EFFECTIVENESS OF DIFFERENT ROOT CANAL DISINFECTION PROTOCOLS ON THE REDUCTION OF BACTERIA IN LARGE ROOT CANALS

Miss Parnwad Sasanakul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Endodontology
Department of Operative Dentistry
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ประสิทธิผลของวิธีกำจัดแบคทีเรียแบบต่าง ๆ ต่อการลดปริมาณแบคทีเรียในคลองรากฟันขนาดใหญ่

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By	Miss Parnwad Sasanakul
Field of Study	Endodontology
Thesis Advisor	Assistant Professor PAVENA CHIVATXARANUKUL, Ph.D.
Thesis Co Advisor	Associate Professor Ruchanee Ampornaramveth, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

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

THESIS COMMITTEE

.... Chairman
(Associate Professor PAIROJ LINSUWANONT, Ph.D.)

.... Thesis Advisor
(Assistant Professor PAVENA CHIVATXARANUKUL, Ph.D.)

.... Thesis Co-Advisor
(Associate Professor Ruchanee Ampornaramveth, Ph.D.)

.... External Examiner
(Assistant Professor Tanida Srisuwan, Ph.D.)

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ปานวาด ศาสนกุล : ประสิทธิผลของวิธีกำจัดแบคทีเรียแบบต่าง ๆ ต่อการลดปริมาณแบคทีเรียในคลองรากฟันขนาดใหญ่. (EFFECTIVENESS OF DIFFERENT ROOT CANAL DISINFECTION PROTOCOLS ON THE REDUCTION OF BACTERIA IN LARGE ROOT CANALS) อ.ที่ปรึกษาหลัก : ผศ. ทญ. ดร.ปวีณา จิวัจฉรานุกูล, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.รัชนี อัมพรอร่ามเวทย์

บทนำ: วัตถุประสงค์ของการศึกษานี้ เพื่อประเมินประสิทธิผลของการกำจัดแบคทีเรียโดยวิธีต่าง ๆ ในคลองรากฟันขนาดใหญ่ เพื่อหาแนวทางที่สามารถเป็นไปได้ในการกำจัดแบคทีเรียในกรณีการรักษารีเจนเนอเรทีฟเอ็นโดดอนติกส์ วิธีวิจัย: เตรียมรากฟันกรามน้อยล่างจำนวน 94 ซี่ ให้มีความยาว 10 มิลลิเมตร และมีเส้นผ่านศูนย์กลางของรูเปิดปลายรากฟัน 0.8 มิลลิเมตร จากนั้นสร้างแผ่นคราบชีวภาพของแบคทีเรียเอ็นเตอโรคอคคัส ฟีคัลลิสบนผิวคลองรากฟันจำนวน 92 ซี่ โดยในจำนวนนี้ รากฟัน 2 ซี่ จะถูกนำไปวิเคราะห์การเกิดแผ่นคราบชีวภาพโดยกล้องอิเลกตรอนแบบส่องกราด ส่วนรากฟันอีก 90 ซี่ จะนำเข้ากลุ่มการทดลองต่าง ๆ 9 กลุ่ม (กลุ่มละ 10 ซี่) ดังนี้ 1) รากฟันไม่ได้รับการชะล้างและการฆ่าเชื้อในคลองรากฟัน (Initial) 2) รากฟันถูกชะล้างด้วยโซเดียมไฮโปคลอไรท์ความเข้มข้น 1.5% เพียงอย่างเดียว (1.5N) 3) รากฟันถูกชะล้างด้วยโซเดียมไฮโปคลอไรท์ความเข้มข้น 2.5% เพียงอย่างเดียว (2.5N) 4) รากฟันถูกชะล้างด้วย 1.5N ร่วมกับการใช้อัลตราโซนิกแบบแพสซีฟ (PUI) 5) รากฟันถูกชะล้างด้วย 1.5N ร่วมกับการใช้แปรงทำความสะอาดรากฟัน (NFX) 6) รากฟันถูกชะล้างด้วย 1.5N ร่วมกับการใช้ไฟล์ชนิด XP-endo Finisher (XPF) 7) รากฟันถูกชะล้างด้วย 1.5N ร่วมกับการทำความสะอาดโดยรอบคลองรากฟัน 1 รอบด้วยเคไฟล์ (Circumferential filing, CF) 8)รากฟันถูกชะล้างด้วย 1.5N ร่วมกับไฟล์ชนิด Self-adjusting File 1 นาที (SAF) 9) รากฟันถูกชะล้างด้วย 1.5N ร่วมกับการเตรียมคลองรากฟันด้วยเคไฟล์ขนาด #90-110 (MI) หลังจากทำความสะอาดคลองรากฟันตามที่กำหนดแล้ว จะนำผงของเนื้อฟันส่วนที่ติดกับคลองรากฟันมาวิเคราะห์ทางจุลชีววิทยาในรูปแบบของหน่วยก่อรูปโคโลนี (CFU Count) และนำค่าเฉลี่ยของ CFU มาวิเคราะห์ทางสถิติโดยการวิเคราะห์ความแปรปรวนทางเดียว (One-way ANOVA) ที่ระดับความเชื่อมั่น 95% ผลการวิจัย: ค่าเฉลี่ยของ CFU ในกลุ่ม MI มีค่าต่ำสุด (63.5 CFU/mL) ตามมาด้วยกลุ่ม NFX XPF SAF 2.5N CF PUI 1.5N และ initial ตามลำดับ โดยพบจำนวนแบคทีเรียที่หลงเหลือในกลุ่ม 1.5N จะสูงกว่ากลุ่ม PUI 3.6 เท่า, สูงกว่า 2.5N CF และ SAF 4-5 เท่า, สูงกว่า XPF และ NFX 22 และ 36 เท่าตามลำดับ สรุปผลการวิจัย: การใช้วิธีการเสริมต่าง ๆ สามารถเพิ่มประสิทธิผลในการกำจัดแบคทีเรียในคลองรากฟันขนาดใหญ่ได้ โดยในการศึกษานี้ การใช้แปรงทำความสะอาดรากฟัน (NFX) สามารถลดจำนวนแบคทีเรียได้มากที่สุดโดยไม่สูญเสียเนื้อฟัน

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		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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Parnwad Sasanakul : EFFECTIVENESS OF DIFFERENT ROOT CANAL DISINFECTION PROTOCOLS ON THE REDUCTION OF BACTERIA IN LARGE ROOT CANALS. Advisor: Asst. Prof. PAVENA CHIVATXARANUKUL, Ph.D. Co-advisor: Assoc. Prof. Ruchanee Ampornaramveth, Ph.D.

Introduction: The aims of this study were to evaluate the effectiveness of disinfection methods and determine the most promising irrigation protocol for regenerative endodontics in teeth with large root canals. Methods: Sterilized root samples with 0.8-mm wide apical foramen (n=94) were prepared from human mandibular premolars. Ninety-two samples were infected with *E. faecalis* for 21 days and biofilm formation was verified using scanning electron microscopy (n=2). The 90 infected samples were randomly assigned into 9 groups: no intervention (initial), 1.5% NaOCl irrigation (1.5N), 2.5% NaOCl irrigation (2.5N), 1.5N + intermittent passive ultrasonic irrigation (PUI), 1.5N + intermittent canal brushing with Navitip FX (NFX), 1.5N + intermittent XP-endo Finisher (XPF), 1.5N+circumferential filing (CF), 1.5N + 1-min Self-adjusting File (SAF), and 1.5N + mechanical instrumentation using #90–110 files (MI). Subsequently, the root canal walls were shaved for microbial analysis. The mean colony forming units were determined and analyzed using one-way ANOVA. Results: The mean CFU count was lowest in the MI group (63.5 CFU/mL), followed by the NFX, XPF, SAF, 2.5N, CF, PUI, 1.5N, and initial groups. The remaining bacteria in the 1.5N group was 3.6-fold higher than that of the PUI group; 4–5-fold higher than that of the 2.5N, CF, and SAF groups; and 22-fold and 36-fold higher than that of the XPF and NFX groups, respectively. The 2.5N and 1.5N groups with adjunctive treatments, excluding the PUI group, had significantly fewer remaining bacteria compared with the 1.5N group ($P < .05$). Conclusions: Performing various procedures supplemental to 1.5N improved large root canal disinfection. Adjunctive NFX most effectively reduced the number of bacteria without dentin removal.

Field of Study: Endodontology
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Student's Signature ....
Advisor's Signature ....
Co-advisor's Signature ....

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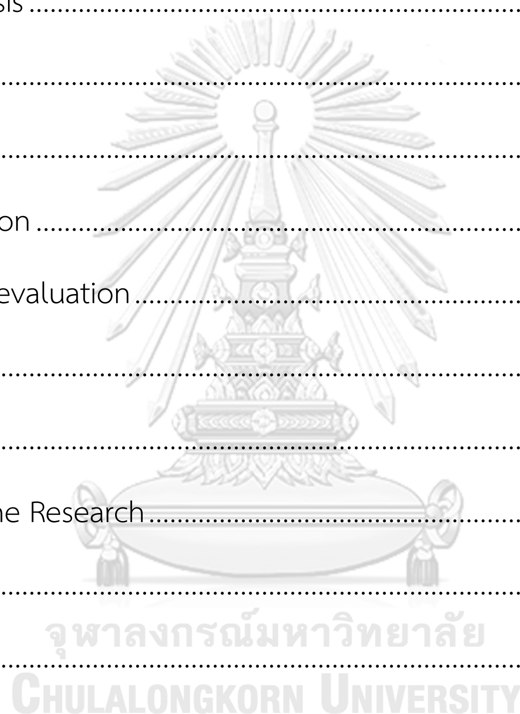
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Chapter 1
Introduction

Background and rationale

The major cause of pulpal and periapical pathosis results from bacteria within a root canal system (1). Therefore, to treat these diseases, creating a sterile system or at least reducing pathologic bacteria to the level that is capable of promoting periapical tissue healing is required for a successful endodontic treatment. Endodontic procedures aiming at disinfecting root canals include processes of mechanical instrumentation, irrigation and medication. Mechanical instrumentation (MI) with endodontic files is the most effective standard method to reduce bacteria (2). After MI, root canals attained a 100 to 1,000 fold bacterial reduction, without using any antibacterial irrigant (2). Moreover, MI together with antibacterial irrigation yielded even lesser residual bacteria (3).

The disinfection steps in regenerative endodontic procedures (REP) are different from that in fully developed teeth. Because thin root canal walls of immature permanent teeth are more susceptible to fracture (4), mechanical instrumentation (MI) which further removes dentin should be avoided (5). Therefore, the disinfection in REP primarily depends on irrigation and medication. Many case reports demonstrated the successful REP treatment where necrotic immature teeth became asymptomatic (6-8). Furthermore, the dentin width and root length are also increased (6-8), which indicate the possibility of disinfection without MI. However, in

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those studies, either full strength of sodium hypochlorite or high concentrations of antibiotics was used as a disinfectant (6-8). While low concentrations of both sodium hypochlorite and antibiotics have been mentioned in the guideline of American Association of Endodotists (AAE) (9).

According to the latest clinical considerations for regenerative procedure launched by AAE in year 2018, the details of disinfection protocol specify the use of 1.5% of NaOCl and root canal medication with 1-5 mg/mL of antibiotic or calcium hydroxide paste (9). These suggestions were given based on advantages in terms of the best survival rate of stem cells residing at apical papilla (SCAPs) and the amount of growth factors released from dentin (10-14). However, previous studies showed that irrigating with 2.5% NaOCl alone or intracanal dressing with 0.1-1 mg/mL of double/triple antibiotic paste was ineffective to remove *E. faecalis* biofilm in simulated root canals (15, 16). It might have took up to 10 mg/mL of double/triple antibiotic paste for a sufficient cleaning (16). Moreover, the failed revitalized teeth closely associated with bacteria left in the root canal system, especially in the form of biofilm (17-19). Therefore, the effectiveness of the disinfection protocols is in doubt if it is adequate to establish an environment suitable for healing clinically.

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Although high concentration NaOCl as well as high concentration antibiotic paste are more effective to eliminate bacteria (16, 20-24), it could lead to a stem cell reduction (14, 25), which may inhibit pulpal regeneration. Using low concentration disinfectants with supplemental procedures such as passive ultrasonic irrigation (PUI)

(15, 26-28) or root canal brushing with Navitip FX (NFX) (29) may result in more bacterial reduction. In addition, minimal dentin removal by XP-endo Finisher (XPF) (30), Self-adjusting File (SAF) or circumferential filing (CF) is possibly be an effective method to enhance bacterial removal in root canals without compromising tooth strength (31-34).

Currently, no study has compared the disinfection effectiveness of those abovementioned procedures applied in addition to 1.5% NaOCl irrigation, using a large root canal model.

Research Objective

The objectives of this study were to evaluate and compare the effectiveness of different disinfection protocols in teeth with large root canals.

Scope of Study

This study is an experimental study performed in extracted human teeth. To simulate immature or large root canal teeth, intact human mandibular premolars were selected with strict criteria. *Enterococcus faecalis* was used as tested species to infect root canal samples. The formation of bacterial biofilm was verified by Scanning Electron Microscope (SEM) inspecting on infected root canal walls. Colony-forming unit (CFU) of remaining bacteria was counted from dentin shavings of inner root canals. Subsequently, the effectiveness on bacterial reduction among experimental

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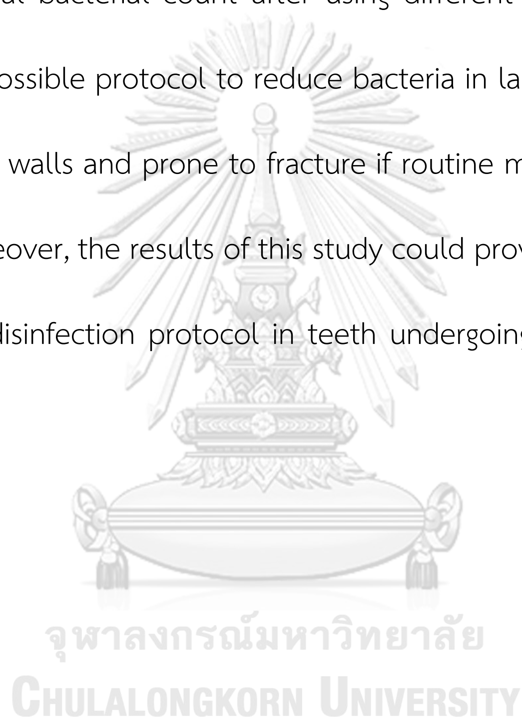
group were compared and statistically evaluated using mean CFU counts of remaining bacteria.

