difference (One-way ANOVA and Tukey post-hoc test, $p<0.05$)

Different uppercase letters in each row demonstrates statistically significant difference (Paired-T-test, $p<0.05$)

The mean lesion depth (±SD) of pre-treatment and post-treatment was shown in Table. 5. There was no significant difference in pre-treatment lesion depth in all groups. The highest lesion depth of post-treatment was 151.57±1.79 in the control group. The lowest lesion depth of post-treatment was 115.43±1.17 in the SDF group. The mean lesion depth of post-treatment in SDF, AgNPs, and control group was significantly different. There was no significant difference in mean lesion depth of post-treatment in group AgNPs+NaF and NaF. Images of typical micro-CT in treatment groups are shown in Figure 8.

Table 4: Mean of lesion depth in 5 experimental groups.

Lesion depth after artificial lesion formation (L_1), and Lesion depth after pH cycling challenge (L_2).

Groups	L_1	L_2
SDF	140.25±1.15 ^{a,A}	115.43±1.01 $^{b, B}$
AgNPs	140.25±1.30 ^{a,A}	125.37±1.17 $^{c, B}$
AgNPs+NaF	140.26±1.35 $^{a, A}$	122.28±1.98 $^{d, B}$
NaF	140.17±0.73 $^{a, A}$	123.02±1.32 $^{d, B}$
Control	140.92±1.41 $^{a, A}$	151.57±1.79 $^{e, B}$

Different lowercase letters in each column presents statistically significant difference (One-way ANOVA and Tukey post-hoc test, $p<0.05$)

Different uppercase letters in each row demonstrates statistically significant difference (Paired-T-test, $p<0.05$)

Chemical composition analysis using EDS revealed in figure 8. Both Ag and F were remained on surface of SDF-treated group. In AgNPs or AgNPs+NaF-treated groups, Ag also

presented on dentin surface after pH cycling challenge yet lower amount than SDF. F also remained on dentin surface in all groups except AgNPs-treated group.

Scanning electron microscopy revealed that exposed dentinal tubules. All treated groups showed either slight or almost complete occlusion in dentinal tubules while the control group showed widely clear patency of dentinal tubules. SDF-treated group showed slight deposition of crystals in dentinal tubules and on dentin surface (Fig. 9A). Meanwhile, AgNPs-treated group demonstrated irregular squamous layer deposits on the dentin surface, together with partial occlusion of dentinal tubules (Fig. 9B). AgNPs+NaF-treated group revealed thorough surface precipitation and almost complete occlusion of dentinal tubules (Fig. 9C). NaF-treated and control groups showed deficits of deposition (Fig. 9D and 9E).

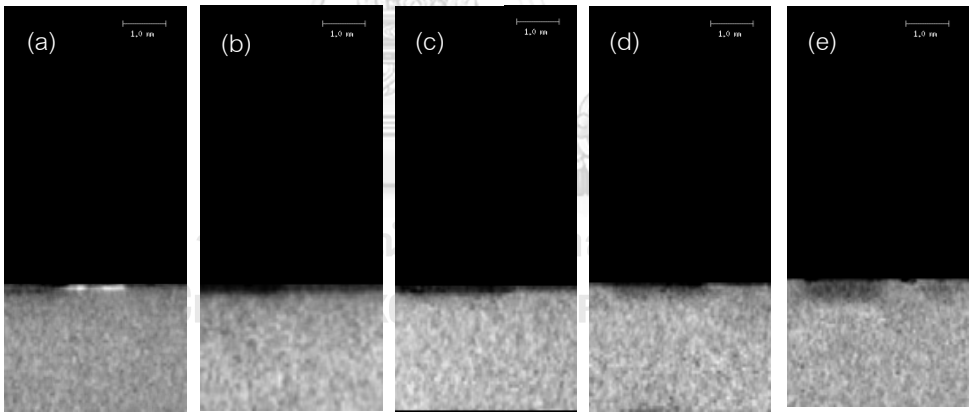

Figure 8: Typical micro-computed tomography images of cross-section of artificial root dentin caries

: left side: lesion body; right side: internal control.(a) Dentine specimen treated with SDF, (b) Dentine specimen treated with AgNPs, (c) Dentine specimen treated with AgNPs+NaF, (d) Dentine specimen treated with NaF, (e) Dentine specimen without treated.

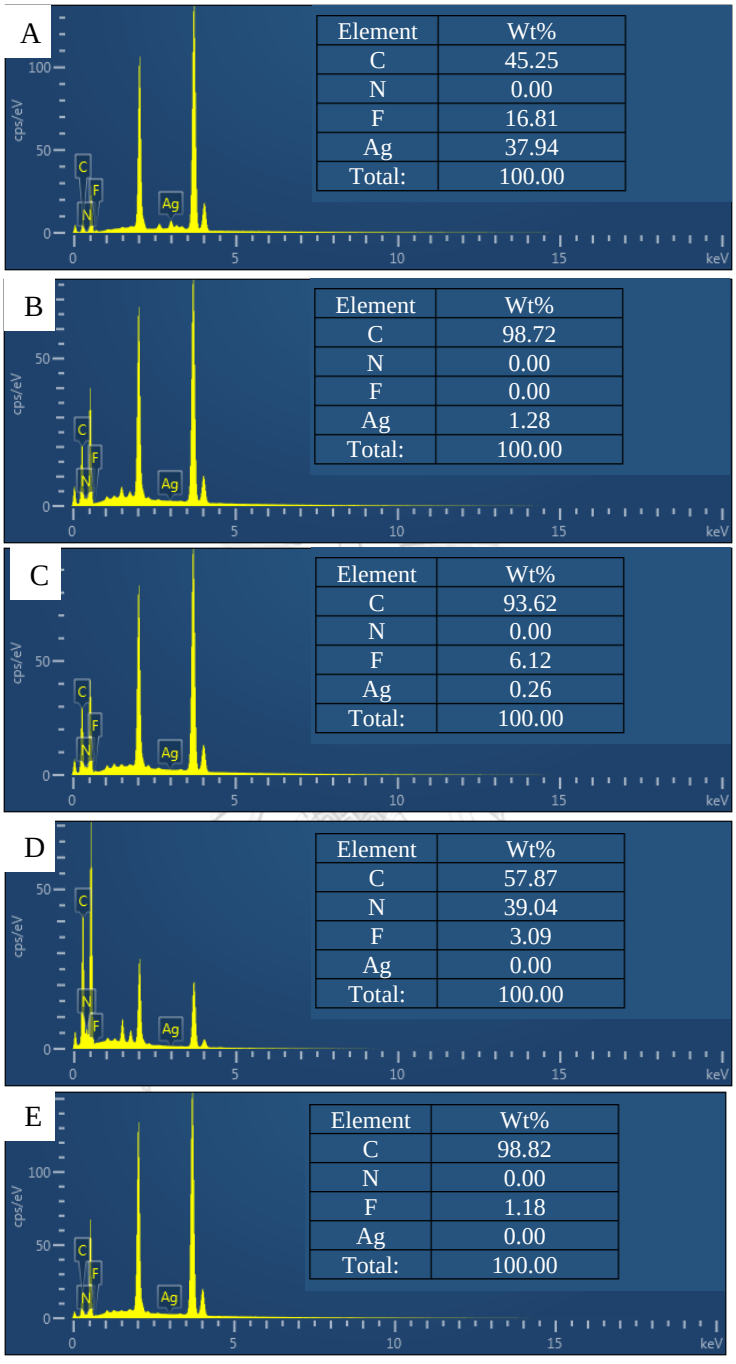

Figure 9: EDS images and elemental analysis on the dentine surface of experimental group showed the detection of percent by weight of Carbon, Nitrogen, Fluoride and Silver, A. Dentine specimen treated with SDF, B. Dentine specimen treated with AgNPs, C. Dentine specimen treated with AgNPs+NaF, D. Dentine specimen treated with NaF, E. Dentine specimen without treated.