Expected Benefits

The findings from this research could be used as supporting information to improve the effectiveness of disinfection protocol in large root canal teeth. The meaning of residual bacterial count after using different supplemental procedures could indicate a possible protocol to reduce bacteria in large root canal teeth which have thin dentinal walls and prone to fracture if routine mechanical instrumentation is performed. Moreover, the results of this study could provide data useful for further development of disinfection protocol in teeth undergoing regenerative endodontic treatment.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Chapter 2
Review of Literature

Arrested root development of immature teeth can cause difficulties in endodontic treatment from two challenged characteristics. First, Thin dentinal walls make the tooth easily fractured (4). Second, open root apices can cause possibility of extruding root canal filling materials during obturation. Therefore, treatment strategies to deal with these challenges have been advocated. Traditionally, inducing apical closure with long-term calcium hydroxide or creating apical barrier with mineral trioxide aggregate (MTA) is a treatment of choice. Nevertheless, in 2004, Banchs and Trope suggested a new technique to treat immature permanent teeth with apical periodontitis called revascularization or regenerative endodontics. This technique has become an alternative option allowing an increase in dentin thickness and root length in addition to reduction of periodontitis, shown by many case reports (7).

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1. Regenerative Endodontics

Regenerative endodontics is a well-known current treatment alternative for necrotic teeth with incomplete root development. According to American Association of Endodontics (AAE) glossary, “regenerative endodontics” was defined as “biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex” (35). This phrase means that the target of regenerative endodontics is not

only for restoring physical functions of immature teeth but also for gaining biological functions of pulp-dentin complex as well (36). Stated by AAE, there are three goals indicating success of the treatment (9). Primary goal is to eliminate symptoms and show the evidence of bony healing. Secondary goal is to increase root wall thickness and/or increase root length. And tertiary goal is to gain positive response to vitality testing which if achieved, could indicate a more organized vital pulp tissue.

Creating pulp-dentin complex relies on harmonious work of three key components of tissue engineering which are stem cells, signaling molecules and scaffolds. Mesenchymal stem cells, mainly stem cell of apical papilla (SCAP) residing at apical papilla in periapical part of human teeth (37), will be proliferated and differentiated in a scaffold by induction of signaling molecules. For example, induced by transforming growth factor-beta (TGF-ß) can result in osteo/odontogenic differentiation of SCAP and will be supported by a scaffold such as blood clot or artificial scaffold which provides a home for cell organization, proliferation, differentiation and vascularization (38). However, such processes can be interfered by bacterial infections and inflammations in root canals (39). As a result, differentiation and maturation of stem cells will be ceased and pulp-dentin complex will not develop (39). Therefore, a good balance between infection control and stem cell survival is significant for success of regenerative endodontics.

The benchmark of revascularization was set since Banchs and Trope had published the successful revitalized case that achieved all three goals of regenerative

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endodontics in year 2004 (7). After 2 years follow-up, the tooth was asymptomatic with healed apical lesion. Moreover, dentin wall was thickened and apical closure was radiographically evidenced. Besides, the tooth responded to electric pulp testing which is the uppermost indicative of organized pulp tissue formation. The treatment details of this case comprised a step of disinfection, blood clot creation and coronal seal. Considering the disinfection steps, the tooth was not mechanically instrumented but irrigated with 5.25% NaOCl and root canals was dressed with triple antibiotic paste (TAP), which contained minocycline, metronidazole and ciprofloxacin (7). The excellent success of this case drew an attention to novel option for treatment of immature necrotic teeth. In addition, the aforementioned steps had been used as a model for regenerative endodontic procedure.

After many successful cases reported (40), regenerative endodontics has been noteworthy as a promising treatment alternative. American Association of Endodontists (AAE) then has continual developed clinical guidelines for regenerative procedure since 2013 and frequently revised. According to the latest clinical considerations for regenerative procedure launched in year 2018, the details of disinfection protocol propose the use of lower concentrations of disinfectants followed by saline or ethylene diamine tetra-acetic acid (EDTA) (9). This recommendation is based on advantageous evidence in terms of stem cell survival and growth factors released from dentin (14, 25, 41).

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According to the latest protocol revised in year 2018, at the first appointment, irrigation with 20 ml of 1.5% sodium hypochlorite (NaOCl) is advised followed by saline or ethylene diamine tetra-acetic acid (EDTA). After drying canals with paper points, the guideline recommends placing intracanal medicament with calcium hydroxide (CH) or 1-5 mg/mL of triple antibiotic paste (TAP). At the second appointment, only irrigating with 17% EDTA is recommended (9). Nevertheless, procedures in the recently revised AAE protocol are different from the protocol initiated by Banch and Trope (7) as well as those performed in many following successful case reports (40), which used either full strength concentrations of NaOCl or high concentration of TAP.

In year 2012, a retrospective study of Jeeruphan *et al.* showed 100% survival rate of revascularization-treated teeth using 2.5% NaOCl and high concentration of TAP, after 21.15±11.70 months follow-up period. This rate is very promising, compared with MTA apexification-treated teeth and calcium hydroxide apexification-treated teeth which had 94.7% and 77.3% survival, respectively (8). Besides, dentin thickness and root length were significantly increased only in revascularized teeth (8). However, teeth in the regenerative group was disinfected by 2.5% NaOCl and high concentration TAP while AAE guideline recommends the lower concentrations of these disinfectants. The discrepancy between NaOCl and TAP concentrations used in Jeeruphan's study and those recommended by AAE may result in different percent

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of success in regenerative endodontic treatment. This assumption would be found out in the future studies.

References	Number of teeth	Disinfectants	Follow-up period	Survival outcomes
Jeeruphan et al, 2012 (8)	61	- 2.5% NaOCl - TAP	21.15±11.7 months	Revas: 100% MTA: 95% CH: 77.3%
Nagy et al, 2014 (31)	36	- 2.6% NaOCl - TAP	18 months	Revas: 90% MTA: 100% FGF: 80%
Alobaid et al, 2014 (17)	31	- Varying concentration of NaOCl and/or CHX - TAP or BAP or calcium hydroxide paste	14.5±8.5 months	Revas: 95% MTA: 100%
Silujjai et al, 2017 (42)	46	- 1.5%-2.5% NaOCl - TAP or CH	12-93 months	Revas: 88.24% MTA: 82.76%

Table 1: Clinical comparison of survival outcomes

Revas, revascularization

MTA, MTA apexification

CH, Apexification with calcium hydroxide

FGF, Fibroblast growth actor

Although clinical outcome of regenerative endodontic treatment treating with high concentration of disinfectants is quite outstanding (Table 1), unsuccessful revascularized teeth were also reported (17). Alobaid’s study reported 100% clinical

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success of apexification (APEX), using either MTA or calcium hydroxide while a regenerative group (REG) that used non-specific disinfection protocols showed only 79% success rate. Most of the failed cases were caused by reinfection (17). Corresponding to 2 case reports in year 2016, both teeth had been strictly disinfected by AAE protocol. Even though periapical lesion sizes of the teeth were decrease and root maturations were seen, they went symptomatic after 3 months and 12 months follow-up without coronal leakage (19). The other findings of unsuccessful case were reported by Lin *et al.* (18). The root canal walls of the tooth were histologically inspected after disinfection with 5.25% NaOCl, calcium hydroxide and triple antibiotic paste. Bacterial biofilm still presented on root canal walls and irregularities, mainly at apical part, even after treated with those disinfectants. Moreover, bacteria also penetrated considerably in dentinal tubules (18). Therefore, inadequate disinfection could be the major cause of the failure in regenerative endodontic treatment. Moreover, Verma *et al.* evidenced an association of infection with the lack of radiographic growth of root dentin in pulp revascularized teeth in ferrets. It is interesting that they found a significantly higher amount of mineralized tissue formed in teeth with no residual bacteria, compared to teeth with presence of bacteria (43), suggesting the importance of bacterial control.

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The paramount importance of root canal treatment and regenerative endodontic is to thoroughly disinfect root canal systems in order to create an environment suitable for apical healing. In general, disinfection in teeth with

complete root formation depends primarily on chemomechanical preparation. However, as a mechanical instrumentation is limited in immature teeth, only chemical means including irrigation with sodium hypochlorite and medication with either calcium hydroxide or triple antibiotic paste play an important role in root canal disinfection. Although high concentration NaOCl is effective on biofilm eradication (20), in terms of regenerative endodontics, a balance of stem cell survival and profound disinfection is a major concern for a successful treatment. Obviously, concentrations of disinfectants used in regenerative procedure suggested by the AAE considerations are lower than the concentrations used in an early treatment period (40). In this review, disinfection protocols, including chemical, mechanical or supplement steps which may improve bacterial biofilm reduction in regenerative procedure, will be highlighted.

2. Effect of disinfection protocol on bacterial reduction

2.1. Mechanical instrumentation

Although, mechanical instrumentation (MI) cannot establish bacterial free environment for root canal system, considerable microbial reduction is attained from MI regardless of techniques used (44). According to a study of Byström and Sundqvist, bacteria reduced from 10^4 -10^6 cells to 10^2 – 10^3 cells or fewer after MI without antibacterial solution (2). This result was preferable because large amount of infected dentin layers were removed by means of MI. However, the more dentin is removed, the thinner root canal wall will get. Consequently, the thin-walled tooth

will be more prone to fracture than the thicker one (5). Therefore, mechanical instrumentation in immature teeth with thin dentinal walls should be avoided. Another debatable point over MI in regenerative cases is a possibility to jeopardize stem cells, which has not yet been confirmed (45). Previously, many regenerative cases that had been received slightly circumferential filing or complete MI were reported to be successful (31, 33, 34, 46-48). These may imply that mechanical cleaning may not be strongly hazardous to stem cells, as a result, regeneration can occur after root canal were mechanically prepared. Accordingly, it is possible that removal of superficial root canal wall can be performed in a regenerative endodontic case in order to clean more bacteria without compromise tooth strength.

2.1.1. Self-Adjusting File (SAF)

Self-Adjusting File (SAF; ReDent-Nova, Ra'anana, Israel; Figure 1) was introduced as a minimally invasive tool for root canal cleaning (49). SAF is designed to have a hollow tube available in 1.5- and 2.0-mm diameter. It made from nickel titanium lattice with an abrasive surface. The operation of SAF needs to work with a rotary motor which has 3000-5000 oscillations per minute and would provide a vertical vibration at amplitude of 0.4 mm. The abrasive surface can adapt to the root canal 3 dimensionally, along with simultaneous irrigation during preparation (50).

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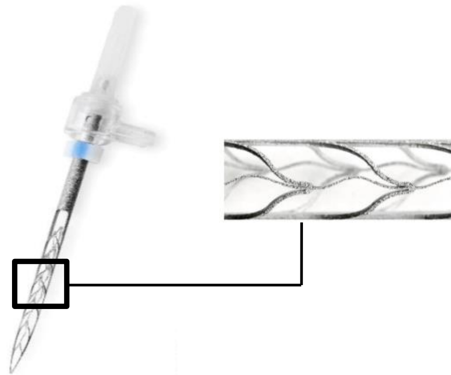

Figure 1: The 1.5-mm hollow tube Self-Adjusting File with an abrasive surface.

Dentin removal ability of SAF is minimal. Starting with the initial canal size of ISO #20, 1.5-mm SAF could remove 60- to 75-μm uniform thickness of dentin layers in 4 minutes. In other words, ISO #35 to #40 of the final canal size was obtained. However, most of dentin removal occurred within the first 2 minutes of operation resulting in an approximate 3-size larger of the root canal (51). According to the same study, a primary canal size has an influence on a compression force created by SAF (51). The force was gradually lesser when increased diameter of root canal. A 300-gram force occurred in 0.25-mm channel while 0.5-mm channel resulted a 150-gram force (51). Consequently, the abrasive ability of SAF would be decreased due to the reduction of compression force (51). Therefore, an initial canal size, file size and working time could affect the amount of dentin removal (51, 52).

Because of an ability to adapt root canal surface, using SAF for 4-5 minutes has been proved that it is more effective in reducing bacterial biofilm in an oval-shaped root canals than other nickle-titanium rotary systems (53-55). Considering the

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shape of an open apex tooth that has a non-conical root canal shape with apical divergence, SAF may be an alternative method for cleaning an irregular-shaped canal of immature tooth without sacrificing too much tooth structure. Although apical extrusion of irrigant can be occurred during SAF instrumentation (56, 57), the incidence was similar to teeth using slot-tipped or close-ended needle (57). However, no study has conducted to evaluate an effectiveness of SAF in large root canal in terms of biofilm reduction and apical extrusion resulted from SAF.

2.1.2. XP-Endo Finisher file (XPF)

The complexity of root canal anatomy is a challenging obstacle for thorough disinfection. Aiming to overcome this difficulty, XP-Endo Finisher file (XPF; FKG Dentaire, La Chaux-de-Fonds, Switzerland; Figure 2) was invented. XPF is made from special nickel-titanium alloy named the NiTi MaxWire® (Martensite-Austenite-electropolish- fleX), available in size of ISO #25 without taper (25/.00). During room temperature, the file is in martensitic phase causing it stays straight. The file is able to transform to a sickle shape due to phase changing into austenitic phase, when temperature raises or it is in use in the patient’s root canal. The curve of XPF file is able to penetrate irregularities and scrape root canal walls 3 dimensionally.

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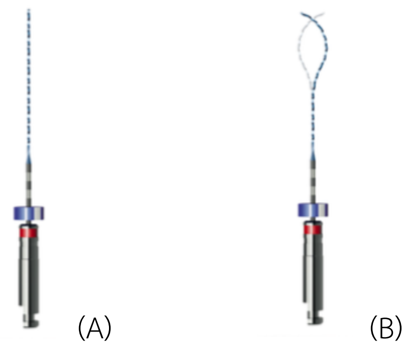

Figure 2: XP-endo Finisher stays straight in martensitic phase (A) and the curve appears during use due to being in austenitic phase (B).

According to the studies evaluated XPF's disinfection ability, they revealed an advantage of XPF over conventional needle irrigation using side-vented needle, Endo Activator (EA; Dentsply, York, PA), Photon-induced photoacoustic streaming (PIPS) or Passive Ultrasonic Irrigation (PUI) (30, 58, 59). XPF significantly reduced more bacterial than PUI even if mechanical instrumentation was given or not (58, 59). Moreover, intermittent use of XPF 20 seconds for 3 cycles provided better efficiency in biofilm removal inside dentin grooves compared with continuous use for 60 seconds, observed by scanning electron microscopy (58). Depicted by confocal laser scanning electron microscopy, less bacteria was found within 50-μm depth dentinal tubules after the use of XPF, compared with side-vented needle, EA and PIPS groups (30). In addition, compared with close-ended needle irrigation and PUI, XPF can remove antibiotic paste in root canals more effectively (60). Therefore, those abilities may be helpful in improving cleaning a large root canal which has limitations in terms of unique anatomy and compromising tooth strength.

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In summary, many minimal mechanical instrumentation protocols, such as circumferential filing with K-file, the use of Self-Adjusting File or XP Endo Finisher, may improve bacterial reduction in infected large root canal tooth or tooth treated by regenerative endodontic procedures without reducing tooth strength (51, 53-55). Effective bacterial reduction may help to create more preferable environment for regeneration of immature necrotic tooth as well as increase a survival outcome of any given endodontic treatments.

2.2. Irrigation

2.2.1 Type of irrigation

Sodium hypochlorite is an effective intracanal irrigant used in endodontic procedures. It is able to dissolve tissue and also inhibit growth of broad range bacteria including obligate and facultative anaerobic bacteria (61). The abilities to dissolve tissue and eliminate bacterial biofilms are dependent on its concentration (20). According to Clegg *et al.*, scanning electron microscopy (SEM) showed that biofilm on dentin sections immersed in 6% NaOCl for 15 minutes were completely removed, as well as negative microbial culture was obtained (20). Despite 15-minute treatment of 3% NaOCl yielded complete biofilm removal, but microbial culture was still positive. The study also showed that the effect of 15-minute 1% NaOCl neither removed biofilm nor gave negative culture (20). These indicated residual bacteria on dentinal walls and within dentinal tubules after exposure to low concentration NaOCl (20).

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In a regenerative endodontic procedure, a low concentration of sodium hypochlorite is more preferable to use as an irrigant (9). This is because 1.5% NaOCl exposure led to greater dentin sialophosphoprotein (DSPP) expression than 3% and 6% NaOCl (25). In addition, the higher concentrations of NaOCl minimized DSPP and survival of SCAP which is a stem cell at apical end of the root important for revascularization (25). Although the final rinse with 17% EDTA could partially reverse such negative effects caused by high concentrations of NaOCl (25), 1.5% NaOCl was still more advantageous to DSPP expression and survival of stem cells (25). However, its effectiveness of bacterial elimination seems to be limited (20). Thus, some additional methods should be applied in order to increase disinfection efficiency without reduction of any markers beneficial to regenerative process.

Ethylenediaminetetraacetic acid (EDTA) has a little effect on killing bacteria (62). In endodontics, EDTA is utilized as a chelating agent that effectively removes smear layer after root canal instrumentation (63). Its capability of demineralizing dentin is useful in regenerative endodontics. Once dentin is demineralized, active molecule such as BMP2, VEGF and TGF-ß which were entombed in dentin during tooth development will be released (10, 64-66). These molecules are important components for regenerative endodontics in terms of dentin formation as well as odontoblast differentiation (65, 67). According to the study reported by Martin *et al*. in year 2014, dentin conditioning with 17% EDTA resulted in a 35% increase in SCAP survival. Moreover, treating dentin with 17% EDTA is able to completely or partially

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reverse the effect of 3% and 6% NaOCl which results in more SCAP survival than those untreated dentine (25). Therefore, EDTA is included in AAE irrigation protocol of regenerative endodontics (68).

Chlorhexidine (CHX) is effective against both gram negative and gram positive bacteria. 0.12% to 2% CHX solution is widely used for antiseptic mouthwash whereas 2% CHX solution is used in endodontic irrigation. CHX is more effective to kill *E. faecalis* than NaOCl in vitro (21, 69). However, CHX is not recommended as the main irrigant used in endodontics because it is not able to dissolved tissue remnants and disrupt bacterial biofilm (20, 70). In terms of stem cell survival, there was no viable SCAP when combination of 2% CHX and 17% EDTA was used. In contrast, 74% stem cell viability was observed in a group irrigated with 6% NaOCl combined with 17% EDTA (12). For these reasons, CHX is not recommended for disinfection in regenerative procedure.

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In summary, although the effectiveness of 1.5% NaOCl on bacterial biofilm reduction is lower than higher concentrations NaOCl (20, 25), AAE recommends the use of 1.5% NaOCl as the main irrigant for regenerative procedure because it is not harmful to stem cells. Therefore, supplement methods to improve bacterial reduction may be necessary. In a regenerative procedure, dentin treatment with EDTA is also recommended since it helps dentin in releasing some signal molecules important for the regenerative process (13, 25).

2.2.2. Delivery techniques

A syringe with needle irrigation is a delivery technique that has been widely used for regenerative endodontic procedure. An advantage of this technique is cost-effective, familiar and controllable. Needle gauge can determine the depth of its tip and also irrigation flow in a root canal (71, 72). For example, the 25-gauge needle could reach the apex of the size 45 root canal, while smaller canal sizes required smaller needle gauges to reach the apices (72). Moreover, a smaller needle tip produces more flow resistance and turbulence which consequences more effectiveness in flushing debris inside root canals (72). Difference of tip designs, a side-vented or open-ended needle, has an effect on apical penetration and extent of apical extrusion. The side-vented needle has a lower risk of apical extrusion because its apical penetration is less than the open-ended needle (73-75). Thus, the side-vented needle probably leads to less effect for stem cells residing at periapical tissue and becomes more preferable for revascularization procedures. However, besides conventional irrigation, there is a variety of irrigation techniques and armamentariums developed for improving effectiveness of irrigants used in root canal treatment, such as sonic/ultrasonic activation systems, negative/positive pressure irrigation devices, laser activating disinfection, brushes, etc.

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2.3. Effect of supplemental protocols on bacterial reduction

2.3.1. Passive ultrasonic irrigation (PUI)

Passive ultrasonic irrigation or PUI is one of the notable ways to improve efficiency of root canal disinfection. With passive ultrasonic irrigation, ultrasonic handpiece passes sound waves to the endodontic file causing vibration at approximately 25,000 times per second. As a result, adjacent irrigant is activated which produces acoustic streaming and cavitation (27). Acoustic streaming is a circular motion of fluid inducing shear stresses that lead to an improvement of root canal cleaning (27) (Figure 3). While cavitation is a bubble in liquid that might create a focus of energy and could help in root canal cleaning (27).

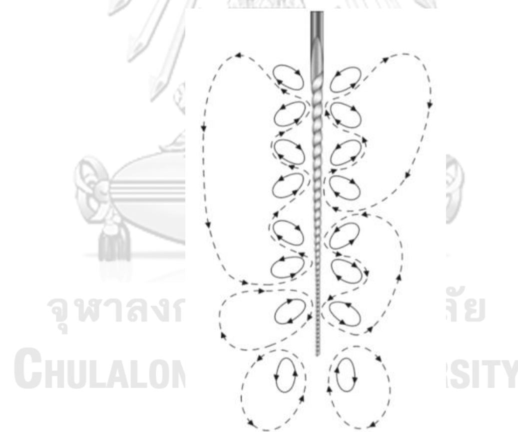

Figure 3: A schematic drawing of acoustic streaming induced around a file (27).

PUI is more effective than syringe irrigation at removing pulpal tissue remnants, debris and bacteria in root canals (15, 76-79). In an in vitro study of Pladisai *et al*. (15), the residual amount of bacteria in *Enterococcus faecalis* biofilm in teeth with large root canals after using different irrigation protocols was evaluated. Without mechanical instrumentation, the number of remaining bacteria in PUI group

was 4.5-fold less than that of the conventional syringe irrigation group (15). In another study, PUI is better in removing calcium hydroxide and triple antibiotic paste in simulated immature roots compared to a side-vented needle, EndoVac and EndoActivator (80). These studies indicate that using PUI in regenerative endodontics could provide benefit to improve bacterial reduction and medicament removal.

Effect of PUI on apical extrusion of irrigation has also been studied. According to study reported by Mitchell *et al*. (81), they investigated and compared the number of teeth that had irrigation extrusion caused by various irrigation techniques in teeth with apical preparation sizes of ISO 30 and 50. The study showed significantly greater extent of apical extrusion in teeth irrigated with PUI, compared to those in teeth using EndoVac irrigating system. These finding occurred in teeth with ISO 30 apical size but did not occur in teeth with ISO 50 apical size. However, PUI had a significantly lower number of teeth with extruded irrigant than that of irrigating using close-ended needle, regardless of apical sizes (81).

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2.3.2. Negative pressure irrigation (EndoVac system)

EndoVac system (Smart Endodontics; Discus Dental, Culver City, CA) is one of the irrigation technique of choices that AAE proposes as a proper delivery method in regenerative endodontic procedures. With EndoVac, the irrigant in the apical part of a root canal is carried away from a root apex by negative pressure of the irrigation system. Therefore, EndoVac minimizes risk of apical extrusion of an irrigant (82-84) which may be harmful to cells at apical area. Because a microcannular has ISO size

of 32, the root canal needs to be enlarged to the ISO size of 35 to facilitate tip penetration (85). However, EndoVac system is not provided in Thailand.

Chemomechanical preparation in close apex teeth cooperated with EndoVac irrigation is able to reduce more than 99% of *E. faecalis* biofilm (86, 87). Although residual bacterial count was lesser than a group of side-vented syringe irrigation, it was not statically significant (86).

In immature teeth with open apices, the benefit of EndoVac in bacterial reduction over conventional irrigation was demonstrated in an in vivo study by Cohenca *et al.* (88) They studied an efficacy of disinfection protocols in immature dog teeth which were induced to form apical periodontitis. The teeth were divided into 2 groups that depended on 2 different protocols. In the first group, 36 immature teeth were disinfected by EndoVac alone, while the teeth in the second group were irrigated with 2.5% NaOCl by side-vented needdle combined with 2-week triple antibiotic paste dressing. The study showed that an EndoVac group yielded more negative culture than the other group significantly. Therefore, EndoVac can be considered to be a promising protocol for root canal disinfection in immature teeth (88).

In terms of apical tissue response and repair, da Silva *et al*. compared histopathological results of immature dog teeth with apical periodontitis after using EndoVac system versus side-vented needle irrigation plus tri-antibiotic intracanal dressing. The results of the negative pressure method presented more intense

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mineralized formations, more structured apical and periapical connective tissue with rich in cells and vessels, and more advanced repair process.

These results determined that negative pressure irrigation could be more preferable than the syringe irrigation in regenerative procedure (89).

2.3.3. Canal brushing technique (Navitip FX)

Navitip FX (Ultradent Products, South Jordan, UT; Figure 4), is a guage-30 open-end irrigation needle covered with a brush. It is used for scrubbing along root canal walls in back-and-forth strokes or clockwise and counter-clockwise movements (29).

Figure 4: Navitip FX, guage-30 open-ended needle covered with bristles

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Navitip FX is beneficial in root canal cleaning evidenced by many reports. It could significantly reduce more debris (90, 91), smear layer (91, 92) and residual calcium hydroxide medication in root canals compared to conventional syringe irrigation (93, 94). In terms of bacterial elimination, Navitip FX combined with 2.5% NaOCl eliminated large amount of *E. faecalis* biofilm (29) and reduced bacterial

count as effective as using 2-minute Self-adjusting file (29). Besides, it was more effective in reducing biofilm and bacterial count than using EndoVac (29).

On our assumptions, the disadvantage of using Navitip FX may occur. Bristles of the brush may dislodge along root canals due to friction created during operation. However, this point has not been proven.

In summary, due to the limitations of immature teeth and regenerative issues considered, a help to improve bacterial elimination in the procedures is necessary. Aiming to maximally reduce bacteria should lead to more preferable treatment outcome. Therefore, minimal dentin removal and supplemental irrigation protocols mentioned earlier are possibly being a promising step used as an adjunct to a conventional irrigation protocol during regenerative procedures.