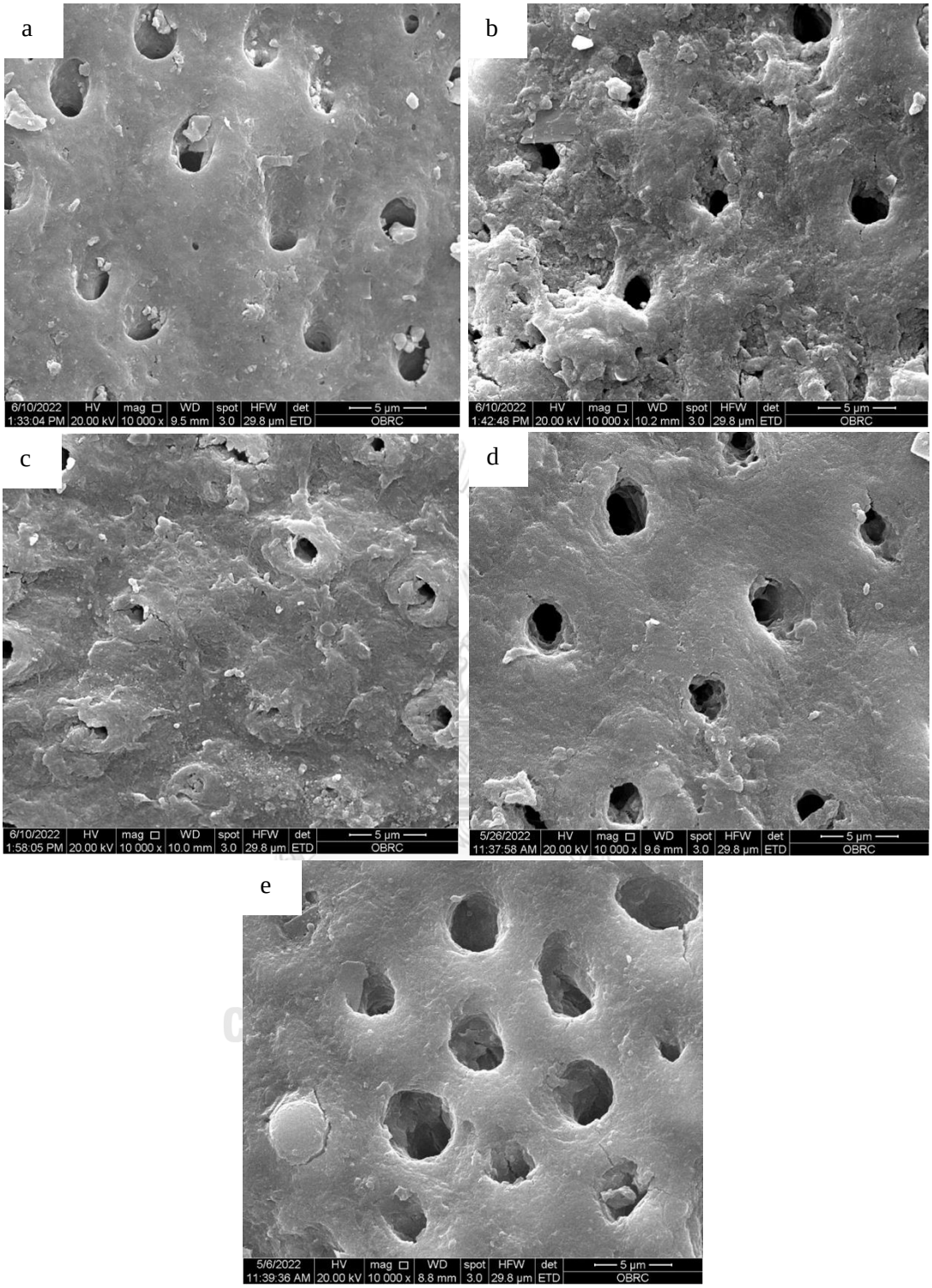

Figure 10 :SEM images showing the dentine surfaces after finished pH cycling process with different material showed the dentine surface with opened tubules and the absence of a smear layer at 10000x magnification, a. Demineralized dentine samples treated with SDF, b. Demineralized dentine samples treated with AgNPs, c. Demineralized dentine samples treated with AgNPs+NaF, d. Demineralized dentine samples treated with NaF, e. Demineralized dentine samples without treated.

CHAPTER V

Discussion

The present study was conducted to evaluate the effect of the applying silver nanoparticles follow by sodium fluoride varnish on remineralization of artificial root caries compared with SDF, AgNPs and sodium fluoride varnish, shown by surface microhardness and lesion depth. The result demonstrated that microhardness of dentin treated with AgNPs followed by NaF varnish-treated and SDF after pH-cycling challenge were similar to pre-treatment value while dentin treated with only AgNPs or NaF showed lower values than pre-treatment value yet higher than non-treated groups. Meanwhile, all treated groups showed reduction of lesion depth over non-treated groups. SDF-treated group provided the lowest lesion depth comparing with other groups. Thus, the null hypothesis was rejected.

This study is a laboratory study based on a chemical model, which is widely used for evaluating dental caries remineralization. The in vitro pH-cycling models are designed to simulate the dynamic variations in mineral saturation and pH associated with the natural caries process. The pH cycling model of the present study is commonly used in caries remineralization studies because they mimic the dynamics of mineral loss and gain occuring in oral cavity.[33, 93] However, the pH cycling model does not include biological factors and the result cannot be extrapolated to the clinical situation and caution should be exercised in their interpretation. The mineral concentration and crystallite size of root dentine changed with age.[73] This study used root dentin from elderly patients to simulate the clinical situation.

Although transverse microradiography (TMR) and polarized light microscope (PLM) are sensitive evaluation methods that can be used for the assessment of remineralization, these methods

are destructive, complicated and specimens will be destroyed in preparation procedures.[94] The advantage of using Micro-CT to measure mineral density are its three-dimensional structure analysis and non-destructive, allowing specimens to be measured again after pH-cycling phase was done. The most common method used to measure the changes in mineral content in laboratory studies was hardness testing.[95] The dentin is anisotropic in a moist environment.[96] The mechanical properties of dentin are greatly influenced by the environment.[97] Knoop hardness testing is recommended for a brittle and/or anisotropic material.[86] In our study, we use the microhardness test to evaluate the superficial hardness of the carious lesions that infer to the remineralization and arrested of caries. The micro-CT was used to show the depth of caries lesions in the cross-sectional view and help to confirm the remineralization which occurs in the root dentine caries lesions.

We were the first study that used root dentine from elderly patients (60 years and more) to simulate the real condition of substrates. The study of Xu et al.[72] demonstrated the old dentin showed a greater hardness, a higher elastic modulus, a greater mineral content and smaller tubular in cervical portion of root than that presented in young dentin. However, the baseline Knoop microhardness of all groups in this study ranges from 64.34±3.49 to 65.58±2.49 which is similar to the normal Knoop microhardness value of dentine ranges from 50-70.[86] The fluoride ions presented using EDS analysis in the control group were probably from natural mineralization.[98] The artificial caries formation in this study can provide a similar hardness value in comparison to naturally caries-affected dentin.[99] According to the study of Tsuda et al. demonstrated the demineralization regimens which is an acetic acid-based solution can replicate the natural root caries lesions.[100]

SDF in this study was employed as a positive control, which was found to be effective in the inhibition of caries lesions. Laboratory studies have found SDF shows good antibacterial action,

increases microhardness, inhibits demineralization, and preserves collagen from degradation in the demineralized dentin.[101] Moreover, many clinical studies have shown that SDF is appropriate for use within patients both coronal caries and root caries.[102-104] SDF is significantly effective in reducing further demineralization and increase the surface microhardness of the dentine lesion compared to other products. This superior effect of the SDF is attributed to its high fluoride concentration (44,800ppmF) and silver component (253,900ppm). Reaction of silver with hydroxyapatite result in the formation of silver phosphate and calcium fluoride. In addition to silver reaction with proteins within the lesion leading to the formation of silver-protein complex which protects collagen from further degradation.[105] SDF showed hyper-remineralization in the body of lesion as estimated from micro-CT. It might because of silver contributed to this high-density mineral profile. EDS analysis revealed that silver was present in this area. The study of Mei revealed the demineralized dentine block treated with 38% SDF had reduced lesion depth after subjected to pH cycling for 8 days because of the interaction between SDF and collagen.[106] Beside, a highly remineralised surface zone, rich in calcium and phosphate was found on the arrested cavitated dentine lesion of the carious primary teeth treated with 38% SDF and the micro-CT examination showed a superficial opaque band on the arrested dentinal lesion.[107] The microhardness of SDF group was increased after subjected to pH cycling process. The study of Chu, reported that the application of SDF increased the microhardness of dentine in primary teeth of preschool children after 30 months as a result arrested dentine formed.[95] Also, the demineralized human dentine blocks treated with SDF which subjected to cariogenic biofilm challenge had increased microhardness and calcium/phosphate weight percentage.[108] Many studies showed

the mean lesion depth of using SDF on carious lesions was less than other materials in the in vitro study.[33, 62, 106] It similar to our study, SDF was found to be successful in increasing the hardness of the artificial root dentine carious lesions. It was thought that the deposition of more silver ions and reaction of silver with hydroxyapatite on the dentine surface of the SDF group may increase surface hardness values and decreased the lesion depth, resulting in a higher microhardness values and lower lesion depth for the SDF group compared to the other groups.

Silver in form of spherical nanoparticles as used in this study showed ability to prevent further demineralization by decelerating the increase of lesion depth after pH-cycle challenge. Silver nanoparticles was proved as antimicrobial agents and have proven to be effective in vitro against cariogenic bacteria such as *Streptococcus mutans*.[109] Many studies showed the good result in antimicrobial properties of using silver nanoparticles in various preparation.[48, 50, 51] The new formulations of silver nanoparticles have been showed the good result in against cariogenic pathogen called nanosilver fluoride (NSF). It proved to be an antimicrobial agent similar to silver diamine fluoride, but less cytotoxicity and not cause tooth staining.[60] The study of Haghgoo et al. reporeted nanosilver fluoride vanish had an antibacterial effect on S.mutans and S.salivarius.[109] For clinical studies, silver nanoparticle in form of nanosilver fluoride showed a good result in arresting caries in six years children.[23] Also, the annual application of 5% nanosilver fluoride showed the same clinical efficacy as SDF in preventing the progression of dental caries in primary posterior teeth.[67] Silver ions are highly reactive and have high polarizing power, consequently part of the silver ions react with hydroxyapatite forming silver phosphate (Ag_3PO_4) and the other part is reduced by the proteins (collagen) resulting in metallic silver attached to the protein (silver-protein complex). Silver nanoparticles was suggested it can decrease dentine demineralization by inhibit the dissolution of

hydroxyapatite and the exposure of collagen by decreasing the production of acid from oral biofilm. This result confirms with the HPO^{2-}_4: amide I ratio was higher on dentine surface treated with AgNPs and NaF than those without AgNPs or NaF.[66] Also, AgNPs can interact with exposed dentine collagen so as to inhibit collagenase activity.[62] Consequently, partially exposed collagen fibers remained as a scaffold for mineral to redeposit into original size of apatite crystals or even delays dissolution of calcium and phosphate as occurred in natural caries-affected dentin.[110] The study of Scarpelli et al. demonstrated AgNPs showed the ability to remineralize enamel 14.63% while SDF was able to remineralize 28.55% of enamel.[111] Also, the study of Yin showed the using silver nanoparticle in addition to sodium fluoride can remineralise dentine caries and the mean lesion depth was lowest.[66]

Efficacy to inhibit the dentin collagenase is depending on the concentration of silver ion. The higher concentration the silver is, the stronger inhibitory effect presents.[112] Silver nanoparticles used in the present study released 0.06 ppm ppb silver ion while SDF released at least 240,000 ppm as implied from our EDS analysis that minimal amount of silver was left on treated surface. Such different released silver ion concentration contributed to difference in lesion depth after pH-cycling challenge as shown in our result (Table 4). With extraordinarily high level of silver ion concentration, SDF showed a promising ability to prevent further demineralization comparing to other agents.

Our results showed that AgNPs group caused a statistically significant higher surface microhardness values and decrease the lesion depth compared to the control group, but significantly higher lesion depth was obtained in comparison with SDF, AgNPs/NaF and NaF groups. This result indicated that AgNPs can decrease dentine demineralization. The study of Zhi et al. demonstrated the silver and fluoride ions can increase the mineral density on demineralized enamel

and dentine, but their synergistic effects were relatively small.[113] We speculate that, if fluoride was incorporated with AgNPs, there will be showed the synergistic effect of silver nanoparticle and fluoride which increase the remineralization of dentine. However, the AgNPs/NaF group showed microhardness values and lesion depth as same as NaF group and still less than SDF group. For this result, it might because the silver concentration of AgNPs solution used in this study was relatively low (0.02 mg/ml) and the non-union of both solutions.

Sodium fluoride varnish is one of the most common topical fluoride products for the prevention of caries. It has been reported prevent and inhibit caries in both permanent and primary tooth. 5% of sodium fluoride varnish was availability, low cost and commonly used in Thailand. The application of topical fluoride can prevent root caries.[114] Topical fluoride applications can arrest or reharden dental caries lesions in dentine.[95] Fluoride deposited on the dentin surface can produce CaF_2 which is a temporary fluoride reservoir. Fluoride ions are released from unstable CaF_2 and subsequently replace the hydroxyl ions in hydroxyapatite, converting to fluorapatites which is much more resistant to acid attack.[115] The high fluoride concentrations can enhance the remineralization of dentin through the formation of fluoro-hydroxyapatite or calcium fluoride-like particles.[116] This remineralization effect will even prevent further demineralization of the carious lesion.[117]

In our result, AgNPs+NaF treated-group showed the microhardness values and lesion depth as same as NaF treated-group. Though AgNPs treated-group showed the Knoop microhardness values similar to NaF treated-group, it had higher lesion depth than NaF treated-group. Some study reported that the remineralization potential of silver nanoparticles was not effective as sodium fluoride varnish and SDF on enamel carious lesions.[63] However, it has been reported that nanosilver fluoride has greater remineralization efficacy than sodium fluoride varnish on the enamel of primary

teeth.[65] The application of fluoride varnish in this study can reduce the demineralization and lesion depth in root dentine carious lesions. The reason for this may be the high concentration of fluoride. While AgNPs had lower concentration, it might be concluded that AgNPs can decrease the demineralization like fluoride varnish but the potential of enhancing the remineralization is still not clear. Besides, the application of AgNPs solution followed with NaF varnish might affect the reaction of the tooth surface and the reagent. The silver ions might react with the tooth surface and leave the by-product that interferes with the reaction of fluoride ions.

Not only releasing of silver ion, availability of fluoride also influences on agents' potential to arrest the caries. Our results clearly indicated fluoride played a crucial role both in maintain microhardness and reducing the lesion depth. Fluoride concentration at higher than 100 ppm is presented in form of calcium fluoride (CaF_2) that acts as a reservoir of calcium and fluoride in low pH condition.[118]The compounds buffer the acidic environment and inhibit further demineralization resulting in lower lesion depth. Concentration and availability of fluoride dictate such buffering capacity. 5% NaF varnish releases fluoride around 22,600 ppm while SDF provides fluoride concentration at 44,800 ppm which means superior potential to arrest caries.

In line with our findings, a highly remineralised surface zone, rich in calcium and phosphate, was found on the arrested cavitated dentine lesion of the carious primary teeth treated with 38% SDF and the micro-CT examination showed a superficial opaque band on the arrested dentinal lesion.[107] Such opaque band should be a regrew apatite crystal since CaF_2 is relatively high water-soluble.[119] The minute amount of fluoride left on surface reacted with calcium and phosphate to form fluorohydroxyapatite[120] and precipitated on partially demineralized crystals left behind in silver-protected collagen scaffold. Moreover, higher pH condition may be favorable for the formation of

apatite crystal, especially fluorohydroxyapatite or fluoroapatite.[121] Together with its alkaine property, higher concentration of SDF also contributes higher availability of fluoride effectively enhanced size of apatite crystal size[120] drawing on the lowest lesion depth and the highest microhardness in SDF group.

The present result also indicated that silver did not play a direct role in increasing microhardness of carious dentin shown as the lowest microhardness in only AgNPs-treated group. Part of the silver ions reacted with hydroxyapatite forming silver phosphate (Ag_3PO_4) and then reacted with chloride precipitating in form of silver chloride (AgCl) as Ag_3PO_4 was hardly detected.[120] Therefore, the precipitation on dentin surfaces in the AgNP-treated group and AgNPs-NaF treated group that was seen on SEM images might be the silver chloride. Also, the EDS analysis can detect the silver ions from both groups. However, the precipitation layer should be investigated with another method. Presence of fluoride in any groups showed favorable results in arresting caries indicated that increase or maintenance of microhardness rather associated with fluoride precipitating on apatite crystal as fluorohydroxyapatite which is higher resistant to acid attack than hydroxyapatite.[118]

In the present study, cariogenic bacteria or biofilm were not involved in either conducting artificial caries or demineralizing challenge. This might limit silver's antimicrobial effect to reduce biofilm formation since AgNPs are well known as the strong antimicrobial properties owing to its small size and enormous surface area and it inhibits the growth and adhesion of cariogenic bacteria.[122] Therefore, the synergistic effect of AgNPs and NaF was not clearly seen as in recent study that showed the use of silver nanoparticle in addition to sodium fluoride increased remineralization in dentinal caries contributing to lowest lesion depth.[66]

Our results suggest either AgNPs or sodium fluoride varnish merely arrests the demineralization process in elderly's root dentin, even though they are not efficient as SDF. Double application of AgNPs and NaF or other types of AgNPs and fluoride may be investigated in further study.

Conclusion

Based on the finding of this in vitro study, silver nanoparticles are not benefit from following application of sodium fluoride varnish in remineralizing artificial root caries. Either silver nanoparticle or sodium fluoride varnish solely remineralize such lesions, yet inferior to SDF.