3. Bacterial biofilm

3.1. Bacterial biofilm related to apical periodontitis

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Pulpal and periapical diseases are mainly caused by bacteria. Many studies demonstrated a relationship between bacteria and these diseases (1, 95). Kakehashi *et al.* (1) found that pulpal tissue exposed to normal flora in the mouths of experimental rats was infected and became completely necrosis after 14 days. In contrast, pathological pulpal and periapical diseases were not present in the group of germ-free rats which bred in germ-free system unit. Many studies demonstrated that infected necrotic pulp contains a large, varying number of bacteria formed in biofilm structure (96-99). In 1987, Nair observed intracanal flora in necrotic teeth with

light and electron microscope (96). He found the colonization of bacteria consisting of rods, cocci, filamentous bacteria and spirochetes with amorphous material in-between (96). This material is an extracellular matrix that helps in co-aggregation or self-aggregation of bacteria (99, 100). Moreover, intraradicular biofilms were found in all root canals with large lesions (>10 mm), while it was found in only 62% of teeth with small lesions (<5 mm) (101). This would be indicated that the presence of bacterial biofilm was associated with the larger sizes of periapical lesions (101).

3.2. Microbial profile in endodontic infections

Endodontic infections is polymicrobial (102). Numerous microorganisms were identified both in primary and persistent endodontic cases. In primary infections, black-pigmented bacteria such as *Prevotella* species and *Prophyromonas* species were frequently found (103). Moreover, *Fusobacterium* species, *Peptostreptococcus* species, *Veillonella* species, *Actinomyces* species, *Eubacterium* and other species were also found and associated with primary infections (102).

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Considering a persistent infection, *Enterococci* is the most frequently found in root canals. The systemic review indicated that the detection rate of *Enterococcus faecalis* using culture method in primary and persistent infections varied from 2% - 13% and 8% - 71%, respectively, whereas the rate using polymerase chain reaction technique varied from 5% - 82% and 10% - 76%, respectively (104). A meta-analysis showed a significantly higher correlation of *E. faecalis* and persistent intraradicular infection compared with primary intraradicular infection (104). *Enterococci* has some

virulence factors which help to withstand harsh environmental conditions. *Enterococcus faecalis* cells are resistant to the antimicrobial effects of calcium hydroxide (105-107) due to an effective proton pump mechanism which maintains optimal cytoplasmic pH levels (108). Besides, it can resist the wide range of antibiotics (109, 110). It can invade dentinal tubules (106, 107, 111, 112) whereas not all bacteria have this ability (111). In addition, it can colonize in the root canals without any support from other bacteria (113). Therefore, *E. faecalis* is the most discussed and studied in endodontic literatures.

Not only was *Enterococci* found but also high prevalence of *streptococci*, *Lactobacilli*, *Actinomyces* and other species were detected in teeth with persistent infection (102, 114-116).

The microbiota of necrotic immature teeth had also been studied (22), with less extent. The profile of bacteria in these immature teeth was similar to that of primary endodontic infection in close apex teeth (22). The frequently found species were *Actinomyces naeslundii* (66.67%), followed by *Porphyromonas endodontalis* (33.34%), *Parvimonas micra* (33.34%), *Fusobacterium nucleatum* (33.34%), *Porphyromonas gingivalis* (26.27%), *Prevotella intermedia* (26.27%), *Tannerella forsythia* (20%), *Filifactor alocis* (13.33%), and *Treponema denticola* (13.33%) (22). Histological section of failed revascularized tooth was also inspected (18). The root canal wall and dentinal tubules in the apical part of the tooth were considerably

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filled with bacterial biofilm (18). This biofilm may be a cause of chronic inflammation and lead to unsuccessful treatment.

4. Study methods for bacterial identification

4.1. Direct visualization

Direct visualization of bacteria can be investigated using electron microscopy. Transmission electron microscope (TEM) and scanning electron microscope (SEM) can visualize bacteria and biofilm trough different processes.

SEM discharges electron beams so that the beams will interact with atoms in a sample. Then the scattered electron will be detected and interpreted into pictures of its surface (117). Therefore, morphological characteristics of bacteria can be visualized by SEM, still, the deeper layers of samples cannot. SEM is widely used to confirm bacterial biofilm formation (117, 118). However, the ability of SEM to visualized bacterial biofilm is often limited by webbing of extracellular matrix masking. Moreover, viability, number and type of bacteria cannot be assessed by SEM.

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Unlike SEM, TEM produces image by transmitting electron beam to the ultrathin specimen so that the primary electron can be detected on the other side of specimen. Therefore, TEM can see through the internal structure of cells because it gives a higher resolution than SEM gives. However, TEM can be costly and only 2 dimensional image is obtained.

Confocal laser scanning electron microscopy (CLSM) is another approach for inspect biofilm directly. Some useful advantages of CLSM over SEM is that both live and dead bacteria can be identified by specific fluorescent probes. Moreover, CLSM provides a topographic data which includes information of both surfaces and deeper layers of dentin. Therefore, it is possible to assess the proportion of live/dead bacteria in biofilm matrix as well as in dentinal tubules (99).

	Advantages	Limitations
TEM	• Very high resolution (< 1nm) • Looking at an internal structure of an object • Looking at relationships between structures	• Cannot looking at living cells • Specimens need to be prepared to an ultrathin section • Costly
SEM	• Looking at object surfaces • Looking at 3D objects	• Not as high resolution as TEM images • Cannot looking at living cells
CLSM	• Looking at living cells • Looking at relationships between cells • Highlighting specific component of cells	• Low resolution • See only objects labeled with fluorescent dye • Artifacts caused by fluorescence

Table 2: Advantages and limitations of TEM, SEM and CLSM

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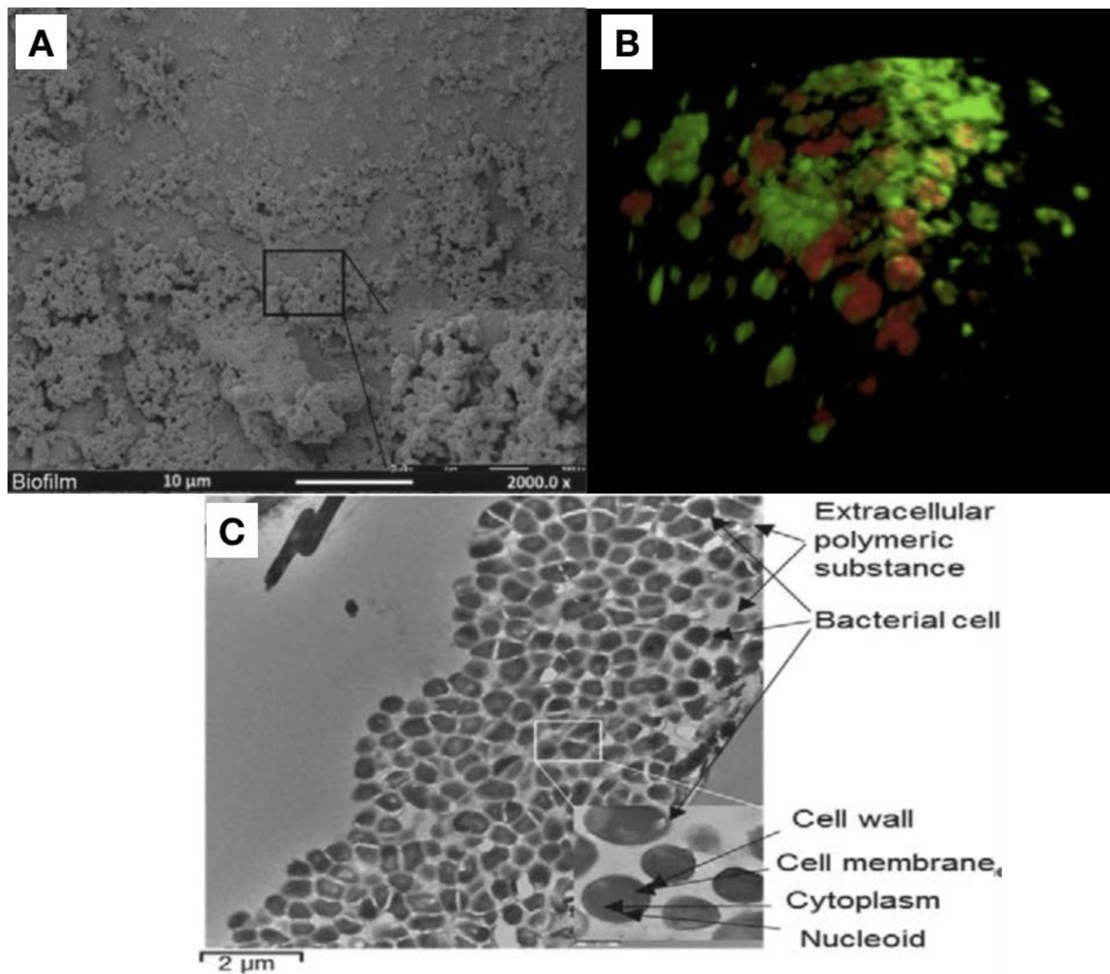

Figure 5: Images of 10-day old E. faecalis depicted by SEM (A), CLSM (B), and TEM (C) (119)

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4.2. Culture method

Culture method is a traditional way to identify bacteria. It comprises many steps; sample collection, transportation, dispersion, dilution, cultivation, isolation, and identification. Bacterial samples must be cultivated in the proper physiochemical environment. Suitable nutrient media, pH, temperature, moisture and oxygen conditions are essential for bacterial growth. Therefore, one of the limitations of culture method is impossible to recover all bacteria in root canals due to many specific requirements for each type of bacteria. Moreover, technique used is sensitive

and requires some expertise. However, culture method allows bacterial quantification of viable cells in the form of colony forming unit count which is useful in a study for comparison between experimental groups (120). The advantages and limitations of culturing method were summarized and presented in table 2 (120).

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Advantages	Limitations
• Broad-range nature, identification of unexpected species • Allow quantification of all major viable cultivable microorganisms in samples • Allow determination of antimicrobial susceptibilities of isolates • Physiologic studies are possible • Pathogenicity studies are possible • Widely available	• Impossibility of culturing a large number of extent bacterial species • Not all viable bacteria can be recovered • Once isolated, bacteria require identification using a number of techniques • Misidentification of strains with ambiguous or aberrant phenotypic behavior • Low sensitivity • Strict dependence on the mode of sample transport • Samples require immediate processing • Costly, time consuming, and laborious, as for cultivation of anaerobes • Specificity is dependent on experience of microbiologist • Extensive expertise and specialized equipment needed to isolate anaerobes • Takes several days to weeks to identify most anaerobes

Table 3: Advantages and limitations of culture method (120)

4.3. Molecular method

Molecular-based methods; such as polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), offer sensitive and direct detection of microorganisms. 16s rRNA gene or 16s rDNA gene have been used for identifying both known and unknown bacteria. Therefore, new strains microorganism can be detected which gives more insight of complexity of bacterial community. Traditionally, molecular methods can detect both viable and non-viable bacteria which are sometimes unwanted. However, reverse transcription-PCR (RT-PCR) was developed to notice only mRNA of the recent dead cells. Only bacterial cells killed by ethanol or heat within 2-16 hours will be detected by RT-PCR. Due to high sensitivity and specificity of these molecular-based methods, some expertise is needed for quality control which is critical for molecular testing. (120).

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Advantages	Limitations
• Detect both cultivable and as-yet-uncultivated species or strains • High specificity and accurate identification of strains with ambiguous or aberrant phenotypic behavior • Detect species directly in clinical samples • High sensitivity Rapid; most assays take no more than minutes to a few hours to identify a microbial species Do not require carefully controlled anaerobic conditions during sampling and transportation • Can be used during antimicrobial treatment • Anaerobic handling and expertise not required • Samples can be stored frozen for later analysis • DNA can be transported easily between laboratories • Detect dead microorganisms	• Most assays are qualitative or semiquantitative (exceptions: real-time PCR) • Most assays only detect one species or a few different species at a time (exceptions: broad-range PCR, checkerboard, microarray) • Most assays detect only the target species and fail to detect unexpected species (exception: broad-range PCR) • Some assays can be laborious and costly (e.g., broad- range PCR) • Biases in broad-range PCR introduced by homogenization procedures, preferential DNA amplification, and differential DNA extraction • Hybridization assays using whole genome probes detect only cultivable species • Can be very expensive

Table 4: Advantages and limitations of molecular-based methods (120)

Chapter 3
Methodology

Target Population

Infected large root canal or immature tooth with thin root canal walls

Sample

Intact mandibular premolars with single and straight root canals

Definition

The term 'Large root canal' in this study refers to intact mandibular premolars which were prepared to obtain 0.8-mm in apical diameter. According to Cvek's study (121), 0.8-4.8 mm apical width were selected for treatment as immature teeth with incomplete root development and wide apical opening. Therefore, this study acquired the large root canal teeth by removing the apical part of those intact premolars without involving root canal preparation. Thus, natural root canal walls and dentinal tubules were preserved. In addition, the coronal parts of the teeth were removed to standardize the length at 10 mm as well as coronal opening at 3-4 mm in bacco-lingual width and 1-2 mm in mesio-distal width (Figure 6).

Independent Variables

Different root canal disinfection procedures were assigned to root samples as follows;

1. No intervention (initial)

2. Syringe irrigation with 1.5% NaOCl (1.5N)

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3. Syringe irrigation with 2.5% NaOCl (2.5N)
4. 1.5N with intermittent passive ultrasonic irrigation (PUI)
5. 1.5N with intermittent canal brushing with Navitip FX (NFX)
6. 1.5N with intermittent operation with XP-Endo Finisher (XPF)
7. 1.5N with circumferential filing (CF)
8. 1.5N with Self-Adjusting File operation (SAF)
9. 1.5N with standard mechanical instrumentation (MI)

Dependent Variables

The number of remaining bacteria in shaving dentin retrieved from root samples after treating with assigned procedures represented by colony-forming unit count (CFU)

Control Variables

Size of samples, initial number of bacteria inoculation, bacteria incubation period, type of needle, type of irrigant, volume of irrigant, irrigation rate, needle penetration dept

Confounding Factors

Laboratory techniques and root canal irregularities

Hypothesis

H_o : There is no significant difference in bacterial reduction among groups treated by 1.5% NaOCl irrigation, 2.5% NaOCl irrigation, 1.5% NaOCl irrigation

supplemented with passive ultrasonic irrigation; NFX brushing; XP-Endo Finisher; circumferential filing; Self-Adjusting File; or standard MI.