CHAPTER VI

Miscellaneous

Budget

	Lists	Amount	Price/Unit	Total (Bath)
1	Silver nanoparticle solution (Sigma-Aldrich, Darmstadt, Germany)	1	9,480	9,480
2	38% Silver diamine fluoride solution (Topamine™ Dental life, Ringwood, Australia)	1	2,000	2,000
3	5% Sodium fluoride varnish (Duraphat®, Colgate-Palmolive Ltd, Guildford, UK)	1	850	850
4	Service fee for low speed cutting machine (IsoMet® 1000, Buchler Ltd., Illinois, USA)	4 hrs	400	4,000
5	Service fee for stereomicroscope (ML 9300®, Meiji Techno Co., Ltd., Saitama, Japan)	4 hrs	100	400
6	Service fee for Automatic polishing machine (Nano 2000, Pace technologies Inc., Arizona, USA)	3 hrs	300	900
7	Service fee for Micro-CT (Scanco Medical, Bassersdorf, Switzerland)	25 hrs	500	12,500
8	Service fee for Micro-hardness Tester, FM810, Future-Tech Corp., Kanagawa, Japan)	450 points	10	4,500
9	Service fee for scanning Electron Microscope (JSM-IT300 InTouchScope™ Scanning Electron Microscope, JEOL Inc., Massachusetts, USA)	10 hrs	660	6,600
10	Service fee for elemental analysis (JSM-IT300 InTouchScope™ Scanning Electron Microscope, JEOL Inc., Massachusetts, USA)	15 samples	500	7,500
	Total			48,730

Appendices

1. Raw data of Knoop microhardness values

1.1 Preliminary data of Knoop microhardness value

SDF group																			
Baseline							Pre-chemical treatment microhardness							Post-pH cycling challenge microhardness					
Sample	KHN0_1	KHN0_2	KHN0_3	KHN0_4	KHN0_5	Mean	Sample	KHN1_1	KHN1_2	KHN1_3	KHN1_4	KHN1_5	Mean	Sample	KHN2_1	KHN2_2	KHN2_3	KHN2_4	KHN2_5	Mean
A1	60.92	62.37	62.37	62.37	60.22	61.65	A1	13.52	10.2	15.32	9.73	16.07	12.968	A1	22.09	18.18	14.14	11.3	10.05	15.152
A2	67.05	59.52	64.65	62.37	54.38	61.594	A2	12.27	13.08	16.56	11.53	16.56	14	A2	23.22	20.33	15.05	18.18	12.34	17.824
A3	63.12	61.64	63.88	63.12	60.22	62.396	A3	14.54	13.45	14.88	19.4	19.66	16.386	A3	12.94	12.67	20.89	19.79	19.66	17.19
A4	61.64	63.12	62.37	60.92	63.12	62.234	A4	13.98	18.07	16.56	18.66	17.51	16.956	A4	8.06	12.67	11.42	14.8	15.87	12.564
A5	62.37	64.65	67.05	68.73	66.24	65.808	A5	13.98	13.08	16.97	15.69	16.26	15.196	A5	11.42	11.83	11.3	13.52	8.84	11.382
A6	74.41	72.36	70.39	72.87	73.25	72.656	A6	15.05	10.86	11.59	12.6	14.14	12.848	A6	14.46	15.32	15.59	14.54	16.66	15.314
A7	63.88	66.24	65.44	63.12	62.37	64.21	A7	10.24	13.75	10.44	13.15	15.41	12.598	A7	13.82	18.66	22.4	14.88	14.54	16.86
A8	68.73	61.64	60.22	62.37	60.92	62.776	A8	18.54	13.59	19.79	18.18	20.47	18.114	A8	12.34	17.4	19.79	12.47	16.76	15.752
A9	64.65	61.64	64.65	63.12	65.44	63.9	A9	15.23	18.18	14.71	18.54	16.56	16.644	A9	16.46	19.53	13.59	16.46	13.08	15.824
A10	71.36	71.36	74.15	73.2	69.59	71.932	A10	13.01	13.82	13.67	13.9	13.08	13.496	A10	11.89	19.79	18.18	20.06	19.15	17.814
A11	68.73	68.73	69.59	67.05	64.65	67.75	A11	10.75	14.14	13.15	12.27	12.87	12.636	A11	13.82	12.47	14.63	11.3	10.81	12.606
Total						65.173	Total						14.712	Total						15.298

AgNPs group																			
Baseline							Pre-chemical treatment microhardness							Post-pH cycling challenge microhardness					
Sample	KHN0_1	KHN0_2	KHN0_3	KHN0_4	KHN0_5	Mean	Sample	KHN1_1	KHN1_2	KHN1_3	KHN1_4	KHN1_5	Mean	Sample	KHN2_1	KHN2_2	KHN2_3	KHN2_4	KHN2_5	Mean
B1	65.44	63.12	64.65	61.64	65.44	64.058	B1	11.02	8.1	11.19	13.82	14.06	11.638	B1	10.6	13.3	12.14	12.47	13.37	12.376
B2	63.12	61.64	63.88	63.12	60.22	62.396	B2	11.65	12.27	12.74	13.15	15.41	13.044	B2	14.63	16.26	14.97	13.67	18.18	15.542
B3	51.84	62.25	64.2	63.48	61.36	60.626	B3	17.4	14.88	16.97	19.4	19.66	17.662	B3	14.14	9.86	14.59	13.52	16.36	13.694
B4	63.88	63.12	60.22	56.23	58.17	60.324	B4	11.53	15.41	14.8	15.87	17.08	14.938	B4	10.2	12.67	13.3	13.15	14.3	12.724
B5	77.52	71.37	71.99	71	69.2	72.216	B5	13.3	11.77	11.25	14.88	13.59	12.958	B5	10.5	14.46	10.44	10.97	11.25	11.524
B6	62.15	65.27	61.86	67.93	66.14	64.67	B6	11.77	10.39	14.38	11.36	12.02	11.984	B6	9	8.96	10	8.35	9.16	9.094
B7	65.44	64.65	62.37	62.37	60.92	63.15	B7	11.59	9.77	10.86	9.55	8.8	10.114	B7	12.47	9.41	7.8	7.04	7.96	8.936
B8	63.88	63.12	60.92	60.92	64.65	62.698	B8	16.66	20.33	14.88	14.14	16.46	16.494	B8	7.54	5.46	7.83	9.37	10.34	8.108
B9	67.88	64.65	63.12	60.92	60.92	63.498	B9	15.23	19.79	19.03	18.66	17.29	18	B9	10.1	10.24	12.94	12.34	14.06	11.936
B10	64.65	67.88	63.12	65.44	65.44	65.306	B10	12.6	12.53	12.53	13.59	17.08	13.666	B10	10.2	8.38	8.2	6.93	11.89	9.12
B11	64.65	66.24	68.73	70.47	74.15	68.848	B11	13.15	14.46	14.71	17.4	15.14	14.972	B11	8.96	10.65	10.15	11.36	10.2	10.264
Total						64.344	Total						14.133	Total						11.210

AgNPs+NaF group																			
Baseline							Pre-chemical treatment microhardness							Post-pH cycling challenge microhardness					
Sample	KHN0_1	KHN0_2	KHN0_3	KHN0_4	KHN0_5	Mean	Sample	KHN1_1	KHN1_2	KHN1_3	KHN1_4	KHN1_5	Mean	Sample	KHN2_1	KHN2_2	KHN2_3	KHN2_4	KHN2_5	Mean
C1	63.88	63.12	63.88	61.64	64.65	63.434	C1	10.65	11.59	11.36	14.3	13.52	12.284	C1	11.59	10.6	19.4	16.16	17.51	15.052
C2	67.88	65.44	65.44	67.05	66.24	66.41	C2	20.33	20.89	13.82	15.78	16.36	17.436	C2	11.77	11.08	9.77	7.3	15.32	11.048
C3	67.88	67.88	69.59	70.47	67.88	68.74	C3	15.97	14.63	15.87	16.76	18.66	16.378	C3	10.55	11.65	11.25	10.5	10.75	10.94
C4	65.44	62.37	63.12	60.22	64.65	63.16	C4	13.01	15.78	16.07	19.15	21.18	17.038	C4	11.42	13.08	15.14	12.02	13.3	12.992
C5	68.73	72.27	65.44	70.47	63.88	68.158	C5	11.02	14.54	15.41	17.41	12.8	14.236	C5	9.96	12.27	18.9	15.87	20.47	15.494
C6	66.24	63.88	66.24	66.24	67.05	65.93	C6	12.8	15.69	8.35	9.59	11.38	11.562	C6	15.05	13.82	13.37	12.08	10.24	12.912
C7	65.44	69.59	56.23	65.44	63.88	64.116	C7	11.53	11.71	13.01	12.74	13.3	12.458	C7	7.27	9	10.75	15.32	8.88	10.244
C8	61.64	62.37	63.12	63.12	63.12	62.674	C8	14.97	15.87	17.51	15.78	18.42	16.51	C8	10.6	10.75	10.6	12.6	12.74	11.458
C9	64.65	63.88	63.12	62.37	63.12	63.428	C9	11.42	12.94	12.14	11.25	14.71	12.492	C9	10.91	12.47	12.74	14.14	11.89	12.43
C10	63.88	64.65	64.65	66.24	67.88	65.46	C10	11.77	13.01	11.77	13.98	14.38	12.982	C10	11.36	8.27	11.02	13.3	13.37	11.464
C11	63.88	72.27	66.24	72.27	75.12	69.956	C11	12.8	12.21	14.38	15.14	15.78	14.062	C11	11.59	15.69	12.74	13.9	12.8	13.344
Total						65.587	Total						14.312	Total						12.488

NaF group																			
Baseline							Pre-chemical treatment microhardness							Post-pH cycling challenge microhardness					
Sample	KHN0_1	KHN0_2	KHN0_3	KHN0_4	KHN0_5	Mean	Sample	KHN1_1	KHN1_2	KHN1_3	KHN1_4	KHN1_5	Mean	Sample	KHN2_1	KHN2_2	KHN2_3	KHN2_4	KHN2_5	Mean
D1	63.12	63.88	50.94	63.12	60.92	60.396	D1	11.02	14.54	13.45	18.66	15.59	14.652	D1	12.67	13.67	14.46	11.96	17.4	14.032
D2	66.24	67.05	70.63	67.59	64.34	67.17	D2	13.75	17.51	15.23	16.36	15.97	15.764	D2	22.09	12.53	10.81	11.25	11.42	13.62
D3	67.14	67.88	68.73	69.59	67.88	68.244	D3	11.96	13.82	12.27	12.6	15.69	13.268	D3	8.1	10.1	11.96	13.52	13.59	11.454
D4	62.37	60.92	60.22	59.52	60.22	60.65	D4	12.47	14.38	17.4	10.65	14.71	13.922	D4	15.41	10.7	9.73	6.93	9.73	10.5
D5	67.88	68.73	69.59	69.59	68.73	68.904	D5	14.14	13.67	17.4	19.15	15.05	15.882	D5	10.15	11.96	14.71	6.73	7.24	10.158
D6	67.05	67.05	65.44	67.05	63.88	66.094	D6	15.5	14.97	18.78	17.08	16.76	16.618	D6	9.41	11.89	10.6	10.86	13.01	11.154
D7	64.65	64.65	71.37	66.14	69.79	67.32	D7	16.26	13.01	11.77	18.66	14.54	14.848	D7	11.08	12.94	14.46	6.85	8.53	10.772
D8	60.22	63.12	62.37	65.44	60.92	62.414	D8	13.3	11.48	13.98	15.87	16.66	14.258	D8	10.44	12.21	15.59	15.69	15.97	13.98
D9	67.88	67.05	61.64	61.64	61.64	63.97	D9	13.67	16.66	17.73	13.3	16.87	15.646	D9	10.86	11.77	13.52	12.53	15.14	12.764
D10	65.44	69.59	67.05	62.37	65.44	65.978	D10	15.69	14.3	15.5	12.8	12.53	14.164	D10	12.53	9.82	12.27	15.32	11.71	12.33
D11	70.47	65.44	65.44	69.59	63.88	66.964	D11	17.84	15.87	15.5	16.26	17.4	16.574	D11	12.8	12.21	10.15	14.3	17.84	13.46
Total						65.282	Total						15.054	Total						12.202

Control group																			
	Baseline							Pre-chemical treatment microhardness							Post-pH cycling challenge microhardness					
Sample	KHN0_1	KHN0_2	KHN0_3	KHN0_4	KHN0_5	Mean	Sample	KHN1_1	KHN1_2	KHN1_3	KHN1_4	KHN1_5	Mean	Sample	KHN2_1	KHN2_2	KHN2_3	KHN2_4	KHN2_5	Mean
E1	62.37	63.88	63.12	64.65	66.58	64.12	E1	9.63	8.35	13.3	12.74	14.38	11.68	E1	9.63	5.7	8.35	8.92	10.34	8.588
E2	57.51	63.88	62.37	64.65	66.24	62.93	E2	11.48	13.59	15.32	12.14	13.15	13.136	E2	5.54	8.64	9.46	6.96	9.96	8.112
E3	67.05	67.05	65.44	62.37	64.65	65.312	E3	9.82	13.3	12.67	13.01	16.76	13.112	E3	5.78	5.56	8.24	11.96	8.84	8.076
E4	63.12	62.37	62.37	62.37	64.65	62.976	E4	13.75	12.6	13.9	14.3	15.59	14.028	E4	8.76	7.1	7.51	7.04	7.48	7.578
E5	65.92	70.88	65.7	69.09	66.47	67.612	E5	11.89	15.87	16.16	14.8	17.18	15.18	E5	11.59	12.02	11.02	9.12	9.73	10.696
E6	64.65	69.59	64.65	68.73	60.92	65.708	E6	12.02	13.75	16.36	11.42	11.59	13.028	E6	9.37	9.73	12.21	10.34	10.65	10.46
E7	61.64	65.44	66.24	66.24	63.12	64.536	E7	12.34	9.96	10.6	12.34	10.5	11.148	E7	7.39	6.85	6.37	5.93	7.3	6.768
E8	63.88	60.22	62.37	61.64	62.37	62.096	E8	13.82	15.59	17.62	20.2	20.06	17.458	E8	8.57	5.58	4.92	6.34	8.1	6.702
E9	66.24	61.64	64.65	65.44	62.37	64.068	E9	17.51	19.15	18.78	17.4	17.18	18.004	E9	7.6	7.73	9.24	10.65	9.82	9.008
E10	69.59	63.12	67.05	63.12	60.22	64.62	E10	11.36	10.29	13.3	14.3	16.56	13.162	E10	6.32	6.85	10.05	6.32	7.21	7.35
E11	64.65	66.24	71.36	66.24	63.12	66.322	E11	14.63	11.89	12.27	16.56	17.84	14.638	E11	10.65	9.73	9.37	10.7	10.7	10.23
Total						64.572	Total						14.052	Total						8.506

1.2 Statistic analysis of Knoop microhardness value

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
KHN0	1	.232	11	.101	.825	11	.020
	2	.210	11	.191	.885	11	.119
	3	.177	11	.200*	.915	11	.280
	4	.229	11	.112	.895	11	.161
	5	.125	11	.200*	.979	11	.963
KHN2	1	.201	11	.200*	.901	11	.190
	2	.180	11	.200*	.950	11	.639
	3	.183	11	.200*	.932	11	.426
	4	.170	11	.200*	.908	11	.231
	5	.158	11	.200*	.917	11	.294
KHN1	1	.184	11	.200*	.882	11	.109
	2	.121	11	.200*	.962	11	.796
	3	.194	11	.200*	.887	11	.127
	4	.157	11	.200*	.948	11	.621
	5	.206	11	.200*	.916	11	.284

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

1.2.1 Statistic analysis of Baseline Knoop microhardness (KHN0)

Descriptive

KHN0

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	11	65.1733	3.98230	1.20071	62.4979	67.8486	61.59	72.66
2	11	64.3445	3.49281	1.05312	61.9980	66.6910	60.32	72.22
3	11	65.5878	2.49574	.75249	63.9112	67.2645	62.67	69.96
4	11	65.2822	2.98091	.89878	63.2796	67.2848	60.40	68.90
5	11	64.5727	1.61177	.48597	63.4899	65.6555	62.10	67.61
Total	55	64.9921	2.94846	.39757	64.1950	65.7892	60.32	72.66

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
KHN0	Based on Mean	1.911	4	50	.123
	Based on Median	.931	4	50	.454
	Based on Median and with adjusted df	.931	4	34.662	.457
	Based on trimmed mean	1.684	4	50	.168

ANOVA

KHN0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.738	4	2.934	.321	.863
Within Groups	457.708	50	9.154		
Total	469.446	54			

1.2.2 Statistic analysis of Pre-chemical treatment Knoop microhardness (KHN1)

Descriptive

KHN1

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	11	14.7129	2.01640	.60797	13.3583	16.0675	12.60	18.11
2	11	14.1336	2.53207	.76345	12.4326	15.8347	10.11	18.00
3	11	14.3125	2.15760	.65054	12.8631	15.7620	11.56	17.44
4	11	15.0542	1.11345	.33572	14.3062	15.8022	13.27	16.62
5	11	14.0522	2.15542	.64988	12.6041	15.5002	11.15	18.00
Total	55	14.4531	2.00926	.27093	13.9099	14.9963	10.11	18.11

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
KHN1	Based on Mean	1.924	4	50	.121
	Based on Median	1.202	4	50	.322
	Based on Median and with adjusted df	1.202	4	38.741	.325
	Based on trimmed mean	1.881	4	50	.128