H_A : There is a significant difference in bacterial reduction among groups treated by 1.5% NaOCl irrigation, 2.5% NaOCl irrigation, 1.5% NaOCl irrigation supplemented with passive ultrasonic irrigation; NFX brushing; XP-Endo Finisher; circumferential filing; Self-Adjusting File; or standard MI.

Ethical Considerations

The study protocol was submitted for approval by the Ethics committees of Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand (#059/2017).

Materials

1. Intact permanent mandibular premolars with straight and single root canals
2. Precision saw (ISOMET 1000, Buehler, USA)
3. Composite resin (Filtek™ Z350; 3M EPSE, MN, USA)
4. Nail polish (OPI Products, CA, USA)
5. Silicone putty (Silagum putty; DMG Chemisch-Pharmazeutische, Hamburg, Germany)
6. *Enterococcus faecalis* (ATCC 29212)
7. Brain heart infusion broth (Himedia, Mumbai, India)
8. Brain heart infusion agar (Himedia, Mumbai, India)

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9. 0.1% Thymol solution (Faculty of Dentistry, Mahidol University, Bangkok, Thailand)
10. 2.5% NaOCl solution (Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)
11. 17% Ethylenediaminetetraacetic acid (EDTA) (Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand)
12. 1% Phosphate buffer saline (PBS)
13. 10% Sodium thiosulphate (Emsure, Darmstadt, Germany)
14. Distilled water
15. Test tubes
16. 1.5 ml Eppendorf tube (Eppendorf North America, Hauppauge, NY)
17. 25-gauge side-vented needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA)
18. Irrisafe tip (K20/21mm; Acteon, NA, USA)
19. Piezoelectric ultrasonic device (P5 Newtron; Satelec, Acteon Group Merignac, France)
20. 30-guage Navitip FX needle (Ultradent Products, UT, USA)
21. XP-Endo Finisher file (XPF; FKG Dentaire, Switzerland)
22. Self-adjusting file (SAF, Redent-Nova, Ra'anana, Israel)
23. Endodontic micromotor system (X-smart; Densply Maillefer, Ballaigues, Switzerland)

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24. #40 barbed broach (Densply Maillefer, Ballaigues, Switzerland)
25. #60 H-type file (Densply Maillefer, Ballaigues, Switzerland)
26. #50, #80, #90, #100, #110 K-type file (Densply Maillefer, Ballaigues, Switzerland)
27. Peeso reamer

Sample size

This research is an in vitro study performing in an open-apex tooth model. Ninety root samples were divided into 9 groups subjected to different interventions. One of these groups was served as an initial control group represented an initial number of bacteria prior to treatments.

The sample size for each group was calculated using G*Power program, version 3.1. To determine a statistical power for one-way ANOVA, an effect size; number of groups; type I error (α); and power (1- type II error) were input. An effect size was calculated using a mean and standard deviation within groups of the previous study (15), which equals 0.8049845. The number of groups was 9. Type I error (α) and power were given as 0.05 and 0.95, respectively.

The computed output suggests that a total sample size is equivalent to 45. In this study, we doubled the suggested sample size to compensate sampling error and increase the level of precision. Therefore, the total sample size for statistical analysis was 90 and the number of samples of each group was 10.

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Sample preparation

Sample collection

Intact human mandibular premolars extracted for orthodontic reason were collected and stored in 0.1% Thymol (Mahidol University, Bangkok, Thailand) solution until used.

Inclusion criteria of samples are intact human mandibular premolars extracted for orthodontic reason from patients under the age of 25 (122), having completely developed roots with single and straight root canals (Schneider angle ≤ 5°) (123). The presence of the single canal was pre-determined by radiographs taken in mesio-distal and bucco-lingual directions.

The teeth with root caries or previous endodontic treatment or having root length less than 13 mm were excluded.

Root canal preparation

Three to four millimeters of apical root portion of 94 selected mandibular premolars was removed with precision saw (ISOMET 1000, Buehler, USA) in order to eliminate ramification and obtain a 0.8-mm apical foramen diameter (Fig. 6C) (121). To confirm a diameter at apical end of root samples, #80 K-type file (Densply Maillefer, Ballaigues, Switzerland) was inserted into root samples so that the tip of the file would fit to the apical end of samples (Fig. 6C). The length of the samples was set at 10 mm (Fig. 6A) standardized by removing a coronal part with ISOMET 1000.

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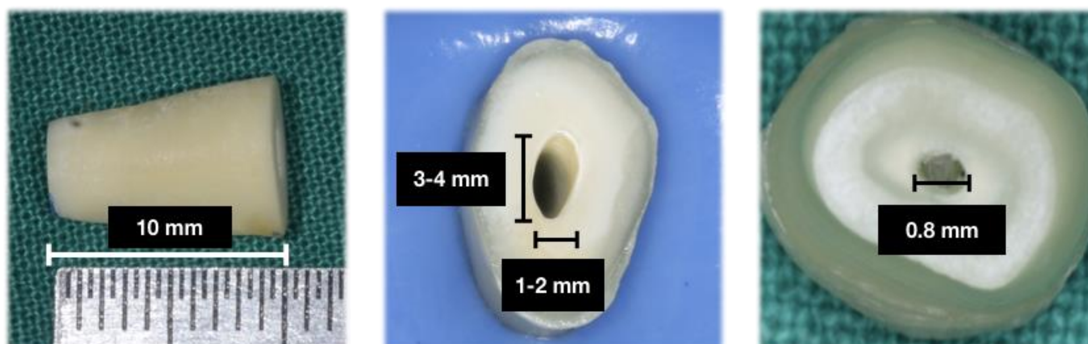

Figure 6: Sample preparation to 10 mm in length (A) 3-4 mm in bacco-lingual width, 1-2 mm in mesio-distal width (B) and 0.8 mm apical foramen diameter (C)

Pulp tissue was gently removed with barbed broach size 40 (Densply Maillefer, Ballaigues, Switzerland) and H-type file (Densply Maillefer, Ballaigues, Switzerland) size 60. An apical end of all samples was capped with composite resin (Filtek^{TM} Z350; 3M EPSE, MN, USA) to create an apical seal. The external root surfaces were coated twice with nail polish (OPI; OPI Products, CA, USA). Silicone blocks (Silagum putty; DMG Chemisch-Pharmazeutische, Hamburg, Germany) were individually customized for each sample to hold it in an upright position. All root canals were sequentially irrigated by 5 mL 2.5% NaOCl, 5 mL 17% EDTA and 5 mL 2.5% NaOCl to remove residual pulp and smear layers. Five milliliters of 10% sodium thiosulfate and 5 mL sterile distilled water were subsequently irrigated to neutralize a NaOCl reaction. Finally, the samples and the silicone blocks were sterilized by means of ethylene oxide gas sterilization.

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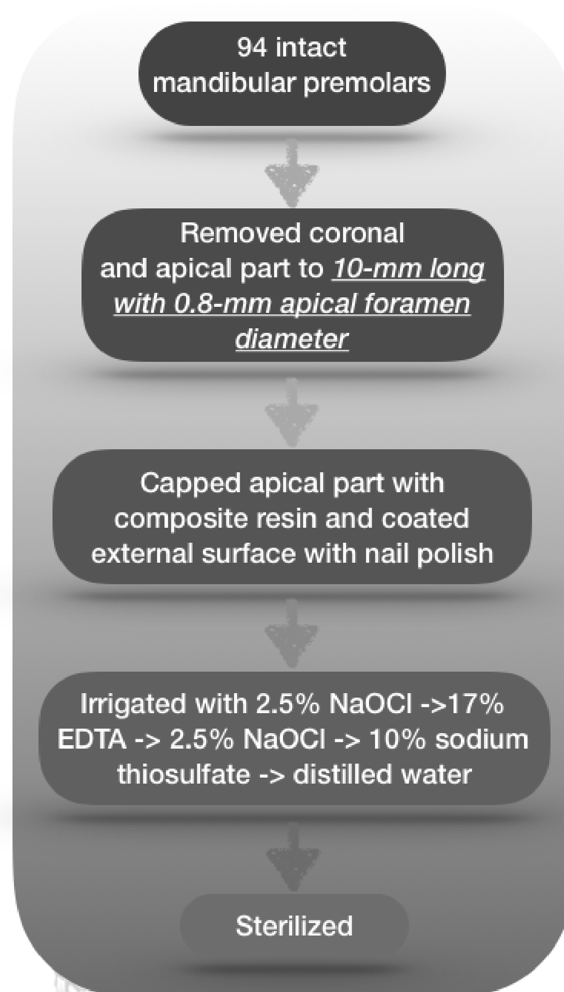

Figure 7: Sample preparation diagram

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Bacterial inoculation

Biofilm of *Enterococcus faecalis* (ATCC 29212) was established in root canals. *E. faecalis* in brain-heart infusion (BHI) broth (Himedia, Mumbai, India) at optical density of 0.5 McFarland at 600 nm (1.65×10^8 CFUs/mL) was used in the inoculation.

Ninety root samples were contaminated with 10 mL of *E. faecalis* suspension, while 2 samples were immersed in sterile BHI media to serve as sterile samples. All samples were incubated for 21 days at 37°C with 5% CO_2 atmosphere. The suspension as well as the broth, in which the sterile samples were immersed, were

refreshed by 9 ml of fresh sterile BHI media every other day. Media contamination was confirmed with gram stain and colony forming morphology on BHI agar plates. After 21 days of incubation, all root samples were washed with 30 ml of 1% phosphate buffered saline (PBS) and subsequently mounted in customized silicone blocks made for each individual ready to use in the experiment.

Experiment

Ninety infected specimens were randomly divided into 9 groups. Each group includes 10 root samples which were treated by assigned protocols as follows:

Group 1: Initial group (Initial)

No intervention was applied for this group representing initial number of bacteria.

Group 2: 1.5% NaOCl irrigation (1.5N)

The irrigation protocol of this group was followed the recommendations of AAE Clinical Considerations for a Regenerative Procedure Revised 4/1/2018 (9).

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The root canals were irrigated with 20 mL 1.5% NaOCl for 5 minutes by 25-gauge side-vented needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) placed at 1 mm short of root apex (68, 74).

Group 3: 2.5% NaOCl irrigation (2.5N)

The irrigation protocol of this group was followed the recommendations of AAE Clinical Considerations for a Regenerative Procedure Revised 4/1/2018 (9), except the concentration of NaOCl.

The root canals were irrigated with 20 mL 2.5% NaOCl for 5 minutes by 25-gauge side-vented needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) placed at 1 mm short of root apex (68, 74).

Group 4: Passive ultrasonic irrigation (PUI)

Prior to the procedures, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, three cycles of ultrasonic activation and syringe irrigation were performed. First, Irrisafe tip (K15/21mm; Acteon, NJ, USA) mounted in a piezoelectric ultrasonic device (P5 Newtron; Satelec, Acteon Group Merignac, France) was inserted in the root canal at 1 mm short of root apex, then the device at power setting of 5 was activated for 20 seconds (26). Then, the root canal was irrigated with 4 mL 1.5% NaOCl for 1 minute. After 3 cycles of the operations, each canal was subsequently rinsed with 4 mL 1.5% NaOCl.

Group 5: Canal brush (Navitip FX, NFX)

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The brush-covered 30-guage needle Navitip FX (Figure 7) or NFX (Ultradent Products, UT, USA) was used in this group. Originally, NFX is designed to be used as an irrigation needle with simultaneous scrubbing along the root canal. However, because it is an open-end needle, only scrubbing action was used in the study to reduce the chance of apical irrigant extrusion.

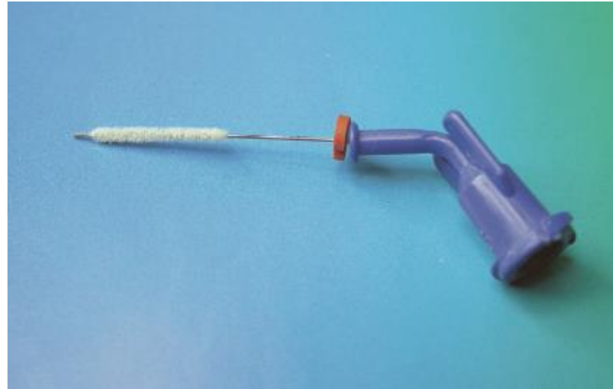

Figure 8: Navitip FX (Ultradent) (90)

Prior to the procedures, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, 3 cycles of scrubbing with NFX and syringe irrigation were performed. First, the brush was inserted in the root canal at 1 mm short of root apex, and scrubbed the root canals with in-and-out motion at 9-mm working length for 20 seconds. Then, the root canal was irrigated with 4 mL 1.5% NaOCl for 1 minute. After 3 cycles of the operations, each canal was subsequently rinsed with 4 mL 1.5% NaOCl.

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Group 6: XP-Endo Finisher (XPF)

XP-Endo Finisher file (XPF; FKG Dentaire, Switzerland) used in the study has 21 mm in length and ISO 25 in diameter with zero taper. Prior to the procedures, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, 3 cycles of XPF operation and syringe irrigation were performed. First, XPF file attached with endodontic micromotor system (X-smart; Densply Maillefer, Ballaigues, Switzerland) was operated at the setting of 800 rpm and 1 Ncm. The file was inserted in the root

canal at 1 mm short of root apex functioned with in-and-out motion at 9-mm working length for 20 seconds. Then, the root canal was irrigated with 4 mL 1.5% NaOCl for 1 minute. After 3 cycles of the operations, each canal was subsequently rinsed with 4 mL 1.5% NaOCl.

Group 7: Circumferential filing (CF)

Prior to the procedures, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, root canals was undergone a circumferential filing performed using #50 K-file (Densply Maillefer, Ballaigues, Switzerland) placed at 9-mm working length, and worked with the pulling stroke along the root canals in clockwise direction for 1 round. Then, all root samples were irrigated with 16 mL 1.5% NaOCl.