ANOVA

KHN1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.825	4	1.956	.465	.761
Within Groups	210.181	50	4.204		
Total	218.006	54			

1.2.3 Statistic analysis of Post-pH cycling challenge Knoop microhardness (KHN2)

Descriptive

KHN2

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	11	15.2984	2.22017	.66941	13.8068	16.7899	11.38	17.82
2	11	11.2107	2.31836	.69901	9.6532	12.7682	8.11	15.54
3	11	12.4889	1.68508	.50807	11.3569	13.6210	10.24	15.49
4	11	12.2022	1.45647	.43914	11.2237	13.1807	10.16	14.03
5	11	8.5062	1.43728	.43336	7.5406	9.4718	6.70	10.70
Total	55	11.9413	2.84472	.38358	11.1722	12.7103	6.70	17.82

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
KHN2	Based on Mean	1.178	4	50	.332
	Based on Median	.944	4	50	.446
	Based on Median and with adjusted df	.944	4	39.647	.449
	Based on trimmed mean	1.177	4	50	.332

ANOVA

KHN2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	263.687	4	65.922	19.019	.000
Within Groups	173.305	50	3.466		
Total	436.993	54			

Post Hoc Tests : Multiple Comparisons Dependent Variable: KHN2

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1	2	4.08764*	.79385	.000	1.8412	6.3341
		3	2.80945*	.79385	.007	.5630	5.0559
		4	3.09618*	.79385	.003	.8497	5.3426
		5	6.79218*	.79385	.000	4.5457	9.0386
	2	1	-4.08764*	.79385	.000	-6.3341	-1.8412
		3	-1.27818	.79385	.498	-3.5246	.9683
		4	-.99145	.79385	.723	-3.2379	1.2550
		5	2.70455*	.79385	.011	.4581	4.9510
	3	1	-2.80945*	.79385	.007	-5.0559	-.5630
		2	1.27818	.79385	.498	-.9683	3.5246
		4	.28673	.79385	.996	-1.9597	2.5332
		5	3.98273*	.79385	.000	1.7363	6.2292
	4	1	-3.09618*	.79385	.003	-5.3426	-.8497
		2	.99145	.79385	.723	-1.2550	3.2379
		3	-.28673	.79385	.996	-2.5332	1.9597
		5	3.69600*	.79385	.000	1.4496	5.9424
	5	1	-6.79218*	.79385	.000	-9.0386	-4.5457
		2	-2.70455*	.79385	.011	-4.9510	-.4581
		3	-3.98273*	.79385	.000	-6.2292	-1.7363

	4	-3.69600*	.79385	.000	-5.9424	-1.4496

Dependent Variable: KHN2

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	1	2	4.08764*	.96784	.003	1.1910	6.9843
		3	2.80945*	.84038	.025	.2776	5.3413
		4	3.09618*	.80059	.009	.6645	5.5279
		5	6.79218*	.79744	.000	4.3680	9.2163
	2	1	-4.08764*	.96784	.003	-6.9843	-1.1910
		3	-1.27818	.86415	.588	-3.8873	1.3309
		4	-.99145	.82551	.751	-3.5059	1.5230
		5	2.70455*	.82244	.031	.1972	5.2119
	3	1	-2.80945*	.84038	.025	-5.3413	-.2776
		2	1.27818	.86415	.588	-1.3309	3.8873
		4	.28673	.67155	.993	-1.7268	2.3002
		5	3.98273*	.66778	.000	1.9798	5.9857
	4	1	-3.09618*	.80059	.009	-5.5279	-.6645
		2	.99145	.82551	.751	-1.5230	3.5059
		3	-.28673	.67155	.993	-2.3002	1.7268
		5	3.69600*	.61696	.000	1.8498	5.5422
	5	1	-6.79218*	.79744	.000	-9.2163	-4.3680
		2	-2.70455*	.82244	.031	-5.2119	-.1972
		3	-3.98273*	.66778	.000	-5.9857	-1.9798
		4	-3.69600*	.61696	.000	-5.5422	-1.8498

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

1.2.4 Statistic analysis of Pre- chemical treatment and Post- pH cycling challenge Knoop microhardness value (T-test)

Paired Samples Test of SDF group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	KHN1 - KHN2	-.58545	3.15037	.94987	-2.70190	1.53099	-.616	10	.551

Paired Samples Test of AgNP group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	KHN1 - KHN2	2.92291	3.08510	.93019	.85031	4.99551	3.142	10	.010

Paired Samples Test of AgNP+NaF group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	KHN1 - KHN2	1.82364	3.07378	.92678	-.24136	3.88863	1.968	10	.077

1.2.4 Statistic analysis of Pre- chemical treatment and Post-pH cycling challenge Knoop microhardness value (T-test)

Paired Samples Test of NaF group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	KHN1 - KHN2	2.85200	1.76595	.53245	1.66562	4.03838	5.356	10	.000

Paired Samples Test of Control group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	KHN1 - KHN2	5.54600	2.43188	.73324	3.91224	7.17976	7.564	10	.000

2. Raw data of lesion depths

2.1 Preliminary data of lesion depths

SDF group																						
Pre-treatment lesion depth												Post-treatment lesion depth										
Sample	L1_1	L1_2	L1_3	L1_4	L1_5	L1_6	L1_7	L1_8	L1_9	L1_10	Mean	Sample	L2_1	L2_2	L2_3	L2_4	L2_5	L2_6	L2_7	L2_8	L2_9	L2_10	Mean
A1	137.88	136.54	132.53	154.47	140.56	142.27	142.27	136.01	138.21	140.44	140.118	A1	115.85	114.04	114.57	114.52	114.47	114.46	119.52	115.05	117.07	114.57	115.412
A2	124.37	139.57	134.32	144.27	125.42	159.2	139.3	134.32	135.42	144.27	138.046	A2	114.52	113.6	110.5	111.5	114.47	119.4	114.47	114.52	117.46	114.47	114.491
A3	139.75	138.88	141.95	138.51	142.27	136.54	146.01	140.95	141.68	140.65	140.719	A3	114.56	117.14	112.45	117.14	114.41	114.41	114.41	111.62	117.14	116.99	115.027
A4	136.26	142.54	141.21	136.26	141.31	136.26	139.3	141.41	139.4	144.37	139.832	A4	116.06	116.16	116.16	112.94	115.85	111.01	114.52	114.47	111.01	116.06	114.424
A5	138.46	137.45	144.37	141.31	143.93	139.35	136.26	139.35	141.21	141.31	140.3	A5	127.27	107.14	115.15	103.25	109.12	113.13	117.22	116.97	119.09	113.13	114.147
A6	143.27	135	130.95	136.9	148.81	136.66	135.75	144.76	142.85	145.45	140.04	A6	117.27	116.98	113.95	125	121.21	114.03	110.11	125	112.12	103.01	115.868
A7	146.66	141.41	140.97	141.51	148.99	136.36	136.36	136.36	133.33	136.36	139.831	A7	113.33	119.04	122.8	121.21	119.04	113.33	115	115	117.06	114.24	117.005
A8	144.17	134.32	139.35	133.33	143.93	146.15	154.04	139.3	136.81	148.99	142.039	A8	118.18	112.12	110.04	112.02	119.04	125.73	116.07	117.06	111.11	121.21	116.258
A9	141.51	146.46	141.31	139.3	138.04	146.46	145.55	138.99	144.98	140.13	142.273	A9	122.89	116.36	114.57	111.11	124.37	107.84	124.37	116.16	116.16	114.42	116.825
A10	139.83	144.78	140.25	141.61	136.56	133.83	138.67	139.3	144.22	141.76	140.081	A10	143.09	109.42	117.64	117.84	106.13	117.94	112.74	111.11	113.63	109.45	115.899
A11	136.36	137.94	137.64	136.03	141.21	132.99	146.26	141.84	144.25	140.52	139.504	A11	112.69	112.37	116.06	111.11	117.74	112.79	110.25	119.42	114.47	117.69	114.459
Total											140.253	Total											115.437

AgNPs group																						
Pre-treatment lesion depth												Post-treatment lesion depth										
Sample	L1_1	L1_2	L1_3	L1_4	L1_5	L1_6	L1_7	L1_8	L1_9	L1_10	Mean	Sample	L2_1	L2_2	L2_3	L2_4	L2_5	L2_6	L2_7	L2_8	L2_9	L2_10	Mean
B1	134.32	144.27	131.71	164.1	134.32	146.76	147.49	144.27	139.3	131.84	141.838	B1	127.57	123.45	123.98	119.34	129.4	130.48	123.45	121.11	121.39	120.71	124.088
B2	142.99	143.93	138.1	139.3	137.69	146.46	144.57	134.32	138.14	135.57	140.107	B2	135.57	123.63	127.42	129.52	126.16	121.11	126.08	128.68	124.47	126.23	126.887
B3	147.59	139.35	133.33	136.15	143.59	140.86	149.25	133.19	133.84	151.28	140.843	B3	121.21	126.26	121.21	123.07	131.21	123.73	128.68	131.11	132.72	129.4	126.86
B4	140.17	140.96	134.32	133.19	144.27	149.66	145.62	138.04	137.4	145.1	140.873	B4	128.46	129.83	132.99	121.51	126.36	122.05	121.11	121.11	119.65	124.53	124.76
B5	141.83	146.46	142.15	139.3	138.24	133.33	143.59	148.71	137.26	131.51	140.238	B5	124.42	127.94	126.16	120.02	127.79	124.37	127.84	132.79	125.42	126.08	126.283
B6	144.42	138.88	136.26	141.61	149.25	144.78	148.35	134.32	143.07	140.93	142.187	B6	130.11	123.09	129.04	125.15	127.17	124.24	124.75	126.66	121.21	121.03	125.245
B7	132.99	136.36	138.14	141.62	139.73	139.2	140.13	139.83	142.52	133.41	138.393	B7	126.36	123.07	119.65	127.74	119.52	124.32	123.67	121.21	119.4	126.03	123.097
B8	131.41	135.98	133.19	131.51	143.59	138.04	142.62	144.27	140.17	142.62	138.34	B8	123.73	123.73	126.26	137.64	127.69	126.26	119.4	114.47	124.35	135.98	125.951
B9	153.02	160.6	143.03	119.19	124.26	148.81	133.72	134.34	129.29	143.43	138.969	B9	125.89	127.43	122.58	127.64	121.36	129.91	127.94	126.86	124.57	126.26	126.044
B10	147.86	141.79	141.02	141.79	135.89	129.63	141.88	141.88	133.83	141.41	139.698	B10	122.25	126.26	123.33	131.62	125.89	131.48	127.35	119.91	118.04	125.04	125.117
B11	123.23	147.47	143.03	141.51	144.44	140.35	144.84	141.51	140.09	146.19	141.266	B11	128.96	122.31	123.45	121.41	120.87	129.39	125.06	122.85	127.37	125.9	124.757
Total											140.250	Total											125.371

AgNPs+NaF group																						
Pre-treatment lesion depth												Post-treatment lesion depth										
Sample	L1_1	L1_2	L1_3	L1_4	L1_5	L1_6	L1_7	L1_8	L1_9	L1_10	Mean	Sample	L2_1	L2_2	L2_3	L2_4	L2_5	L2_6	L2_7	L2_8	L2_9	L2_10	Mean
C1	138.46	138.14	139.83	144.78	134.61	137.64	148.99	148.18	137.26	142.62	141.051	C1	123.59	126.49	121.41	125.94	138.04	124.35	132.99	125.04	114.42	126.49	125.876
C2	139.56	146.68	128.04	148.14	147.35	127.37	139.22	142.27	143.34	143.57	140.554	C2	124.24	134.14	134.92	120.27	120.59	100.27	118.57	119.06	113.85	115.12	120.103
C3	136.36	134.32	148.14	140.96	132.67	148.14	131.31	138.71	141.01	134.04	138.566	C3	143.51	143.44	112.76	111.11	113.63	121.21	114.48	109.84	126.03	127.28	122.329
C4	131.84	137.62	138.58	149.25	139.3	141.37	138.1	140.05	148.14	143.09	140.734	C4	121.11	122.79	123.44	129.23	125.67	126.26	128.2	125.42	123.71	112.37	123.82
C5	138.39	146.46	144.44	136.9	147.47	144.84	136.36	147.47	137.89	142.85	142.307	C5	136.36	128.2	109.45	114.47	119.52	117.42	129.63	129.35	109.42	118.15	121.197
C6	143.59	139.83	147	141.41	145.52	135.1	138.71	143.84	141.88	134.61	141.149	C6	126.49	126.26	118.2	124.35	120.22	126.92	125.62	126.26	123.07	127.52	124.491
C7	143.33	139.4	144.84	140.33	135.25	142.82	136.36	139.63	140.44	136.54	139.894	C7	136.36	121.21	112.37	117.84	127.94	120.22	114.42	122.89	121.36	114.1	120.871
C8	131.31	129.63	150	129.91	153.84	136.75	146.46	137.64	129.06	136.36	138.096	C8	119.4	123.27	121.36	114.47	119.65	111.11	111.53	126.49	128.2	122.89	119.837
C9	141.41	137.69	145.62	138.04	144.37	137.62	143.33	144.57	136.08	135.98	140.471	C9	141.88	114.53	124.57	117.84	124.57	122.89	112.76	124.78	109.42	111.11	120.435
C10	133.33	141.97	128.96	131.68	151.51	128.78	147.47	130.95	148.81	141.97	138.543	C10	133.74	104.93	115.15	133.33	127.27	115.74	123.23	144.84	118.18	120.53	123.694
C11	143.07	138.68	138.2	138.89	140.72	138.04	137.2	142.15	149.83	148.71	141.549	C11	129.35	131.31	124.78	117.94	124.57	136.36	116.16	109.42	122.29	112.82	122.5
Total											140.264	Total											122.286

NaF group																						
Pre-treatment lesion depth												Post-treatment lesion depth										
Sample	L1_1	L1_2	L1_3	L1_4	L1_5	L1_6	L1_7	L1_8	L1_9	L1_10	Mean	Sample	L2_1	L2_2	L2_3	L2_4	L2_5	L2_6	L2_7	L2_8	L2_9	L2_10	Mean
D1	143.59	139.39	136.46	142.57	140.59	143.03	141.41	140.76	141.02	140.59	140.941	D1	130.59	124.89	118.2	119.52	118.98	122.79	127.74	129.63	124.37	121.21	123.792
D2	141.91	141.36	145.45	145.55	140.9	139.4	142.85	140.5	134.84	138.14	141.09	D2	143.93	119.94	121.11	121.89	117.94	122.79	121.21	116.66	122.27	117.84	122.558
D3	144.03	133.3	139.85	134.48	135.16	141.97	135.54	142.53	138.21	147.57	139.264	D3	118.58	121.79	125.98	122.76	127.1	122.27	117.32	128.2	122.72	122.87	122.959
D4	135	135.1	142.35	146.26	135	143.08	139.74	136.26	142.57	140.37	139.573	D4	134.32	130.34	119.52	119.06	118.87	116.61	121.94	114.1	126.26	116.16	121.718
D5	136.85	139.56	138.21	139.85	142.11	138.25	135.5	139.91	134.77	146.5	139.151	D5	123.13	121.89	129.63	119.45	121.11	124.79	124.89	118.2	126.16	123.63	123.288
D6	145.62	139.73	136.86	140.54	140.91	142.67	135.89	136.36	140.54	135.1	139.422	D6	126.26	125	125.72	133.33	116.16	118.58	120.69	117.32	123.31	117.32	122.369
D7	135	143.93	140.3	142.85	145.83	138.88	140.95	135.75	136.9	142.85	140.324	D7	119.69	126.15	126.56	123.63	120.6	123.63	119.04	123.63	122.72	115.15	122.08
D8	137.62	138.78	134.32	138.88	153.62	136.36	134.32	145.1	138.88	152.57	141.045	D8	125.38	124.42	125.74	122.79	116.16	116.16	119.94	121.11	124.35	126.26	122.231
D9	136.98	144.44	136.9	140.95	144.34	137.87	140.3	139.88	141.78	136.36	139.98	D9	138.63	123.27	116.95	128.78	125.25	131.81	131.81	124.24	121.21	124.16	126.611
D10	139.63	142.25	140.05	141.31	136.24	141.62	138.88	140.05	144.68	139.08	140.379	D10	119.52	131.31	122.89	127.69	128.78	121.21	121.21	112.79	121.36	124.1	123.086
D11	147	141.41	138.88	139.3	142.67	141.84	135.57	137.25	140.05	143.73	140.77	D11	118.97	121.21	121.79	117	127.69	128.68	117.42	123.07	123.73	125.91	122.547
Total											140.176	Total											123.021