Group 8: Self-adjusting file (SAF)

Prior to the procedures, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, root canals were obtained a minimal debridement by SAF (51). The 1.5-mm Self-Adjusting File (SAF; Redent-Nova, Ra'anana, Israel) attached with endodontic micromotor system (X-smart; Densply Maillefer, Ballaigues, Switzerland) was operated at the setting of 5000 rpm. The file was inserted in the root canal by pecking motion for 1 minute along with simultaneous irrigation at the rate of 4 ml/minute until the file reached the 9-mm working length. Then, all root samples were irrigated with 12 mL 1.5% NaOCl.

Group 9: Standard mechanical instrumentation (MI)

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Prior to MI, the root samples had been rinsed with 4 mL 1.5% NaOCl. Afterward, root canals were obtained standard MI using #90, 100, and 110 K-files (Densply Maillefer, Ballaigues, Switzerland) at 9-mm working length. Circumferential filing was performed until the subsequent file could fit the apical diameter. Root canals were irrigated with 4 ml of 1.5% NaOCl for 1 minute after each increasing files (124). Finally, each canal was rinsed with 4 mL 1.5% NaOCl.

In group 2-9, all irrigants was delivered by 25-gauge side-vented needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) and irrigation rate was equivalent to 4 mL/min. The working length for tips of needles and supplementary applications was 9 mm. Finally, the total volume of NaOCl irrigation for each sample in group 2-9 was 20 mL. After their respective procedures, each root canal was flushed with 5 mL 10% sodium thiosulfate to inactivate NaOCl activity. In these groups, the files and instrument tips were single-use, except for the ultrasonic file tips that were reused after sterilization in the PUI group.

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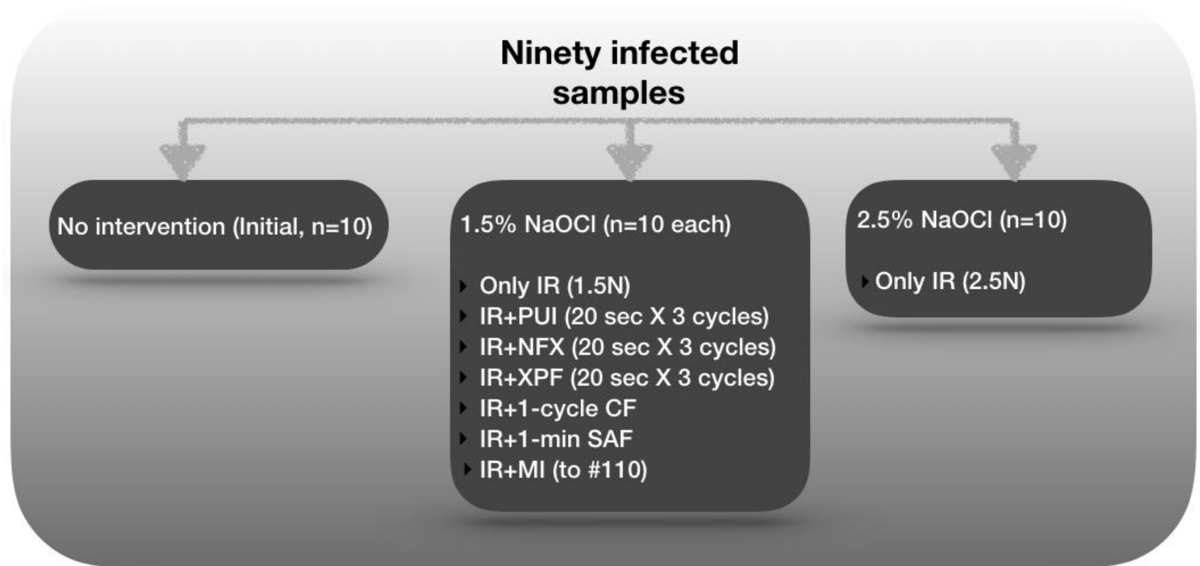

Figure 9: The protocols used for each group, IR = Syringe irrigation

Quantitative Evaluation of Residual Bacteria

The remaining bacteria in the root canal and inner dentin of the experimental and sterile samples were collected. The root canal were drilled using a No.4 Peeso reamer (Densply Maillefer, Ballaigues, Switzerland) and the dentin shavings on the Peeso reamer were transferred into an Eppendorf tube for each root sample (Eppendorf North America, Hauppauge, NY) containing 1 mL phosphate buffered saline (PBS). Three sterile paper points were sequentially placed into the root canal to absorb the remaining fluid and transferred to the same Eppendorf tube. The contents were sonicated (Microson ultrasonic cell disruption; Misonix Inc, Farmingdale, NY) at 22.5 kHz and 20% intensity for 30 seconds, 10-fold serially diluted, spread onto BHI agar plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours and the colony forming units (CFUs) were counted and calculated to the mean CFUs. All procedures were performed by one operator.

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SEM Evaluation of Biofilm Formation

To confirm bacterial biofilm formation in the root canals, 2 root samples were prepared in the similar fashion of other samples. However, the teeth were given 2 longitudinal grooves on buccal and lingual sides of external root surface (Fig. 10A) with diamond disc in order to split the samples into 2 halves (Fig. 10B).

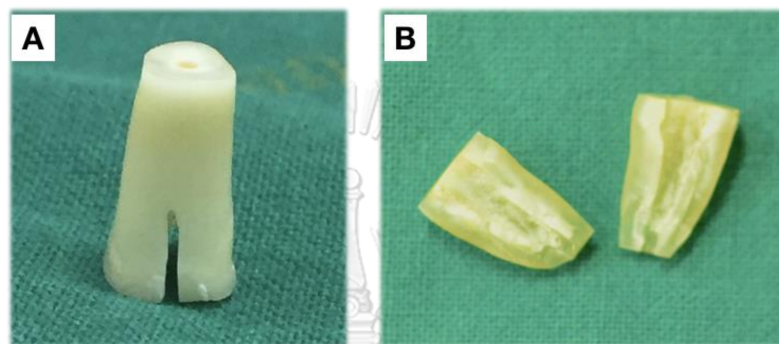

Figure 10: Sample preparation for SEM inspection

After 21-day incubation, these root samples were washed with 10 mL 1% phosphate buffer saline (PBS), fixed in 2.5% glutaraldehyde for 24 hours and split longitudinally with a cutting blade. The samples were serially dehydrated, critical point dried, gold sputter coated, and examined using scanning electron microscope (SEM, Quanta 250 FEG, FEI, Oregon, USA). Root canal wall and dentinal tubule images at random apical third areas were captured at 5000X and 10,000X magnification.

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Statistical analysis

The CFU counts were transformed into Log_{10} values that were analyzed using SPSS Statistics software version 21.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test indicated normal data distribution. The differences between groups

were evaluated using one-way analysis of variance (ANOVA). Subsequently, the post hoc Tukey HSD test was performed for pairwise comparisons. The significance level was set at $P < .05$. The ratios of remaining bacteria between groups were calculated from the mean CFUs of each group to represent the magnitude of bacterial reduction.

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Chapter IV
Research Results

Biofilm verification

The SEM images illustrated a biofilm consisting of large bacterial clusters on the infected root canal walls (Fig. 9A). Bacterial penetration into the dentinal tubules was also observed (Fig. 9B).

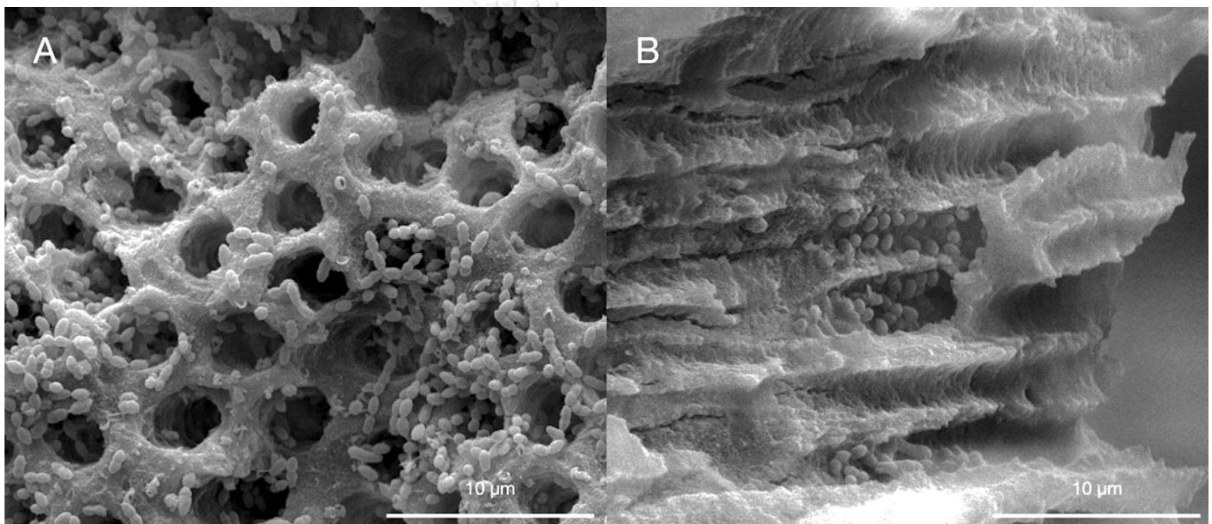

Figure 11: SEM images of infected root canals show clusters of bacteria on the root canal wall (A) and bacteria penetrated into the dentinal tubules (B).

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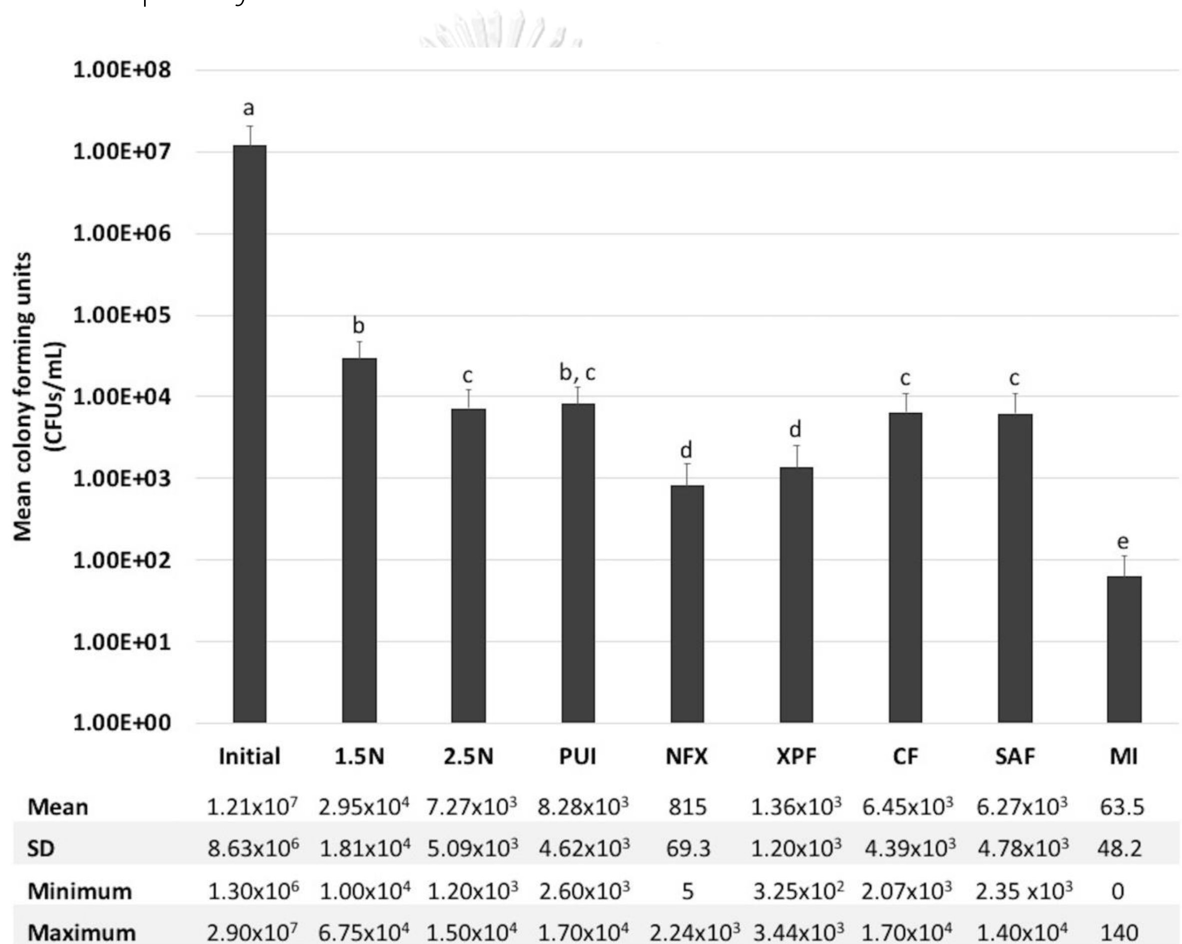
Microbiological evaluation

The mean of the CFUs was determined from the remaining bacteria in each group (Figure 10). No bacteria were detected in the sterile samples (data not shown). The mean CFUs in the initial group was 1.21×10^{7} CFUs/mL. The mean CFUs in each treatment group was significantly lower compared with the initial group ($P < .05$). The 2.95×10^{4} CFUs/mL in the 1.5N group was the highest among the irrigation groups. The mean CFUs of the 1.5N group was approximately 3.6-fold higher than that of the PUI

group; 4-5-fold higher than that of the 2.5N, CF, and SAF groups; and 22-, 36-, and 464-fold higher than that of the XPF, NFX, and MI groups, respectively (Figure 10). The 2.5N group and 1.5N groups with adjunctive treatments, excluding the PUI group, had a significantly lower mean CFUs compared with the 1.5N group ($P < .05$) (Table 4). When bacterial growth was present, gram staining and colony forming morphology indicated a pure *E. faecalis* culture.