Control group																						
Pre-treatment lesion depth												Post-treatment lesion depth										
Sample	L1_1	L1_2	L1_3	L1_4	L1_5	L1_6	L1_7	L1_8	L1_9	L1_10	Mean	Sample	L2_1	L2_2	L2_3	L2_4	L2_5	L2_6	L2_7	L2_8	L2_9	L2_10	Mean
E1	137.84	138.88	148.37	147.35	136.17	139.22	136.17	135.8	141.26	140.24	140.13	E1	149.72	151.74	148.99	154.32	155.47	151.51	161.53	148.99	143.93	144.32	151.052
E2	132.94	136.85	137.86	139.22	145.32	134.14	137.19	138.88	149.06	140.2	139.166	E2	150.17	146.36	149.14	150.15	151.41	152.62	148.88	146.46	147.52	148.98	149.169
E3	139.2	140.25	141.41	135.1	139.74	148.99	143.09	148.99	151.51	131.31	141.959	E3	149.25	146.81	152.47	146.36	153.33	151.31	146.46	150.15	150	156.46	150.26
E4	149.93	149.83	149.66	141.51	142.67	135.94	140.67	138.99	140	131.74	142.094	E4	147.87	148.38	145.73	166.66	151.78	151.24	144.21	154.97	148.48	152.42	151.174
E5	146.06	149.59	149.59	140.09	141.91	133.63	147.47	129.96	149.55	134.33	142.218	E5	145.52	147.72	158.97	141.88	145.15	149.73	154.27	144.61	155.63	145	148.848
E6	145.29	149.57	133.19	147	144.78	144.78	148.18	138.88	140.25	143.19	143.511	E6	153.44	145.2	155.57	148.99	152.6	150.25	154.87	151.51	157.96	153.83	152.422
E7	135.89	130.67	141.53	135.72	148.99	133.19	143.03	149.25	147.77	139.09	140.513	E7	153.93	153.33	155.57	151.31	156.3	158.88	148.14	155.15	150.25	147.43	153.029
E8	146.66	146.06	133.5	146.06	141.51	141.51	143.84	136.46	143.56	134.04	141.32	E8	151.41	169.23	156.56	167.91	146.26	149.73	151.61	148.99	156.46	151.31	154.947
E9	136.81	143.44	141.79	138.48	144.78	141.73	135.1	144.27	136.66	141.41	140.447	E9	153.71	151.51	153.33	150.54	147.94	147.3	149.25	148.04	151.02	158.2	151.084
E10	138.81	141.41	136.06	154.54	139.39	132.12	136.66	137.57	137.87	143.76	139.819	E10	156.36	147	153.33	147	153.33	150.42	148.88	153.44	158.88	157.62	152.626
E11	133.73	137.83	137.87	145.83	141.91	130.95	141.62	140	139.64	140.6	138.998	E11	140.9	174.24	159.59	157.57	157.57	143.93	147.75	152.27	150.79	142.85	152.746
Total											140.925	Total											151.577

2.2 Statistic analysis of lesion depth

Tests of Normality

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Pre- chemical Treatment	1	.211	11	.185	.906	11	.220
	2	.129	11	.200*	.952	11	.667
	3	.197	11	.200*	.931	11	.420
	4	.157	11	.200*	.904	11	.209
	5	.160	11	.200*	.957	11	.734
Post-pH cycling challenge	1	.190	11	.200*	.923	11	.344
	2	.144	11	.200*	.955	11	.710
	3	.163	11	.200*	.941	11	.531
	4	.239	11	.080	.767	11	.003
	5	.135	11	.200*	.962	11	.801

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

a. Lilliefors Significance Correction

2.2.1 Statistic analysis of Pre-treatment lesion depth

Descriptive

Pre-chemical treatment lesion depth

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	11	140.2530	1.15628	.34863	139.4762	141.0298	138.05	142.27
2	11	140.2502	1.30800	.39438	139.3715	141.1289	138.34	142.19
3	11	140.2649	1.35121	.40741	139.3572	141.1727	138.10	142.31
4	11	140.1763	.73788	.22248	139.6806	140.6720	139.15	141.09
5	11	140.9250	1.41463	.42653	139.9746	141.8754	139.00	143.51
Total	55	140.3739	1.20510	.16250	140.0481	140.6997	138.05	143.51

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
Pre-treatment	Based on Mean	1.241	4	50	.305
	Based on Median	.906	4	50	.468
	Based on Median and with adjusted df	.906	4	42.701	.469
	Based on trimmed mean	1.219	4	50	.314

ANOVA

Pre-treatment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.230	4	1.058	.713	.587
Within Groups	74.193	50	1.484		
Total	78.423	54			

2.2.2 Statistic analysis of post-pH cycling challenge lesion depth

Descriptive

Post-treatment

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	11	115.4377	1.00840	.30405	114.7603	116.1152	114.15	117.01
2	11	125.3717	1.17407	.35399	124.5830	126.1605	123.10	126.89
3	11	122.2866	1.98773	.59932	120.9513	123.6220	119.84	125.88
4	11	123.0217	1.32791	.40038	122.1296	123.9138	121.72	126.61
5	11	151.5779	1.79743	.54195	150.3704	152.7854	148.85	154.95
Total	55	127.5391	12.66404	1.70762	124.1156	130.9627	114.15	154.95

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
Post-treatment	Based on Mean	2.066	4	50	.099
	Based on Median	1.777	4	50	.148
	Based on Median and with adjusted df	1.777	4	39.671	.153
	Based on trimmed mean	2.100	4	50	.095

ANOVA

Post-treatment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8547.001	4	2136.750	942.088	.000
Within Groups	113.405	50	2.268		
Total	8660.406	54			

Post Hoc Tests Multiple Comparisons Dependent Variable: Post-treatment

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1	2	-9.93400*	.64217	.000	-11.7512	-8.1168
		3	-6.84891*	.64217	.000	-8.6661	-5.0317
		4	-7.58400*	.64217	.000	-9.4012	-5.7668
		5	-36.14018*	.64217	.000	-37.9574	-34.3230
	2	1	9.93400*	.64217	.000	8.1168	11.7512
		3	3.08509*	.64217	.000	1.2679	4.9023
		4	2.35000*	.64217	.005	.5328	4.1672
		5	-26.20618*	.64217	.000	-28.0234	-24.3890
	3	1	6.84891*	.64217	.000	5.0317	8.6661
		2	-3.08509*	.64217	.000	-4.9023	-1.2679
		4	-.73509	.64217	.782	-2.5523	1.0821
		5	-29.29127*	.64217	.000	-31.1085	-27.4741
	4	1	7.58400*	.64217	.000	5.7668	9.4012
		2	-2.35000*	.64217	.005	-4.1672	-.5328
		3	.73509	.64217	.782	-1.0821	2.5523
		5	-28.55618*	.64217	.000	-30.3734	-26.7390
	5	1	36.14018*	.64217	.000	34.3230	37.9574
		2	26.20618*	.64217	.000	24.3890	28.0234
		3	29.29127*	.64217	.000	27.4741	31.1085

	4	28.55618*	.64217	.000	26.7390	30.3734

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	1	2	-9.93400*	.46664	.000	-11.3334	-8.5346
		3	-6.84891*	.67204	.000	-8.9272	-4.7707
		4	-7.58400*	.50274	.000	-9.0986	-6.0694
		5	-36.14018*	.62141	.000	-38.0479	-34.2325
	2	1	9.93400*	.46664	.000	8.5346	11.3334
		3	3.08509*	.69606	.003	.9560	5.2142
		4	2.35000*	.53443	.002	.7485	3.9515
		5	-26.20618*	.64732	.000	-28.1728	-24.2395
	3	1	6.84891*	.67204	.000	4.7707	8.9272
		2	-3.08509*	.69606	.003	-5.2142	-.9560
		4	-.73509	.72076	.843	-2.9218	1.4516
		5	-29.29127*	.80802	.000	-31.7115	-26.8711
	4	1	7.58400*	.50274	.000	6.0694	9.0986
		2	-2.35000*	.53443	.002	-3.9515	-.7485
		3	.73509	.72076	.843	-1.4516	2.9218
		5	-28.55618*	.67380	.000	-30.5889	-26.5235
	5	1	36.14018*	.62141	.000	34.2325	38.0479
		2	26.20618*	.64732	.000	24.2395	28.1728
		3	29.29127*	.80802	.000	26.8711	31.7115
		4	28.55618*	.67380	.000	26.5235	30.5889

2.2.3 Statistic analysis of Pre-chemical treatment and Post-pH cycling challenge lesion depth (T-test)

Paired Samples Test of SDF group

		Paired Differences					t		
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper		df	Sig.2tailed
Pair 1	Pre-Post	24.81527	1.03284	.31141	24.12140	25.50914	79.686	10	.000

Paired Samples Test of AgNP group

		Paired Differences							
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper	t	df	Sig.2tailed
Pair 1	Pre-Post	14.87845	1.77110	.53401	13.68861	16.06830	27.862	10	.000

Paired Samples Test of AgNP+NaF group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Pre-Post	17.97827	2.14550	.64689	16.53691	19.41964	27.792	10	.000

Paired Samples Test of NaF group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Pre-Post	17.15455	1.55628	.46924	16.10902	18.20007	36.559	10	.000

Paired Samples Test of Control group

		Paired Differences					t	df	Sig.2tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Pre-Post	-10.65291	2.32926	.70230	-12.21773	-9.08809	-15.169	10	.000

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