	Initial	1.5N	2.5N	PUI	NFX	XPF	CF	SAF	MI
Mean	1.21×10^7	2.95×10^4	7.27×10^3	8.28×10^3	815	1.36×10^3	6.45×10^3	6.27×10^3	63.5
SD	8.63×10^6	1.81×10^4	5.09×10^3	4.62×10^3	69.3	1.20×10^3	4.39×10^3	4.78×10^3	48.2
Minimum	1.30×10^6	1.00×10^4	1.20×10^3	2.60×10^3	5	3.25×10^2	2.07×10^3	2.35×10^3	0
Maximum	2.90×10^7	6.75×10^4	1.50×10^4	1.70×10^4	2.24×10^3	3.44×10^3	1.70×10^4	1.40×10^4	140

Figure 12 Mean numbers of remaining bacteria (CFUs/mL) of each group, showing in log scale. Different lowercase letters indicate significant difference between the groups ($P < .05$). SD, standard deviation.

Group (A)	Group (B)	Mean Difference (A-B)	*P* Value	95% Confidence Interval	
				Lower Bound	Upper Bound
Initial	1.5N	2.54	<.001	1.96	3.12
	2.5N	3.22	<.001	2.64	3.80
	PUI	3.09	<.001	2.51	3.67
	NFX	4.25	<.001	3.67	4.83
	XPF	3.96	<.001	3.38	4.54
	CF	3.21	<.001	2.63	3.79
	SAF	3.24	<.001	2.66	3.83
	MI	5.35	<.001	4.77	5.93
1.5N	2.5N	0.68	<.05	0.10	1.26
	PUI	0.55	0.08	-0.03	1.13
	NFX	1.71	<.001	1.13	2.29
	XPF	1.42	<.001	0.84	2.00
	CF	0.67	<.05	0.09	1.25
	SAF	0.71	<.01	0.12	1.29
	MI	2.81	<.001	2.23	3.39
2.5N	PUI	-0.13	1.00	-0.71	0.45
	NFX	1.03	<.001	0.45	1.61
	XPF	0.74	<.001	0.16	1.32
	CF	-0.01	1.00	-0.59	0.57
	SAF	0.03	1.00	-0.55	0.61
	MI	2.13	<.001	1.55	2.71
PUI	NFX	1.16	<.001	0.58	1.74
	XPF	0.87	<.001	0.29	1.45
	CF	0.12	1.00	-0.46	0.70
	SAF	0.16	0.99	-0.42	0.74
	MI	2.26	<.001	1.68	2.84
NFX	XPF	-0.29	0.81	-0.87	0.29
	CF	-1.04	<.001	-1.62	-0.46
	SAF	-1.01	<.001	-1.59	-0.42
	MI	1.10	<.001	0.52	1.68
XPF	CF	-0.75	<.001	-1.33	-0.17
	SAF	-0.72	<.01	-1.30	-0.13
	MI	1.39	<.001	0.81	1.97
CF	SAF	0.04	1.00	-0.54	0.62
	MI	2.14	<.001	1.56	2.72
SAF	MI	2.10	<.001	1.52	2.69

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Table 5: Mean difference in remaining bacteria (Log_{10} values) between each group, P Value, and 95% confidence interval (Post-hoc Tukey HSD analysis).

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Chapter V
Discussion

Effective bacterial reduction contributes to successful REP. Therefore, this study evaluated the effectiveness of different disinfection methods in teeth with large root canals. The results revealed that adjunctive procedures, with no or minimal dentin removal, improved the antibacterial effectiveness of the REP irrigation protocol.

In this study, root samples were prepared to mimic infected necrotic immature teeth. The apical portion of intact mandibular premolars was removed to obtain a large root canal with a natural surface of the root canal wall. The length of the root samples was standardized to 10-mm representing 2/3 of the mature premolar's root length (125). Although root canal infection comprises multi-species bacteria, the single-species biofilm model was employed to limit any variations that may be caused by bacterial interaction. *Enterococcus faecalis* was chosen as a test species to represent bacteria with abilities to form a biofilm, invade dentinal tubules, resist harsh environments and withstand the antimicrobial effects of calcium hydroxide (105). Moreover, *E. faecalis* is commonly found in failed endodontic cases (104).

To explore the procedures that could improve bacterial reduction during REP, the adjunct protocols were evaluated and compared with 1.5% NaOCl irrigation without mechanical instrumentation. Although 1.5% NaOCl irrigation is mentioned in

the AAE clinical considerations for a REP (9), 2.5% NaOCl is within the concentration range recommended by the European Society of Endodontology (126) and has been used in previous studies showing a high success rate (8, 40). Mechanical instrumentation representing the standard chemomechanical approach was also included as a positive control (15).

The results confirmed those of previous studies where 1.5% NaOCl irrigation alone was less effective than 2.5% NaOCl and far less effective than standard mechanical instrumentation (15, 20, 127). However, standard mechanical instrumentation should be avoided in REP because this technique may make immature teeth more susceptible to fracture (5). Although 2.5% NaOCl irrigation was statistically more effective than 1.5% NaOCl, the magnitude of the difference was small. Moreover, the toxicity to stem cells of 2.5% NaOCl was also higher than 1.5% NaOCl (25).

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Our results aligned with those of previous studies demonstrating that various minimal mechanical instrumentation procedures effectively enhanced bacterial removal from the root canal at different magnitudes (29, 30, 53, 58). Compared with the 1.5N group, the CF and SAF groups showed similarly improved disinfection, comparable to that of the 2.5N group, while the XPF group had much lower number of remaining bacteria. These differences may be due to their instrument designs. The XPF's flexible and fine working tips effectively contact and remove bacterial biofilm from non-uniform root canals, while the SAF and K-type files are more rigid and less

effective (128). Moreover, previous studies showed that, compared with traditional irrigation or sonic and ultrasonic activation, XPF was also more effective in decreasing dentinal tubule bacteria (30, 58).

PUI and NFX are supplemental procedures that do not remove dentin. Our results indicated that PUI was 3.6-fold more effective than 1.5N; however, this difference was not significant. Although previous studies demonstrated that PUI significantly improved bacterial elimination from the root canal and dentinal tubules (15, 28), the results were not consistent (129, 130). These disparities may be caused by differences in experimental design, such as root canal size, irrigant concentration, and irrigation time (15, 28, 129, 130). Theoretically, the shear stress produced by PUI is inverse to the boundary layer thickness or root canal width (27), showed in the following equation.

$$\tau = \eta \frac{V}{\delta}$$

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where τ is shear stress, η is kinematic viscosity of the liquid, V is the streaming velocity and δ is the boundary layer thickness. Therefore, the bigger canal size is, the lower shear stresses will occur (27). Regarding to our experimental design, the large canal size in our study may have contributed to a non-significant effect of PUI in removing bacteria.

NFX was the most effective method in eliminating bacteria, except for the standard MI. The NFX brushing action mechanically dispersed the bacterial biofilm,

allowing the NaOCl solution to penetrate into exposed dentinal tubules and exert its antibacterial effect without further dentin removal (29). In the present study, NFX was applied with a brushing stroke without simultaneous irrigation to avoid irrigant extrusion from open-ended needles. However, our results were similar to those of previous studies where NFX significantly improved canal cleanliness and biofilm reduction compared with conventional syringe irrigation and EndoVac™ (29, 90, 92). These results suggest the possibility of using NFX as a supplemental procedure to improve biofilm removal in REP.

In the present study, the statistical improvement of bacterial reduction by adjunct protocols was demonstrated in a laboratory setting. However, the results should be interpreted with caution. The small magnitudes of improvement, especially in the 2.5N, PUI, CF and SAF groups, may not have a marked clinical effect on treatment outcome. Clinically, other procedures such as the use of additional irrigants and medicaments may also be beneficial for bacterial control. A robust immune response also contributes to periapical healing and treatment success in immature teeth with open apices (131).

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Minimizing the extent of apical irrigant extrusion is another concern in immature teeth. Previous studies on teeth with closed apices demonstrated a similar amount of extruded irrigant among conventional needle irrigation, PUI, XPF, and SAF (57, 81, 132). However, the irrigant extrusion in teeth with large open apices may be different. Large-sized root canals may have higher irrigant extrusion that could have a

deleterious effect on periapical stem cells or result in a NaOCl accident (25, 57, 81). Although NFX and XPF are non-invasive adjuncts that could improve root canal disinfection, they may generate turbulent irrigant flow and pressure towards the apical area. In this study, to maintain the irrigants within the root canals during irrigation, the root samples were apically sealed and irrigant extrusion was not evaluated. However, further investigation of the effect of these adjuncts on irrigant extrusion in an immature root canal model is required prior to clinical consideration. Furthermore, alternative disinfectants that are effective and non-toxic to stem cells and periapical tissue should also be evaluated.

Limitations of the Research

As this study was an in vitro study, it was unable to create a human periapical tissue environment such as periapical pressure and immunological involvement. In addition, multi-species infection could not be used in this study due to complexity of endodontic bacterial system. However, *Enterococcus faecalis* biofilm was used to represent frequently-found bacteria in failed endodontic cases. Another limitation of this study was to collect an open apex tooth. Therefore closed apex teeth that 3-4 mm of apical end was removed, were selected to simulate teeth with an open apex in this study.

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Conclusion

Under the conditions of this study, using the large root canal model, the results indicated that root canal disinfection using 1.5% NaOCl irrigation was

significantly improved using supplemental procedures such as NFX, XPF, CF or SAF. Adjunctive root canal brushing using NFX was the most promising alternative for improving root canal disinfection without dentin removal.

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Appendix

A. Growth curve

A growth curve of *Enterococcus faecalis* (ATCC 29212) was observed prior to the experiment. The optical density (OD) of *E. faecalis* suspension was identified at 600 nm every hour until it had reached a stationary phase (Figure 11). *E. faecalis* in log phase at OD 0.5, approximately equivalent to 1.65×10^8 CFUs/mL, was used for tooth inoculation.

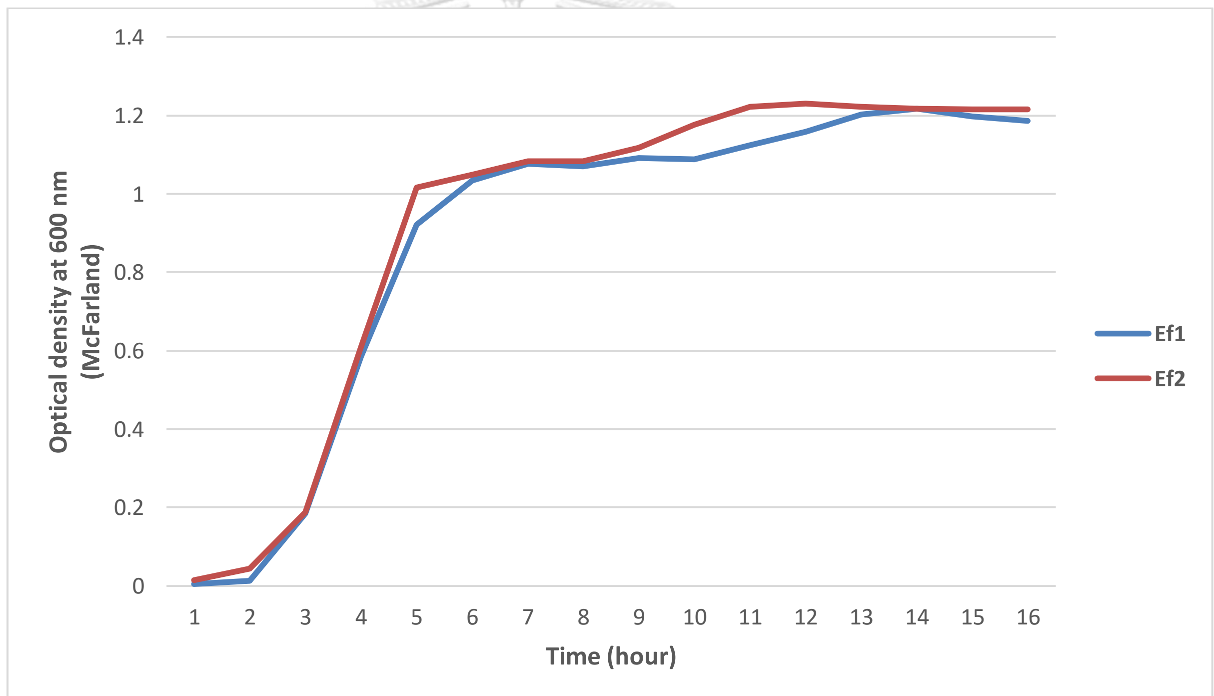

Figure 13: A growth curve of Enterococcus faecalis was observed twice. (Ef 1 = 1st observed, Ef 2 = 2nd observed)

B. Raw data results of each experimental group

Specimen number of group 1 (Initial group)	CFU count (CFUs/mL)			Log$_{10}$ of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	3.10E+06	4.10E+06	3.60E+06	6.56
2	1.90E+07	1.70E+07	1.80E+07	7.26
3	1.11E+07	9.80E+06	1.05E+07	7.02
4	2.80E+07	3.00E+06	2.90E+07	7.46
5	1.20E+07	1.20E+07	1.20E+07	7.08
6	2.90E+06	3.00E+06	3.00E+06	6.48
7	1.30E+06	1.30E+06	1.30E+06	6.11
8	1.87E+07	1.92E+07	1.90E+07	7.28
9	1.70E+07	1.50E+07	1.60E+07	7.20
10	8.90E+06	8.10E+06	8.50E+06	6.93

Table 6: CFU count of each sample of the initial group

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Specimen number of group 2 (1.5N group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	1.40E+04	1.40E+04	1.40E+04	4.15
2	1.00E+04	1.00E+04	1.00E+04	4.00
3	2.30E+04	2.10E+04	2.20E+04	4.34
4	1.50E+04	1.50E+04	1.50E+04	4.18
5	5.00E+04	5.10E+04	5.05E+04	4.70
6	1.90E+04	2.10E+04	2.00E+04	4.30
7	4.00E+04	3.60E+04	3.80E+04	4.58
8	3.09E+04	3.19E+04	3.14E+04	4.50
9	6.30E+04	7.20E+04	6.75E+04	4.83
10	2.70E+04	2.60E+04	2.65E+04	4.42

Table 7: CFU count of each sample of the 1.5N group

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Specimen number of group 3 (2.5N group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	7.00E+03	8.00E+03	7.50E+03	3.88
2	1.30E+03	1.30E+03	1.30E+03	3.11
3	1.10E+03	1.30E+03	1.20E+03	3.08
4	9.60E+03	8.40E+04	9.00E+03	3.95
5	1.30E+03	1.70E+03	1.50E+04	4.18
6	4.10E+03	4.80E+03	4.45E+03	3.65
7	2.30E+03	1.90E+03	2.10E+03	3.32
8	7.80E+03	8.40E+03	8.10E+03	3.91
9	9.50E+03	8.50E+03	9.00E+03	3.95
10	1.50E+04	1.50E+04	1.50E+04	4.18

Table 8: CFU count of each sample of the 2.5N group

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Specimen number of group 4 (PUI group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	5.50E+03	6.80E+03	6.15E+03	3.79
2	2.60E+03	2.60E+03	2.60E+03	3.41
3	3.80E+03	4.20E+03	4.00E+03	3.60
4	1.00E+04	1.00E+04	1.00E+04	4.00
5	1.30E+04	1.10E+04	1.20E+04	4.08
6	6.00E+03	6.00E+03	6.00E+03	3.78
7	4.10E+03	3.60E+03	3.85E+03	3.59
8	9.00E+03	8.00E+03	8.50E+03	3.93
9	1.50E+04	1.90E+04	1.70E+04	4.23
10	1.20E+04	1.34E+04	1.27E+04	4.10

Table 9: CFU count of each sample of the PUI group

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Specimen number of group 5 (NFX group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	7.00E+01	6.00E+01	6.50E+01	1.81
2	5.00E+01	5.00E+01	5.00E+01	1.70
3	3.80E+02	5.20E+02	4.50E+02	2.65
4	5.20E+02	4.80E+02	5.00E+02	2.70
5	1.14E+03	9.40E+02	1.04E+03	3.02
6	2.90E+02	3.10E+02	3.00E+02	2.48
7	1.05E+03	7.70E+02	9.10E+02	2.96
8	1.42E+03	1.66E+03	1.54E+03	3.19
9	2.24E+03	2.23E+03	2.24E+03	3.35
10	9.80E+02	1.12E+03	1.05E+03	3.02

Table 10: CFU count of each sample of the NFX group

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Specimen number of group 6 (XPF group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	4.60E+02	4.00E+02	4.30E+02	2.63
2	5.00E+02	5.50E+02	5.25E+02	2.72
3	1.53E+03	1.63E+03	1.58E+03	3.20
4	4.90E+02	5.10E+02	5.00E+02	2.70
5	3.32E+03	3.56E+03	3.44E+03	3.54
6	6.00E+02	5.40E+02	5.70E+02	2.76
7	3.50E+02	3.00E+02	3.25E+02	2.51
8	2.30E+03	2.50E+03	2.40E+03	3.38
9	3.10E+03	3.10E+03	3.10E+03	3.49
10	7.20E+02	6.80E+02	7.00E+02	2.85

Table 11: CFU count of each sample of the XPF group

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Specimen number of group 7 (CF group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	8.30E+03	7.61E+03	7.96E+03	3.90
2	2.14E+03	1.99E+03	2.07E+03	3.32
3	5.01E+03	4.83E+03	4.92E+03	3.69
4	9.50E+03	9.50E+03	9.50E+03	3.98
5	4.40E+03	5.40E+03	4.90E+03	3.69
6	3.60E+02	3.60E+02	3.60E+03	3.56
7	2.30E+02	2.50E+02	2.40E+03	3.38
8	7.90E+03	6.10E+03	7.00E+03	3.85
9	5.00E+03	5.20E+03	5.10E+03	3.71
10	1.70E+04	1.70E+04	1.70E+04	4.23

Table 12: CFU count of each sample of the CF group

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Specimen number of group 8 (SAF group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	3.31E+03	3.29E+03	3.30E+03	3.52
2	2.70E+03	3.00E+03	2.85E+03	3.45
3	4.10E+03	3.80E+03	3.95E+03	3.60
4	2.70E+03	3.10E+03	2.90E+03	3.46
5	3.80E+03	4.20E+03	4.00E+03	3.60
6	2.30E+02	2.40E+03	2.35E+03	3.37
7	1.20E+04	1.60E+04	1.40E+04	4.15
8	1.30E+04	1.30E+04	1.30E+04	4.11
9	1.20E+04	1.28E+04	1.24E+04	4.09
10	4.00E+03	3.80E+03	3.90E+03	3.59

Table 13: CFU count of each sample of the SAF group

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Specimen number of group 8 (MI group)	CFU count (CFUs/mL)			Log_{10} of average (CFUs/mL)
	1st technical duplication	2nd technical duplication	Average	
1	0.00E+00	0.00E+00	0.00E+00	0.00
2	4.00E+01	2.00E+01	3.00E+01	1.48
3	4.00E+01	4.00E+01	4.00E+01	1.60
4	4.00E+01	7.00E+01	5.50E+01	1.74
5	5.00E+01	7.00E+01	6.00E+01	1.78
6	1.40E+02	1.40E+02	1.40E+02	2.15
7	2.00E+01	2.00E+01	2.00E+01	1.30
8	5.00E+01	5.00E+01	5.00E+01	1.70
9	1.20E+02	8.00E+01	1.00E+02	2.00
10	1.10E+02	1.70E+02	1.40E+02	2.15

Table 14: CFU count of each sample of the MI group

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C. Statistic test (SPSS test)

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
CFU	Initial	.192	10	.200*	.920	10	.355
	1.5N	.102	10	.200*	.986	10	.990
	2.5N	.250	10	.077	.870	10	.099
	PUI	.130	10	.200*	.964	10	.833
	NFX	.188	10	.200*	.890	10	.171
	XPF	.230	10	.142	.876	10	.117
	CF	.141	10	.200*	.973	10	.918
	SAF	.323	10	.004	.804	10	.016
	MI	.230	10	.143	.778	10	.008

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

a. Lilliefors Significance Correction

Table 15: Test of normality

CFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	169.870	8	21.234	127.537	.000
Within Groups	13.486	81	.166		
Total	183.356	89			

Table 16: ANOVA test

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Dependent Variable: CFU

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Initial	1.5N	2.53800*	.18248	.000	1.9564	3.1196
	2.5N	3.21700*	.18248	.000	2.6354	3.7986
	PUI	3.08700*	.18248	.000	2.5054	3.6686
	NFX	4.25000*	.18248	.000	3.6684	4.8316
	XPF	3.96000*	.18248	.000	3.3784	4.5416
	CF	3.2070*	.18248	.000	2.6254	3.7886
	SAF	3.24400*	.18248	.000	2.6624	3.8256
	MI	5.34800*	.18248	.000	4.7664	5.9296
1.5N	Initial	-2.53800*	.18248	.000	-3.1196	-1.9564
	2.5N	.67900*	.18248	.010	.0974	1.2606
	PUI	.54900	.18248	.080	-.0326	1.1306
	NFX	1.71200*	.18248	.000	1.1304	2.2936
	XPF	1.42200*	.18248	.000	.8404	2.0036
	CF	.66900*	.18248	.012	.0874	1.2506

	SAF	.70600*	.18248	.006	.1244	1.2876
	MI	2.81000*	.18248	.000	2.2284	3.3916
2.5N	Initial	-3.21700*	.18248	.000	-3.7986	-2.6354
	1.5N	-.67900*	.18248	.010	-1.2606	-.0974
	PUI	-.13000	.18248	.998	-.7116	.4516
	NFX	1.03300*	.18248	.000	.4514	1.6146
	XPF	.74300*	.18248	.003	.1614	1.3246
	CF	-.01000	.18248	1.000	-.5916	.5716
	SAF	.02700	.18248	1.000	-.5546	.6086
	MI	2.13100*	.18248	.000	1.5494	2.7126
PUI	Initial	-3.08700*	.18248	.000	-3.6686	-2.5054
	1.5N	-.54900	.18248	.080	-1.1306	.0326
	2.5N	.13000	.18248	.998	-.4516	.7116
	NFX	1.16300*	.18248	.000	.5814	1.7446
	XPF	.87300*	.18248	.000	.2914	1.4546
	CF	.12000	.18248	.999	-.4616	.7016
	SAF	.15700	.18248	.994	-.4246	.7386
	MI	2.26100*	.18248	.000	1.6794	2.8426
NFX	Initial	-4.25000*	.18248	.000	-4.8316	-3.6684
	1.5N	-1.71200*	.18248	.000	-2.2936	-1.1304

	2.5N	-1.03300*	.18248	.000	-1.6146	-.4514
	PUI	-1.16300*	.18248	.000	-1.7446	-.5814
	XPF	-.29000	.18248	.808	-.8716	.2916
	CF	-1.04300*	.18248	.000	-1.6246	-.4614
	SAF	-1.00600*	.18248	.000	-1.5876	-.4244
	FMI	1.09800*	.18248	.000	.5164	1.6796
XPF	Initial	-3.96000*	.18248	.000	-4.5416	-3.3784
	1.5N	-1.42200*	.18248	.000	-2.0036	-.8404
	2.5N	-.74300*	.18248	.003	-1.3246	-.1614
	PUI	-.87300*	.18248	.000	-1.4546	-.2914
	NFX	.29000	.18248	.808	-.2916	.8716
	CF	-.75300*	.18248	.003	-1.3346	-.1714
	SAF	-.71600*	.18248	.005	-1.2976	-.1344
	MI	1.38800*	.18248	.000	.8064	1.9696
CF	Initial	-3.20700*	.18248	.000	-3.7886	-2.6254
	1.5N	-.66900*	.18248	.012	-1.2506	-.0874
	2.5N	.01000	.18248	1.000	-.5716	.5916
	PUI	-.12000	.18248	.999	-.7016	.4616
	NFX	1.04300*	.18248	.000	.4614	1.6246
	XPF	.75300*	.18248	.003	.1714	1.3346

	SAF	.03700	.18248	1.000	-.5446	.6186
	MI	2.14100*	.18248	.000	1.5594	2.7226
SAF	Initial	-3.24400*	.18248	.000	-3.8256	-2.6624
	1.5N	-.70600*	.18248	.006	-1.2876	-.1244
	2.5N	-.02700	.18248	1.000	-.6086	.5546
	PUI	-.15700	.18248	.994	-.7386	.4246
	NFX	1.00600*	.18248	.000	.4244	1.5876
	XPF	.71600*	.18248	.005	.1344	1.2976
	CF	-.03700	.18248	1.000	-.6186	.5446
	MI	2.10400*	.18248	.000	1.5224	2.6856
MI	Initial	-5.34800*	.18248	.000	-5.9296	-4.7664
	1.5N	-2.81000*	.18248	.000	-3.3916	-2.2284
	2.5N	-2.13100*	.18248	.000	-2.7126	-1.5494
	PUI	-2.26100*	.18248	.000	-2.8426	-1.6794
	NFX	-1.09800*	.18248	.000	-1.6796	-.5164
	XPF	-1.38800*	.18248	.000	-1.9696	-.8064
	CF	-2.14100*	.18248	.000	-2.7226	-1.5594
	SAF	-2.10400*	.18248	.000	-2.6856	-1.5224

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

Table 17: Post hoc test: Multiple Comparisons with Tukey HSD

CFU

	Group	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	MI	10	1.5900				
	NFX	10		2.6880			
	XPF	10		2.9780			
	SAF	10			3.6940		
	2.5N	10			3.7210		
	CF	10			3.7310		
	PUI	10			3.8510	3.8510	
	1.5N	10				4.4000	
	Initial	10					6.9380
	Sig.		1.000	.808	.994	.080	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Table 18: Homogeneous Subsets

Table 19: Ratios of bacterial reduction calculated from mean CFUs of each group

Ratio	Initial	1.5N	2.5N	PUI	NFX	XPF	CF	SAF	MI
Initial	1	4.10E+02	1.66E+03	1.46E+03	1.48E+04	8.91E+03	1.88E+03	1.93E+03	1.90E+05
1.5N	-	1	4.06E+00	3.56E+00	3.62E+01	2.17E+01	4.58E+00	4.71E+00	4.64E+02
2.5N	-	-	1	-	8.92E+00	5.35E+00	1.13E+00	1.16E+00	1.14E+02
PUI	-	-	1.14E+00	1	1.02E+01	6.10E+00	1.28E+00	1.32E+00	1.30E+02
NFX	-	-	-	-	1	-	-	-	1.28E+01
XPF	-	-	-	-	1.67E+00	1	-	-	2.14E+01
CF	-	-	-	-	7.91E+00	4.75E+00	1	1.03E+00	1.01E+02
SAF	-	-	-	-	7.69E+00	4.62E+00	-	1	9.87E+01
MI	-	-	-	-	-	-	-	-	1

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

VITA

NAME	Parnwad Sasanakul
DATE OF BIRTH	23 August 1988
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Department of Operative Dentistry, Chulalongkorn University
HOME ADDRESS	28/5 M2, Bangbuathong-Suphanburi road, Namai, Ladlumkaew, Pathumthani

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY