ประสิทธิผลของเกณฑ์วิธีการฆ่าเชื้อที่แตกต่างกันในการลดปริมาณแบคทีเรียในแผ่นคราบชีวภาพของเชื้อเอ็นเตอโรคอคคัสฟีคัลลิส ในฟันที่มีคลองรากขนาดใหญ่

นางสาวพาทินี พลาดิสัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2558

EFFECTIVENESS OF DIFFERENT DISINFECTION PROTOCOLS IN THE REDUCTION OF BACTERIA IN *ENTEROCOCCUS FAECALIS* BIOFILM IN LARGE ROOT CANAL TEETH

Miss Patinee Pladisai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Endodontology
Department of Operative Dentistry
Faculty of Dentistry
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Thesis Title	EFFECTIVENESS OF DIFFERENT DISINFECTION PROTOCOLS IN THE REDUCTION OF BACTERIA IN *ENTEROCOCCUS FAECALIS* BIOFILM IN LARGE ROOT CANAL TEETH
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พาทินี พลาดิสัย : ประสิทธิผลของเกณฑ์วิธีการฆ่าเชื้อที่แตกต่างกันในการลดปริมาณแบคทีเรียในแผ่นคราบชีวภาพของเชื้อเอ็นเตอโรคอคคัสฟีคัลลิส ในฟันที่มีคลองรากขนาดใหญ่ (EFFECTIVENESS OF DIFFERENT DISINFECTION PROTOCOLS IN THE REDUCTION OF BACTERIA IN *ENTEROCOCCUS FAECALIS* BIOFILM IN LARGE ROOT CANAL TEETH) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ทญ. ดร.ปวีณา จิวัจฉรานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ทญ. ดร. รัชนี อัมพรอร่ามเวทย์, หน้า.

บทนำ: การศึกษานี้มีจุดประสงค์เพื่อเปรียบเทียบประสิทธิผลของเกณฑ์วิธีการฆ่าเชื้อที่แตกต่างกันในการลดปริมาณแบคทีเรียในแผ่นคราบชีวภาพของเชื้อเอ็นเตอโรคอคคัส ฟีคัลลิส ในฟันที่มีคลองรากขนาดใหญ่ วิธีวิจัย: เตรียมรากฟันกรามน้อยล่างของมนุษย์ที่มี 1 คลองรากฟันและมีคลองรากขนาดใหญ่จำนวน 55 ซี่ โดยรากฟัน 50 ซี่จะถูกนำไปเพาะเลี้ยงเชื้อเอ็นเตอโรคอคคัส ฟีคัลลิสให้เข้าสู่คลองรากฟันเป็นเวลา 21 วัน ในงานวิจัยนี้ รากฟัน 4 ซี่จะถูกนำไปวิเคราะห์การเกิดแผ่นคราบชีวภาพบนผนังคลองรากฟันด้วยกล้องอิเลกตรอนแบบส่องกราด ส่วนรากฟันอีก 51 ซี่จะถูกแบ่งเป็นกลุ่มควบคุมที่ปราศจากเชื้อ (sterile control) จำนวน 3 ซี่ และกลุ่มที่ทดสอบเกณฑ์วิธีการฆ่าเชื้อที่แตกต่างกันจำนวน 48 ซี่ คือ 1) กลุ่มเตรียมคลองรากฟันโดยใช้เคไฟล์เบอร์ 60-90 (MI) 2) กลุ่มชะล้างคลองรากฟันเพียงอย่างเดียวด้วยโซเดียมไฮโปคลอไรท์ความเข้มข้น 2.5% (IRN) 3) กลุ่มชะล้างคลองรากฟันด้วยโซเดียมไฮโปคลอไรท์ความเข้มข้น 2.5% ร่วมกับการใช้อัลตราโซนิก (PUI) 4) กลุ่มชะล้างคลองรากฟันเพียงอย่างเดียวด้วยน้ำเกลือ (IRS) และ 5) กลุ่มที่ไม่ได้รับการฆ่าเชื้อในคลองรากฟัน (initial) หลังจากทำการฆ่าเชื้อในคลองรากฟันแล้ว จะเก็บเนื้อฟันในส่วนผนังคลองรากฟันเพื่อนำมาวิเคราะห์ทางจุลชีววิทยาโดยเปรียบเทียบจากค่าเฉลี่ย (mean) ของหน่วยก่อรูปโคโลนี (CFU count) และนำมาวิเคราะห์โดยใช้สถิติวิเคราะห์ความแปรปรวนทางเดียว (one-way ANOVA)ที่ระดับความเชื่อมั่น 95% ผลการวิจัย: กลุ่ม MI พบปริมาณแบคทีเรียที่หลงเหลืออยู่ในคลองรากฟันน้อยที่สุด (24.5 CFU/mL) ตามด้วยกลุ่ม PUI และกลุ่ม IRN ตามลำดับ ปริมาณแบคทีเรียที่หลงเหลืออยู่ในกลุ่ม IRS ไม่แตกต่างจากกลุ่ม initial แต่พบว่าปริมาณของแบคทีเรียที่หลงเหลืออยู่ในคลองรากฟันในกลุ่มอื่นๆ มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P<.01$) โดยมีปริมาณของแบคทีเรียที่หลงเหลืออยู่ในกลุ่ม PUI น้อยกว่ากลุ่ม IRN 4.5 เท่า และมากกว่ากลุ่ม MI 1,862 เท่า สรุปผลวิจัย: เกณฑ์วิธีการฆ่าเชื้อที่มีประสิทธิผลสูงสุดในฟันคลองรากขนาดใหญ่คือ วิธีการเตรียมคลองรากฟัน ถึงแม้ว่าอัลตราโซนิกจะช่วยเพิ่มประสิทธิภาพของการชะล้างคลองรากฟันขนาดใหญ่ที่ไม่มีข้อจำกัดของการเข้าถึงของน้ำยาล้างคลองรากฟัน แต่ก็ไม่สามารถทดแทนวิธีการเตรียมคลองรากฟันได้

ภาควิชา ทันตกรรมหัตถการ
สาขาวิชา วิทยาเอ็นโดดอนต์
ปีการศึกษา 2558

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: LARGE ROOT CANAL / DISINFECTION / ENTEROCOCCUS FAECALIS / BIOFILM / PASSIVE ULTRASONIC IRRIGATION / BACTERIAL REDUCTION

PATINEE PLADISAI: EFFECTIVENESS OF DIFFERENT DISINFECTION PROTOCOLS IN THE REDUCTION OF BACTERIA IN *ENTEROCOCCUS FAECALIS* BIOFILM IN LARGE ROOT CANAL TEETH. ADVISOR: PAVENA CHIVATXARANUKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. RUCHANEE AMPORNARAMVETH, Ph.D., pp.

Introduction: This study compared the effectiveness of different disinfection protocols in reducing bacteria in an *Enterococcus faecalis* biofilm in teeth with large root canals. Methods: Fifty-five roots were prepared from human mandibular premolars with large single root canals and 50 roots were infected with *E. faecalis* for 21 days. Four roots were observed using scanning electron microscopy (SEM) to verify biofilm formation. The remaining specimens were assigned into 5 experimental groups and sterile control group: mechanical instrumentation using files size 60–90 (MI); irrigation with 2.5% NaOCl (IRN), irrigation with 2.5% NaOCl followed by intermittent passive ultrasonic irrigation (PUI), irrigation with normal saline (IRS), and no intervention (initial). After root canal disinfection, dentin specimens were collected for microbial analysis. Mean colony forming units (CFU) counts were calculated and compared between groups using one-way ANOVA. Results: The lowest number of intracanal bacteria (24.5 CFU/mL) was recovered from the MI group followed by the PUI and IRN groups. IRS alone did not demonstrate a significant reduction compared with the initial group. However, there were significant differences between groups (P <.01). The remaining bacteria in the PUI group was 4.5 fold lower compared with the IRN group, however, it was 1862 fold higher than that in the MI group. Conclusions: MI was the most effective method to disinfect large root canals. Although PUI enhanced the efficacy of root canal irrigation, it could not substitute for MI, even in large root canals where irrigant access to the apical portion was unlimited.

Department:	Operative Dentistry	Student's Signature	.....
Field of Study:	Endodontology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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CHAPTER I

INTRODUCTION

Background and Rationale

Bacteria are the main cause of apical periodontitis. Recent findings showed that biofilm is the form of microorganism that associated with persistent infection (1). Biofilm are bacterial community in self-made polysaccharide matrix, the matrix can act as a physical barrier against host immune response and restrict the penetration of disinfecting agents. Bacterial biofilm are also reported to be more resistant to antimicrobial agents compared to bacteria in planktonic form (2, 3). The fact that bacteria in biofilm were found in irregular or complicated anatomy of root canal (4) make it very difficult to be managed. Association of remaining bacteria in the form of biofilm in failed endodontically treated cases was demonstrated in histological study of extracted teeth that intraradicular biofilm was observed at the apical part of root canal (1, 4). Difficulties in bacterial biofilm removal still be the problem in bacterial management because no complete eradication could be achieved (5).

The goal of endodontic treatment is prevention or elimination of microbial infection in root canal system. The procedures that are generally applied for bacterial elimination in root canal are mechanical instrumentation

(MI), antibacterial irrigation (IR) and intracanal medication (Med). In regard to MI, the removal of infected root dentin without antibacterial agent can reduce bacteria in root canal up to 100-1000 folds (6). However, the effectiveness of MI can also be improved by the use of antibacterial irrigant during MI (7) and other supplemental techniques (8, 9).

In regard to the extent of MI, studies demonstrated that; the more root canal enlargement, the more reduction of intraradicular bacteria can be achieved. However, Extensive MI may lead to the reduction of dentin thickness and make the tooth prone to fracture (10-12). Although the former concept introduced by Weine recommending the preparation of "three sizes larger than the initial apical file (IAF)" for routine mechanical preparation is still being used (13). Opinions about the extent of MI in teeth with large root canal are still inconclusive. For example, some studies on regenerative cases suggested to omit MI for disinfection protocol in immature teeth, only irrigation with antimicrobial agent follow by intracanal medication is enough (14, 15). However, in retreatment case, apical preparation to larger sizes was recommended to remove infected dentin and allow antimicrobial agents to penetrate dentinal tubule effectively (16),

In the chemo-mechanical preparation, it was proven that antibacterial irrigation improves the effect of MI to remove bacteria from root canal system (7, 17). This effect becomes more obvious after irrigant access to the apical root

canal is gained (7). It was showed that NaOCl eliminated *E. faecalis* biofilms on dentin (18) and penetrated into dentinal tubules (19). An accessibility of irrigants to the apical part of root canal could be enhanced by the increase in size of root canal and depth of needle insertion (7, 20).

Passive ultrasonic irrigation (PUI) is one of supplementary techniques that was found to be effective in bacterial eradication and flushing of dentine debris in root canal (21). It has shown that ultrasonic energy allows better permeation of irrigant to complex anatomical recess in root canal system including dentinal tubules (9, 18) and has effect on biofilm disruption (18, 22).

In large root canals where irrigants can initially access the apical part without prior canal enlargement required, the effect of MI may be less important and bacterial reduction may be achieved solely with antibacterial irrigation. Bacterial eradication and debris removal from the root canal was also improved using supplemental passive ultrasonic irrigation (PUI), without further dentin removal (9, 18, 21). Previously, the favorable outcomes were reported when treating infected immature teeth with 1-5% NaOCl followed by intracanal medication, without MI (14, 15, 23, 24). However, there is scant evidence of the efficacy of non-invasive protocols, such as irrigation with or without PUI, compared to MI in large root canals.

This study aims to compare the effectiveness of chemo-mechanical preparation and other non-invasive disinfection protocols on bacteria

reduction in teeth with large root canals. The results of this study would lead to a better understanding of the effect of disinfection protocol on bacterial reduction in generally large root canal teeth which may prone to fracture if routine MI is applied.

Objective

To evaluate and compare effectiveness of applying different root canal disinfection protocols on the reduction of viable bacteria in *E. faecalis* biofilm in teeth with large root canals.

Scope of Study

This study was scoped in experimental study. Human teeth with strictly inclusion criteria were used to stimulate large root canal teeth. Mono-specie bacterial biofilm of *E. faecalis* was used as representative of bacteria biofilm in root canal wall. Verification of bacterial biofilm was done by SEM study. The effectiveness of different disinfection protocols were tested and evaluated by comparing CFU counts in quantitative data.

Expected Benefits and Application

The results of the research project can lead to clinical application of disinfection protocol for treatment in teeth with large root canals which may have thin dentinal wall and prone to fracture if routine mechanical instrumentation is applied.

CHAPTER II

LITERATURE REVIEW

The purpose of this study was to evaluate and compare effectiveness of different disinfection protocols on bacterial reduction in teeth with large root canal. Literature reviews are consists of microorganism in root canal and effect of endodontic procedure on the reduction of intraradicular bacteria.

Microorganism in Root Canal

Oral cavity consist of more than 500 different kinds of microorganisms (25). Pulpal tissue, the most venerable and vital part of tooth, is protected by harder tooth structure called dentin and enamel or cementum. As long as the hard tissue are still intact, the pulp are protected from microorganism invasion (26).

Bacteria are the primary cause of pulp and periapical inflammation. They invaded root canal space via caries, crack or trauma. In primary apical periodontitis, mixed bacterial infection plays an important role on inducing apical inflammation. Compositions of bacterial community in infected root canal are partly determined by nutrients in root canal under a circumstance (27). The micro-environment in root canal favors ecological selection of strictly anaerobic bacteria. Species that were frequently found in primary root canal infection usually belong to the genera *Bacteroides, Fusobacterium,*

Prevotella, Porphylomonas, Treponema, Peptostreptococcus, Eubacterium, Actinomyces, and *Streptococcus* (28).

Although the majority of microorganism resides in the main root canal system, they were also found in root canal isthmus, lateral canal, furcation and dentinal tubules. Penetration of bacteria from main canal into dentinal tubule occurs seemingly at random (29). Bacteria have different ability to invade dentinal tubule and the invasion does not seem to be dependent on bacterial mobility (30). The invading bacteria are dominantly gram-positive facultative and anaerobic cocci and rods. Gram-negative species have also been reports *such as Fusobacterium nucleatum, Eubacterium alactolyticum, Eubacterium nodatum, Lactobacillus casei*, and *Peptostreptococcus spp*. (31). Among to root canal bacteria microflora, the best invaders are *Enterococci, Streptococcus* and *Actinomyces* species (32). It has also been indicated that the invasion is more extensive at the coronal and middle portion of the root canal (30). Bacteria that have penetrated deep into tooth structure are obviously more difficult to eradicate directly by instrumentation (33).

After mechanical instrumentation was accomplished, as high as 65% of teeth were found to have bacteria in dentinal tubules (31). Compared to facultative anaerobic bacteria, anaerobic bacteria are more easily to be eliminated and less likely to survive after endodontic treatment procedure. It

was found that gram-positives are predominated (85%). *Lactobacillus spp*.(22%), *non-mutans streptococci* (18%) and *Enterococcus spp*.(12%) were the most common isolate after chemo-mechanical treatment was performed in teeth with apical periodontitis (34)

A. <u>*Enterococcus faecalis*</u>

Enterococcus faecalis, facultative anaerobic gram-positive cocci, is a normal commensal flora that can adapt to complex environment in oral cavity. Siqueira et al. (2002) detected *E. faecalis* in 11.5% of the cases with asymptomatic primary root canal infection. Although it was detected in primary root canal infection, several evidences indicated that *E. faecalis* is one of bacterial species that is often found in cases with endodontic failure (28, 35). Studies show that *E. faecalis* could form biofilm inside the medicated root canal (36) commonly survived after chemo-mechanical disinfection (16) and survived in high alkaline environment such as calcium hydroxide (34, 36). Although high prevalence of *E. faecalis* has been found in case of persistent or secondary endodontic infection, the current finding revealed that no significant difference in prevalence was observed when comparing *E. faecalis* in root-filled teeth with and without periradicular lesions (37). Moreover, other bacterial taxa including as-yet-uncultivated bacteria may be involved in post-endodontic treatment failure. It was indicated that mixed bacterial infection, other than *E. faecalis*, may play an

important role in post-treatment apical periodontitis (38). However, *E. faecalis* was commonly used as bacterial model in *in vitro* study because it can be grown in both aerobic and anaerobic condition, penetrate into dentinal tubule and resist to bacterial eradication by chemo-mechanical procedure.

B. Bacterial Biofilm

Biofilm are a complex dynamic communities of bacteria embedded in a self-made polysaccharide matrix established on various surface structures (36, 39, 40). The three major components involved in biofilm formation are bacteria cells, a solid surface and a fluid medium. Bacteria in biofilm are originated from free-floating bacteria existing in an aqueous environment or so called planktonic bacteria. Biofilm formation occurs in three consecutive stages (39, 40).

- Stage 1: Adsorption of macromolecule such as protein, glycoprotein from saliva or gingival crevicular fluid and some secreted bacterial products to the solid surface creating a conditioning layer.
- Stage 2: Adhesion and co-adhesion of planktonic bacteria to the conditioned layer. There are many factors that affect bacterial attachment to solid surface include pH, temperature, nutritional availability, bacterial growth stage, bacterial contact time and physiochemical properties of initial colonizer bacteria (40).

- Stage 3: Monolayer of microbes attracts secondary colonizers. The growth and metabolism of attached bacteria result in structurally organized mixed microbial community. During this phase, environment has an effect to characteristic of bacteria in biofilm (39).

Stages of biofilm formation are illustrated as seen in figure 1.

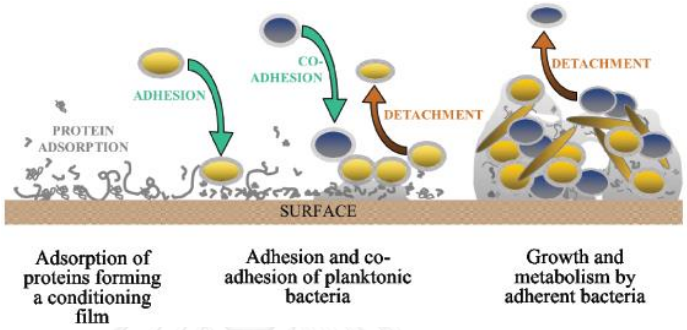


Figure 1: Stage of biofilm formation (39)

The nature of biofilm structure and physiological characteristics of resident microorganisms offer an inherent resistance to antimicrobial agents, such as antibiotics and disinfectants (41). The resistance of microbes in biofilm to antimicrobial agents has been found to be 1000 times more than microbes in planktonic form (42). Biofilm-grown bacteria might develop a biofilm-specific biocide resistant phenotype (2, 3).

1. *Evidence for Biofilm Study in Endodontic Infection*

Biofilm in root canal infection is different from biofilm on caries or periodontitis because root canal is originally a sterile compartment (43). Progression of root canal infection alters the nutritional and environmental

status within root canal. This sequential alteration introduced more anaerobic bacteria which change ecological niche for surviving microorganisms (40).

Endodontic bacterial biofilms can be categorized as intraradicular, extraradicular and periapical biofilms (40). It is assumed that preconditions for biofilm formation in the root canal vary depend on the cause of pulpal breakdown and inflammatory exudate toward the apex. Inflammatory exudate provides the fluid vehicle and source of nutrient for bacterial colonization (39). Hubble et al. (2003) demonstrated that serine protease and collagen binding protein (Ace) of *E. faecalis* contributed to the adhesion on root canal wall of extracted human teeth (44). In apical periodontitis, Ricucci et al. (2010) evaluated the prevalence of bacterial biofilm in extracted teeth with apical periodontitis by histopathological study and found that intraradicular biofilms were observed in 77% of apical segment (80% were from untreated canals and 74% from treated canals). The difference of bacterial biofilm between untreated and treated canal in terms of bacterial arrangement as intraradicular biofilm was not significant. In contrast, extraradicular biofilm were observed only 6%. Biofilm was often confined to the root canal and faced by inflammatory cell near the root apex because exudate seepage from apical part provide nutrient to form biofilm. In addition, the dentinal tubules subjacent to the biofilm were also heavily invaded to varying depths as shown in figure 2 (45).

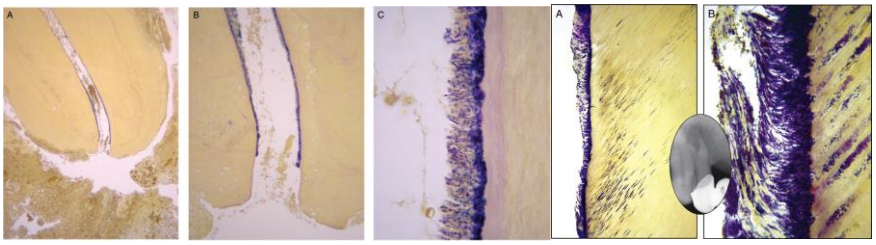


Figure 2: Talor-modified Brown and Brenn method show bacteria biofilm cover the dentinal wall in apical part of root with apical periodontitis. Dentinal tubules were invaded by bacteria covering with biofilm (45).

2. *Observation of Biofilm in vitro Study*

Biofilm in root canal was observed by examination of extracted teeth with periapical lesion. For example, when root sections were examined by electron microscope, densely aggregate cocci and rods embedded in extracellular matrix were observed along the root canal wall (45, 46). Many studies demonstrated morphology of endodontic biofilm using different experimental methodology such as histopathological study, scanning electron microscope or confocal laser scanning microscopy. The details of each method applied for biofilm studies in various aspects are described as follow:

a. Histological study (Talor-modified Brown and Brenn stain): Section of apical third of root with necrotic pulp and apical periodontitis lesion was typically observed in histological study. It demonstrated that bacterial cells attached to dentin surface and enmeshed in self-produced extracellular matrix as shown in figure 2. However, quantitative data of cultivated bacterial cells and viability which

perform of all cell function of bacterial cells could not be identified (39, 43, 45).

b. Scanning Electron Microscopy (SEM): This method allows the study of characteristics of bacterial biofilm adhered on root canal wall. SEM could demonstrate clumps *of E. faecalis* biofilm colonized on root canal (47). However, examining specimen with SEM cannot provide quantitative data of cultivated bacterial cells.

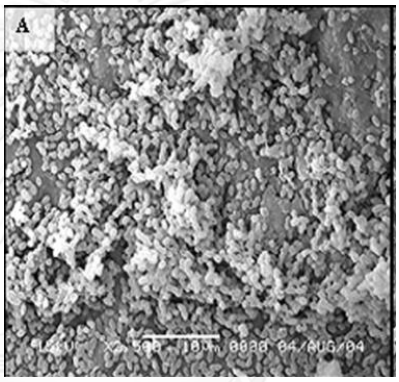


Figure 3: There are clumps of coaggregated bacterial cells of *E. faecalis* biofilm formed on root canal wall (47).

c. Transmission Electron Microscopy (TEM): This method identified the nature of the extracellular fibers in biofilms and be able to elucidate their association with the cells (48, 49).

d. Atomic Force Microscopy (AFM): AFM was used for imaging the hydrated freshwater bacterial biofilms on copper surfaces. Specimens were placed on an XYZ piezoelectric translator. A true 3D image of the sample surface is reconstructed from the collected data (48, 50). AFM

studies attempt to understand the more realistic properties such as interaction and attachment to surface of biofilm in figure 4 (51). The image from AFM provides height information which determined the slope surface of specimen compare to SEM (51).

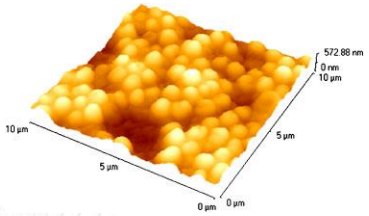


Figure 4: AFM image of *Staphylococcus epidermis* biofilm showing the structure and complete surface coverage of the biofilm (51).

e. Confocal Laser Scanning Microscopy (CLSM): Distel et al. (2002) firstly introduced CLSM to demonstrate bacteria biofilm architecture in root canal wall. The viability of bacterial cell in biofilm can also be evaluated by fluorescence viability staining (36, 43, 47). This method can detect viability of bacterial cell in biofilm. The volume ratio of red fluorescence to green and red fluorescence could indicate the proportion of killed cells (52).

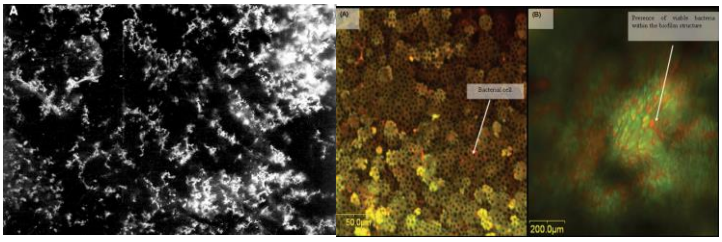


Figure 5: (left) BacLight stain containing SYTO9 and propidium iodine (36), (right) Acridine orange fluorescence staining of *E. faecalis* biofilm on root canal wall (47).

f. Viable plate count procedure: The most common technique for investigating bacterial viability in quantitative data. Dispersed bacterial cells in biofilm are plated onto a solid microbiological medium, incubated, and counted (53).

Among experimental studies examining structure of biofilm formed on root canal wall, *E. faecalis* is one of the most common microorganism used in bacterial model (36, 43, 45, 47).

3. Eradication of Root Canal Biofilm

Microbial community in biofilms is difficult to eradicate (39). The complex structure and dense organization of polymeric matrix might restrict the penetration of antimicrobial agent (39). Many studies mimic biofilm formation in laboratory in order to analyze the effectiveness of bacterial biofilm eradication process. Medicaments containing Chlorhexidine and human beta-defensin-3 peptide were more effective than calcium hydroxide, against *E. faecalis* biofilm (54, 55). The results of antimicrobial irrigation

demonstrated that NaOCl was the most effective irrigant in bacterial biofilm reduction (5, 56-59). However, the effect of NaOCl concentration in its efficacy in eliminating bacterial biofilm is still inconclusive. Both 1% and 6% NaOCl demonstrated same efficient in eliminating biofilm (5). However, disinfection of polymicrobial biofilm on apical root of primary endodontic infection indicated that 6% NaOCl was capable to disrupt and remove biofilm effectively, while 3% and 1% NaOCl were able to partially disrupt biofilm and they still resulted in positive culture (60). Moreover, the combination of 2.5% NaOCl and 17% EDTA significantly decreased *E. faecalis* biofilm in SEM study (61, 62). Recently, CLSM studies indicated that 3-week old biofilm model on root dentin were resistant to the eradication by antimicrobial agent (63, 64). The effectiveness of killing bacteria in biofilm are depend on time, type of irrigants and concentration of irrigants (63-66). The details of studies on bacterial biofilm eradication were shown in table 1.

Table 1: Studies of the effects of antimicrobial agent on endodontic biofilm

Study	Bacteria	Biofilm formation technique	Evaluated by	Time of bacterial incubation	Anti-microbial agent	Results
Spratt 2001	Single-species biofilms -*P.intermedia* -*P.micros* -*S.intermedius* -*F.nucleatum* -*E.faecalis*	cellulose nitrate membrane filters	CFU count	48 hr	15 and 60 min test in -5 p.p.m. colloidal silver - 2.25% NaOCl -0.2% CHX -10% iodine	-Effectiveness was dependent on the nature of organism and contact time. -NaOCl was generally the most effective agent tested.
Giardino 2007	*E.faecalis* (ATCC 29212)	cellulose nitrate membrane filters	CFU count	48 hr	5, 30, 60 min test in -5.25% NaOCl -BioPure MTAD -Tetraclean	5.25% NaOCl can disgregate and remove the biofilm at every time point.

Study	Bacteria	Biofilm formation technique	Evaluated by	Time of bacterial incubation	Anti-microbial agent	Results
Dunavant 2006	*E.faecalis* (OG1X)	Flow cell system	Percentage kill from CFU count	24 hr	1 and 5 min test in -6% NaOCl -1% NaOCl -Smear clear -2% CHX -BioPure MTAD	Both 1% and 6%NaOCl were more efficient in eliminating biofilm than other solutions test.
Clegg 2006	Bacteria from patient saliva	Incubated with apical root section	-SEM -culture	7 days	15 min. immersed in -6% NaOCl -3% NaOCl -1% NaOCl -1% NaOCl/MTAD	-6% NaOCl was capable disrupted biofilm and resulted in negative culture. -3% and 1% NaOCl were able partial disrupted biofilm and resulted in 20-90% positive culture.

Study	Bacteria	Biofilm formation technique	Evaluated by	Time of bacterial incubation	Anti-microbial agent	Results
Chavez de Paz 2010	Single species biofilm -*E.faecalis* -*L.paracasei* -*S.anginosus* -*S.gordornii*	Miniflow chamber system	% survival of biofilm cell on fluorescent marker	24 hr	5 min test in -2.5% CHX -17% EDTA -1% NaOCl	1% NaOCl affected the membrane integrity of all organisms and remove most biofilm cell.
Ozdemir 2010	*E.faecalis* (ATCC 29212)	Incubated with Single root -young(<30y) -old(>60 y)	-SEM -CLSM -CFU count	24 hr	-2.5% NaOCl -17% EDTA -NaOCl + EDTA	-Combine application with NaOCl and EDTA reduced biofilm significantly. -Biofilm formation is thicker in elderly group.
Soares 2010	*E.faecalis* (ATCC 19433)	Incubated with canine	-SEM -CFU count	21 days	5 min. irrigation of 5.25% NaOCl 17% EDTA -conventional IR -alternating IR	-Alternating IR yield 0 CFU/ml after experimental period. -SEM of alternating IR confirm several bacterial-free site.

Study	Bacteria	Biofilm formation technique	Evaluated by	Time of bacterial incubation	Anti-microbial agent	Results
Ordinola-Zapata 2012	subgingival plaque of healthy volunteer	Incubated with Bovine root dentine	CLSM (percent of biovolume, surviving cell, biofilm thickness)	12 hr	5 min. immersed in -1% NaOCl -2% CHX -10% citric acid -17% EDTA -sterile water	1% NaOCl was the only irrigant that had a significant effect on biofilm viability.
Wang2012	*E.faecalis* VP3-181	Incubated with single root tooth	CLSM (LIVE/DEAD bacterial viability stain)	-1 day (young biofilm) -3 wk (old biofilm)	1 and 3 min. immersed in -6% NaOCl -2% NaOCl -2% CHX	- Significantly fewer bacteria were killed in the 3-week-old dentin biofilm than in the 1-day-old biofilm. - 6% NaOCl was the most effective followed by QMiX in 3-week-old dentin biofilm

Study	Bacteria	Biofilm formation technique	Evaluated by	Time of bacterial incubation	Anti-microbial agent	Results
Stojicic 2013	Plaque bacteria from 6 donors	Incubated with collagen-coated hydroxyapatite disk	CLSM (LIVE/DEAD viablility staining)	1, 2, 3, 4 and 8 wk	1 and 3 min. exposed to -1% NaOCl -0.2/0.4% IPI -2% CHX	-After 3 weeks of growth, the biofilm bacteria were more resistant to the same agents. -1% NaOCl was the most effective agent in killing bacteria in biofilm.
Du 2014	*E.faecalis* VP3-181	Incubated with cylinder root dentin	CLSM (LIVE/DEAD viablility staining)	-1 day (young biofilm) -3 wk (old biofilm)	3, 10 and 30 min. immersed in -6% NaOCl -2% NaOCl -2% CHX	6% NaOCl was the most effective against both the 1-day-old and 3-week-old biofilms.

Effect of Endodontic Procedure on the Reduction of Intraradicular Bacteria

A. Effect of Mechanical Instrumentation on Bacterial Reduction

1. Disinfection Protocol of Mature teeth

In clinical practice, MI has been considered to be the most important phase of root canal therapy (33). Bystrom and Sunqvist (1981) found that mechanical instrumentation follow by IR with physiologic saline was able to eliminate more than half of bacteria in root canal system (6). According to Dalton's study, increasing root canal debridement while using saline as an irrigant readily resulted in substantial bacterial reduction (67). Subsequently, the study of Shuping et al. (2000) using 1.25% NaOCl as an irrigant was compared with Dalton's study. The results indicated that there was a significantly greater extent of intracanal bacterial reduction after irrigation with NaOCl, compared with sterile saline. The results of Dalton et al. (1998) and Shuping et al. (2000) studies demonstrated that although intracanal bacteria were greatly reduced during the initial phase of MI, the effect of antibacterial irrigant appeared to be minimal. The antibacterial effect of NaOCl and calcium hydroxide medication in premolar and molar were more significant in root canal with larger canal preparation (size 35-60), as shown in figure 6 (7). However, it was extremely difficult or impossible to completely eradicate root canal bacteria because of complexity of root canal system.

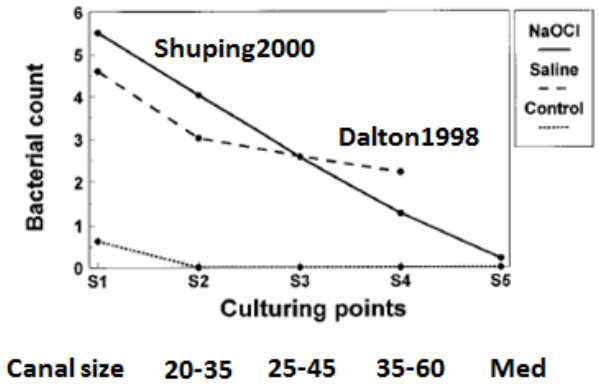


Figure 6: The relation between bacterial count in log_{10} mean values and culturing point of increasing instrumentation number.
Solid line of the graph is from Shuping et al. (2000) study, dash line is from Dalton et al. (1998) saline study and dotted line is negative control. The teeth in negative control were diagnosed with irreversible pulpitis and no periapical lesion. These served were presumably “uninfected canals” (7). S1, S2, S3, S4 and S5 referred to microbiological sample collection of pre-instrumentation, after initial instrumentation, during instrumentation, final instrumentation and post-medication, respectively.

Effect of root canal preparation to the larger size on bacterial reduction has been evaluated by Card et al. (2002). The initial root canals size were ISO size 10-20. After initial MI of canine, premolar and molar, the authors found that increasing canal size preparation, from ISO size 60 to 100 result in no significant difference in bacterial elimination (17). Other study used bioluminescent bacteria culture technique to compare the efficacy of distilled water irrigation in the removal of intracanal bacteria among teeth with different root canal preparation sizes. The results show that size of canal preparation influences the cleansing efficacy of irrigation. While Irrigation 1

mm from WL was significantly less effective in canals prepared to size 36, an increase in apical size of root canal enlargement, from size 60 to 77, in canine, resulted in no significant difference in bacterial reduction as shown in figure 7 (68).

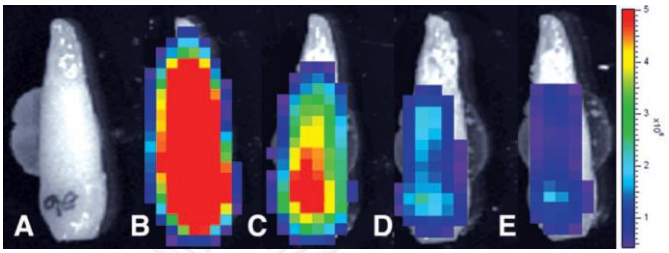


Figure 7: Image of representative tooth. (A) no bacteria, (B) *P. fluorescens* 5RL in root canal, (C-E) after sterile water irrigation in canal (C) size 36, (D) size 60, (E) size 77. Color bar on the right side gives bioluminescence image units (68).

According to above studies, root canal preparation was an effective means to remove infected dentin. However, after the root canals were prepared to some extent, extension of mechanical instrumentation through deeper layer of dentine demonstrated no significant difference in bacterial reduction (7, 17, 67, 68).

2. *Disinfection protocol in Immature Teeth with Open Apex*

In immature teeth with incomplete root formation, the complete removal of necrotic tissue and intraradicular bacteria is difficult (69). When compared with mature teeth, bacterial reduction efficiency of root canal debridement and antibacterial irrigation in immature teeth could be more challenged (70). MI could not effectively eradicate necrotic and infected pulp

tissue in compromised fragile immature root canals (70). According to minimal to no MI protocol of immature teeth, in order to preserve vital tissue, antimicrobial irrigation and intracanal medication were used to achieve the root canal disinfection (71). In regenerative endodontics procedure, 1-5.25% NaOCl has been used for root canal irrigation (14, 15, 23, 24).

The disinfection procedure solely relied on irrigation and medication to reduce the number of bacteria in pulpal space. Although the mechanical instrumentation was omitted, the favorable outcome including the continuation of root development and periapical healing were observed in many cases (14, 15, 23, 24). Moreover, the recent study applied EndoVac, the newer irrigation protocol, for regenerative endodontics in dog teeth (72). This alternative protocol provided similar bacterial reduction compared with conventional irrigation plus intracanal antibiotic medication (22, 72).

B. Effect of Mechanical Instrumentation Extent on Fracture Resistance of Teeth

Although an increase in size of root canal preparation effectively reduced bacteria in root canal, aggressive MI in large root canal with thin dentinal wall thickness could lead to the weaken and fractured teeth (12). Despite the effectiveness of canal debridement in reducing intracanal bacteria, the limitations in MI in large root canal teeth need to be concerned.

Evidences indicated that the more increase in size of root canal preparation, the more decrease in fracture resistance of teeth. Wilcox et al.

(1997) assess the correlation between amount of remaining root dentin and the development of vertical root fracture by preparing root canal to 20%, 30%, 40% and 50% of root canal width. The results demonstrated that the more tooth structure was removed, the more likely a root fracture and craze line also developed during testing procedure as shown in figure 8 (12). Furthermore, Trope et al. (1992) and Ricks Williamson et al. (1995) also demonstrated that extensive root canal preparation (ISO size 55-100) can lead to the higher stress concentration and the weaken roots (11, 73).

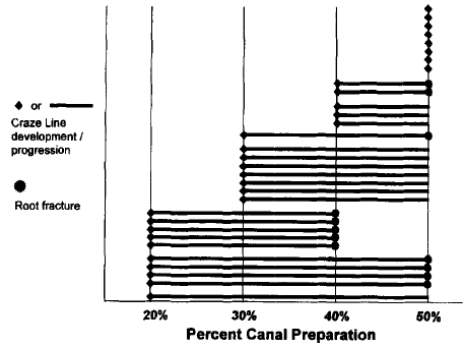


Figure 8: Five teeth developed VRF at 40% of root width and seven at 50% of root width all of teeth had evidence of root craze lines.
The remaining 19 teeth all developed craze lines at the end of experiment. Rhomboid dot is initiation of craze line and continue progression (solid line) until separate during testing (circle dot) (12)

The factor affected fracture resistance of mechanical instrument teeth are list as follow:

1. *Canal shape*: Stress concentration is increase in bucco-lingual side of oval canal shape and enlargement of oval root canal may significantly weaken the tooth (74, 75).

2. *Larger preparation*: The more tooth structure removed, the more likely a root is to fracture (12, 73).
3. *Instrument taper*: The root was significantly weaken by the preparation with greater taper instrument (76).
4. *Retreatment procedure*: During re-instrumentation, the mean fracture resistance is decrease significantly (10).

For all of the reasons, it can be concluded that an increasing in size of root canal preparation can lead to a weaken tooth structure, thus decrease fracture resistance of the tooth. Therefore, thin root canal wall seemed to make MI more challenged, especially in initial large root canal teeth or retreated teeth with infected root canal space. In these cases, bacterial reduction by mechanical instrumentation may be limited and could not be performed as much as it should be. Although there is no protocol specifically suggested for bacterial reduction in teeth with large root canals in general, the non-invasive protocols including a minimal or no MI and copious IR with low concentration of NaOCl follow by medication with calcium hydroxide or triple antibiotic paste was recommended as protocol for root canal disinfection for immature teeth undergone regenerative procedures (77-79).

C. Effect of Supplementary Technique on Bacterial Reduction: Ultrasonic Irrigation

The use of irrigating solution is an important part of effective chemo-mechanical instrumentation. The goal of irrigation is to facilitate removal of pulp tissue remnant, microorganism, smear layer and dentine debris (33). The effectiveness of irrigation can be enhanced physically by using together with ultrasonic energy (21). This was first investigated in root canal by Martin in 1976. Cavitation effect of ultrasonic energy helps scrubbing and dislodging debris and organic component from root canal surface (80). Martin's study also demonstrated that the use of ultrasonic alone can reduce microorganism but coupling it with antibacterial agent leading to a more efficient bactericidal synergism (81).

1. Mechanism of Passive Ultrasonic Irrigation

The ultrasonic device converts electrical energy into ultrasonic waves of a certain frequency by magnetostriction or by piezoelectricity (21). The properties of the ultrasonic are determined by the frequency 25-40 kHz of oscillating instrument (82). The transverse oscillation of file consist of antinodes (A) where the greatest oscillation occurs and nodes (N) where minimal oscillation occurs (83). Frequency and intensity of ultrasonic power setting play a role in transmission of energy from the ultrasonically oscillating

file to irrigant. A higher frequency was result in a higher streaming velocity of irrigant and more powerful acoustic streaming (21).

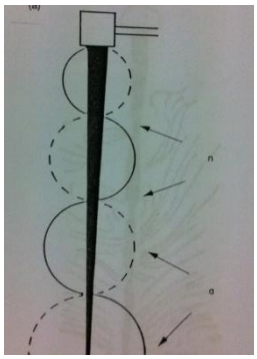


Figure 9: the transverse oscillation of ultrasonic file (83)

1.1 Acoustic streaming

Acoustic streaming is the rapid movement of fluid in circular or vortex-like motion around a vibrating file when applying the ultrasonic energy. It allows the irrigant to penetrate more easily in apical part of canal isthmus (83). The characteristic streaming pattern is nodes and antinodes along the length of the oscillating file as shown in figure 10. When the file is unable to vibrate freely, acoustic streaming will become less intense (21).

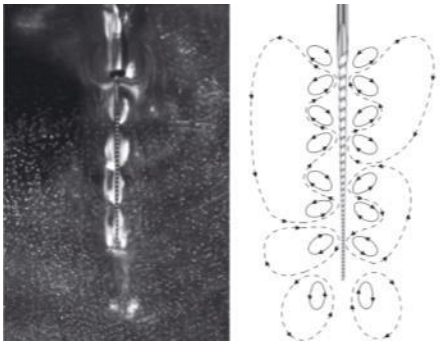


Figure 10: (left) acoustic streaming around file in free water, (right) schematic drawing (21)

Moreover, the effect of different size of endosonic file at different power setting was investigated by Ahmad et al. (1987). The results indicated that smaller files generated relatively greater acoustic streaming and increased the streaming velocity according to the equation. The shear flow caused by acoustic streaming produces shear stress which can remove debris and bacterial along the root canal wall (84).

$$v = \frac{\omega \epsilon_0^2}{a}$$

v: liquid streaming velocity **ω**: 2¶ times the driving ultrasonic frequency

ϵ_0: displacement amplitude **a**: the radius of the file/wire

1.2 Cavitation

Cavitation is the impulsive formation of cavities in a liquid through tensile forces induced by high-speed flows or flow gradients. Acoustic cavitation can be defined as the creation of bubble or expansion, contraction and/or distortion of pre-existing bubbles in liquid (figure 11) (80). The effect creates bubbles under extreme hydrodynamic pressure caused radiation shock waves that can disrupt cell wall or create effective scrubbing and cleaning mechanism due to the irregular agitation (81). It beneficially improves the chemical and mechanical efficacy of root canal cleansing by promote tissue dissolution and intracanal bacterial eradication (21, 80).

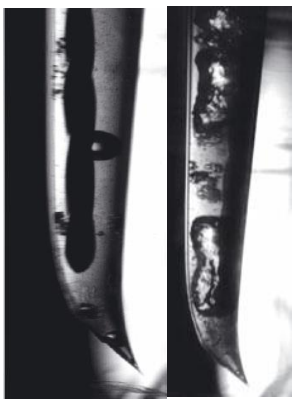


Figure 11: (left) cavitation phenomenon and streaming pattern (right) vigorous microstreaming and collapsing cavitation bubbles in glass root canal model (21)

The surface of file also plays an important role in enhancement of cavitation. The smooth file with sharp edges and square cross-section produced significant more cavitation than a normal K-file. When the file was in contact with the canal wall, stable cavitation was less effective (21, 85).

However, it was showed in many studies that cavitation has no or minimal effect on mechanism of root canal debridement. The phenomenon of cavitation was investigated by Ahmad et al. (1988). SEM observation revealed no significant difference in debris score removal implying that cavitation did not play an important role in debridement mechanism (86), while Walmsley et al. (1987) claimed that cavitation provides only minor benefit in ultrasonic irrigation (83). However, the ultrasonic power generated bubbles which convert into heat and hydrodynamic shear field and able to disrupt biological tissues (83).

2. *Ultrasonic irrigation techniques*

According to the irrigant flushing techniques, there are 2 types of ultrasonic irrigation techniques (87).

2.1 *Passive ultrasonic irrigation using intermittent flushing technique (I-PUI):* The irrigation and ultrasonic tips are seperately applied into the root canal.

2.2 *Passive ultrasonic irrigation using continuous flushing technique (C-PUI):* The irrigation technique allows simultaneous continuous irrigant delivery and ultrasonic activation at the same time. For C-PUI, the irrigant outlet could be located either at the location closed to the hub of ultrasonic file (88) or at the tip of irrigating needle (8, 89)

3. *The Effects of Passive Ultrasonic Irrigation (PUI) on Disinfection and Cleanliness of Root Canal*

Van der Sluis et al. (2010) exhibited that intermittent flush with three cycles of ultrasonic activation and irrigant refreshment could reduce dentin debris effectively (88, 90). Recently, Guerreiro-Tanomaru et al. (2015) exhibited that intermittent flush with three cycles of ultrasonic activation and irrigant refreshment (PUI) with 1% NaOCl could reduce intraradicular bacteria effectively (91). This flushing technique was less likely to push the irrigant out of the root apex (92).

In regard to the C-PUI where irrigant outlet is located closed to the hub of needle, the efficacy in bacterial reduction was not significantly different from conventional irrigation (93-95). However, Carver et al. (2007) reported that 1-minute application of C-PUI, with continuous flushing from the ultrasonically activated needle, was effective in reducing the number of bacterial-positive culture (8). In addition, C-PUI could effectively introduce irrigant into the apical third of root canal (87, 96)

Mechanical instrumentation results in cleaner root canal. However, untouched area such as root canal irregularities, isthmus and apical delta were not be able to debrided completely with MI alone (21). Ultrasonic device was used as an adjunctive method for debris and bacterial removal. After shaping the root canal, final flush with syringe irrigation and PUI were found to be effective in bacterial eradication and flushing of dentine debris (8, 9, 21, 97). Two parameters, bacterial and debris removal were used to evaluate the effectiveness of ultrasonic irrigation.

2.1. *Debris and smear layer removal in root canal system*

Several studies demonstrated that PUI could remove pulp tissue and debris effectively in the area that is untouchable by endodontic instruments as a result of acoustic streaming. The taper and diameter of root canal have an influence on the efficacy of

removing dentine debris. The more taper of root canal, the more debris was able to removed (98).

The efficacy of different types and concentrations of irrigant solution used in ultrasonic irrigation on debris removal has been tested (21). Applying PUI with water as an irrigant was unable to remove smear layer effectively (99). NaOCl activated by ultrasound generates greater number of small bubbles which increase efficiency of organic tissues dissolving, compared to distilled water (90). Many studies concluded that PUI with NaOCl was significantly more effective in removing dentine debris than syringe irrigation (99, 100). The use of one minutes of ultrasonic activation after hand/rotary instrumentation resulted in significantly cleaner canals in histologic evaluation (97). Furthermore, van der Sluis et al. (2010) evaluated the effect of irrigants on dentine debris removal during refreshment and activation cycle of ultrasonic irrigation. The results show that intermittent flush method of three refreshment/activation cycles in two minutes produces a cumulative effect in dentine debris removal. PUI with NaOCl demonstrated a statistically significant difference in debris score reduction compared to distilled water (90).

2.2 Bacterial removal in root canal system

A general problem of cleaning and shaping is that endodontic file cannot access every part of root canal wall, especially in oval shape or isthmus. Spoleti et al. (2003) evaluated the influence of ultrasonic activation with saline irrigation in lower incisors, canines and first molars and found a significant difference in reducing of bacterial colonies after using ultrasonic activation for 10 seconds (101). According to *in vivo* study of mandibular teeth by Carver et al. (2007), the addition of one minute ultrasonic irrigation using 6% NaOCl resulted in a significant reduction in CFU count and positive cultures, compared to conventional irrigation. Moreover, logistic regression analysis indicated that the addition of PUI was seven times more likely to yield a negative culture than normal irrigation (8). In addition, Harrison et al. (2010) demonstrated that PUI supplementary was as effective as one week calcium hydroxide medication in bacterial reduction, after routine chemo-mechanical instrumentation. It also reduced bacteria within dentinal tubule up to 12-18% from baseline samples in histologic examination in *in vitro* study (9). The summarized studies of bacterial removal enhancing by PUI were concluded in table 2.

While benefit of PUI in root canal cleaning has been demonstrated as mentioned, many studies revealed that using PUI with 1-2.5% NaOCl did not

enhance bacterial reduction beyond usual chemo-mechanical instrumentation (18, 93-95, 102). Therefore, the result of PUI in term of bacterial reduction may be still inconclusive due to the difference of each study design.

Table 2: Bacterial evaluation studies compare PUI with syringe irrigation

study	Study design	Evaluation	Tooth type	Preparation size	irrigation	Exposer time	result
Spoleti 2003	In *vitro*	CFU count	Incisor Canine DB of 1st molar	Gate no.1,2,3 K-file no.50 K-file no.35 (molar)	NSS	10 s	Significant difference in reducing of survival colonies after ultrasonic activation.
Carver 2007	In *vivo*	CFU count	Mesial root of mandibular molar	Profile GT 30/06 VS K-file no.30	6% NaOCl	1 min.	The addition of 1 min. of PUI resulted in significant reduction in CFU count
Harrison 2010	In *vitro*	LM, SEM	Straight roots with single canal	ProTaperF3	1% NaOCl	1 min.	One minute ultrasonic activation after routine preparation might enhance bacterial reduction in root canal.

study	Study design	Evaluation	Tooth type	Preparation size	irrigation	Exposer time	result
Alves 2011	In *vitro*	culture test	Mandibular Incisor/ Premolar	BioRace 40/04	2.5% NaOCl	1 min.	PUI did not significantly increase the incidence of negative culture.
Paiva 2012	In *vivo*	Culture and PCR	Single root	BioRace 40/04 MI to size 40-50	2.5% NaOCl	1 min.	PUI did not significantly increase the incidence of positive culture.
Paiva 2013	In *vivo*	Culture and PCR	Single root	BioRace 40/04 MI to size 40-50	2.5% NaOCl	1 min.	PUI did not significantly reduce the positive result of bacterial culture.

study	Study design	Evaluation	Tooth type	Preparation size	irrigation	Exposer time	result
Bhuva 2010	In *vitro*	biofilm score	Single root	ProTaper F3 Apical size 50	1% NaOCl	20 s Intermittent flush (2 cycles)	Both syringe and PUI were effective in removal of *E.faecalis* biofilm (no significant difference).
Grundling 2011	In *vitro*	SEM CFU count	Bovine incisor	K-file no.60	2% NaOCl	15 s	No significant difference between syringe and PUI in CFU count.
Beus 2012	In *vivo*	Culture test	Posterior teeth	ProTaper MI to size 25-50	1% NaOCl	30 s Intermittent flush	No significant difference between multi-irrigation protocol using PUI and non-activated syringe irrigation.

CHAPTER III

RESEARCH METHODOLOGY

Target Population

Large root canal teeth with thin dentinal root canal wall

Sample

Human mandibular premolar with intact single root and single root canal

Definition

The definition of “large root canal teeth” in this study referred to the intact single root canals of human mandibular premolar which had apical root canal size of 0.6 mm. In this study, apical portion of root canals were prepared according to the definition of larger root canal of immature teeth described by Cvek et al. (1976) (103). Cvek et al. (1976) described larger root canal of immature teeth as root canal with apical size equal or greater than 0.6 mm. Therefore, only prepared root samples with apical root canal size of 0.6 mm were included in this study. Moreover, root canal size at the level of cemento-enamel junction (CEJ) was controlled to be 3-4 mm in bucco-lingual width and 1-2 mm in mesio-distal width to standardize all root samples.

Independent Variable

Different disinfection protocols

1. Mechanical instrumentation (MI)

2. Conventional irrigation with 2.5% NaOCl (IRN)
3. Passive ultrasonic irrigation (PUI)
4. Conventional irrigation with 0.9% normal saline (IRS)
5. Initial bacterial count (initial)

Dependent Variables

The number of bacterial cell count (CFU counts)

Control variables

Type and irrigant concentration, irrigation time, rate of irrigation and bacteria inoculation period

Confounding Factors

Root canal irregularities of each tooth, error from laboratory technique

Hypothesis

Ho: There is no difference in the number of bacterial cell count among four disinfection protocols applied in large root canal teeth.

H_1: There is a difference in the number of bacterial cell count among four disinfection protocols applied in large root canal teeth.

Ethical Consideration

This research was approved from the Ethics Review Committee for Research Involving Human Research Subjects, Chulalongkorn University (HREC-DCU 2014-012) because of using extracted human teeth.

Materials

1. Straight, intact human mandibular premolar with complete root formation
2. *Enterococcus faecalis* (standard strain ATCC 29212)
3. Brain heart infusion broth (Himedia, Mumbai, India)
4. Blood agar base (Himedia, Mumbai, India)
5. Chemical agents
    a. 0.1% Thymol (Faculty of Dentistry, Mahidol University, Thailand)
    b. 2.5% Sodium hypochlorite (Faculty of Dentistry, Chulalongkorn University, Thailand)
    c. 17% Ethylenediaminetetraacetic acid (EDTA) (Faculty of Dentistry, Chulalongkorn University, Thailand)
    d. 1% Phosphate buffer saline (PBS)
    e. 10% Sodium thiosulphate (Emsure®, Darmstadt, Germany)
    f. Distilled water
    g. 0.9% Normal saline solution (Faculty of Dentistry, Chulalongkorn University, Thailand)
    h. 50% Glutaraldehyde EM grade distillation purified (Electron Microscopy Sciences, Pennsylvania, USA)
6. 6-well plate, 24-well plate (Costar®, New York, USA)
7. Test tube
8. 1.5 ml Eppendoft tube (Sarstedt, Germany)

9. K-file no. 60, 70, 80 and 90 (Dentsply Maillefer, Ballaigues Switzerland)
10. Diamond disc 270D (Intensive, Montagnola, Switzerland)
11. Irrisafe ultrasonic tip K20/21 mm (Acteon, NA, USA)
12. P5 Newtron Satelec (Acteon, NA, USA)
13. Peeso reamers no.3 (Dentsply Maillefer, Ballaigues Switzerland)
14. Nail varnish (OPI®, USA)
15. Putty silicone (Detaseal®, NuvoDent, Ettlingen, Germany)
16. 25-gauge needle syringe and 10 ml sterile plastic syringe (Nipro, Osaka, Japan)
17. paper point size L (Faculty of Dentistry, Chulalongkorn University, Thailand)
18. Micropipette, 5 ml pipette (Corning incorporated, Reynosa, Mexico)
19. Composite resin (3M EPSE Filtek™ Z350, MN, USA)
20. ISOMET™ 1000 precision saw (Buehler, Illinois USA)
21. Incubator (Forma Scientific, NJ, USA)
22. Spectrophotometer (Thermo spectronic genessys 20, NJ, USA)
23. Light-cured composite (Elipar Trilight, 3M, MN, USA)
24. Scanning Electron Microscope (JSM-5410 LV, JEOL, Japan)
25. Microson™ ultrasonic cell disruption (Heat system, New York, USA)

Methods

A. Sample Preparation and Selection

Intact human mandibular premolar teeth extracted for orthodontic reason from young subjects (<25 years old) were stored in 0.1%Thymol (Mahidol university, Bangkok, Thailand). After radiographic examination, teeth with a single root canal with curvature less than 5° (104),15–18 mm long, and complete root formation were selected.

For the experimental groups and sterile control, the roots were sectioned using a precision saw (ISOMET 1000, Buehler, USA) perpendicular to the long axis into samples of 13 mm long from cemento-enamel junction (CEJ) and at 13 mm to the apical end (figure 12A). The pulp tissue was removed using an H-file (Dentsply Maillefer, Ballaigues, Switzerland). Only specimens with 0.6 mm apical root canal diameters (figure 12B) and root canal width of 3-4 mm bucco-lingually and 1-2 mm mesio-distally at the level of CEJ (figure 12C) were selected.

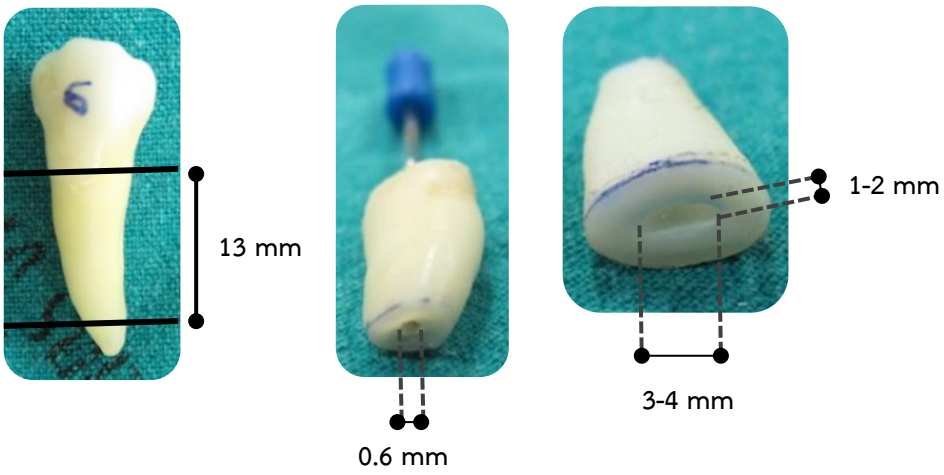


Figure 12: Transversely tooth sectioned at the level of 13 mm from CEJ by ISOMET 1000 (A), apical size of sectioned roots should fit with K-file size 60 (B) and root canal width of 3-4 mm bucco-lingually and 1-2 mm mesio-distally at the level of CEJ (C)

Figure 13: Coronal end of root was cut at the level of CEJ (A), apical end of root was seal with composite resin (B) and the root was fixed in customized putty silicone in upright position (C).

Apical size of sectioned roots must fit with K-file size 60 to mimic wide root apex of large root canal and get rid of apical ramification. Fifty-one roots were capped with composite resin (3M EPSE FiltekTM Z350, MN, USA) to

create an apical seal and external root surfaces were coated with nail varnish (figure 13B). Customized silicone blocks were made to secure the roots in an upright position (figure 13C).

For biofilm verification, 4 root specimens were cross-sectionally cut into 6 mm pieces in the middle third of root (figure 14A) and the pulp tissue was removed with an H-file. Guiding grooves were created at the top and bottom end in bucco-lingual direction of the specimens using diamond disc (figure 14B). The outer root surface was coated with nail varnish.

(A) (B)

Figure 14: Specimens were cross-sectionally cut into 6 mm at the level of 5 mm above root apex by ISOMET 1000 (A) and grooves at coronal end and apical end of specimen were created in bucco-lingual direction on the surface of cross-sectional area (B).

The smear layer was removed from the root canal of 55 root specimens by irrigating with 5 mL of 17% EDTA followed by 2.5% NaOCl. The bactericidal effect of NaOCl was inactivated by rinsing with 5 mL of 10% sodium thiosulphate (Emsure®, Darmstadt, Germany). The specimens and silicone blocks were sterilized using ethylene oxide gas.

B. Verification of *E. faecalis* Biofilm

In order to ensure the model of biofilm formation of root canal, two root specimens were incubated in sterile BHI broth as sterile controls, while 2 specimens were infected with *E. faecalis*.

Two days before experiment, all prepared roots were separately immersed in 5 ml of sterilized BHI broth in 6 well plate and incubated for 24 hours at 37℃ for sterile check of each sample. At the beginning of the experiment, *E. faecalis* ATCC 29212 from -80 ℃ glycerol stock was plated on blood agar. On the following day, one colony of bacteria was inoculated in BHI broth and cultured overnight at 37 ℃ with 5% CO_2. Bacterial culture was adjusted to optical density (OD) 0.5 at 600 nm which approximate to 7.4 X 10^8 CFU/ml of bacteria (see appendix B). Sterile BHI broth was removed and replaced with 5 ml of bacterial suspension in each well. All roots were incubated at 37 ℃ with 5% CO_2 for 21 days. During incubation period, 4.5 ml of bacterial suspension was refreshed with fresh BHI broth 3 times weekly. Contamination was periodically checked by gram-staining and plating of cultures onto blood agar. After incubation, the 4 specimens were gently washed with 1% phosphate buffer saline (PBS). The specimens were split longitudinally with sharp blade and mallet, fixed in 2.5% glutaraldehyde for 24 hours, and washed with 1% PBS. The specimens were serially dehydrated, critical point dried at 31.1℃ to replace alcohol with liquid carbon dioxide,

gold sputter coated, and examined using scanning electron microscopy (SEM) (JSM-5410 LV, JEOL, Japan) at magnification levels of X3500, X5000, and X10000.

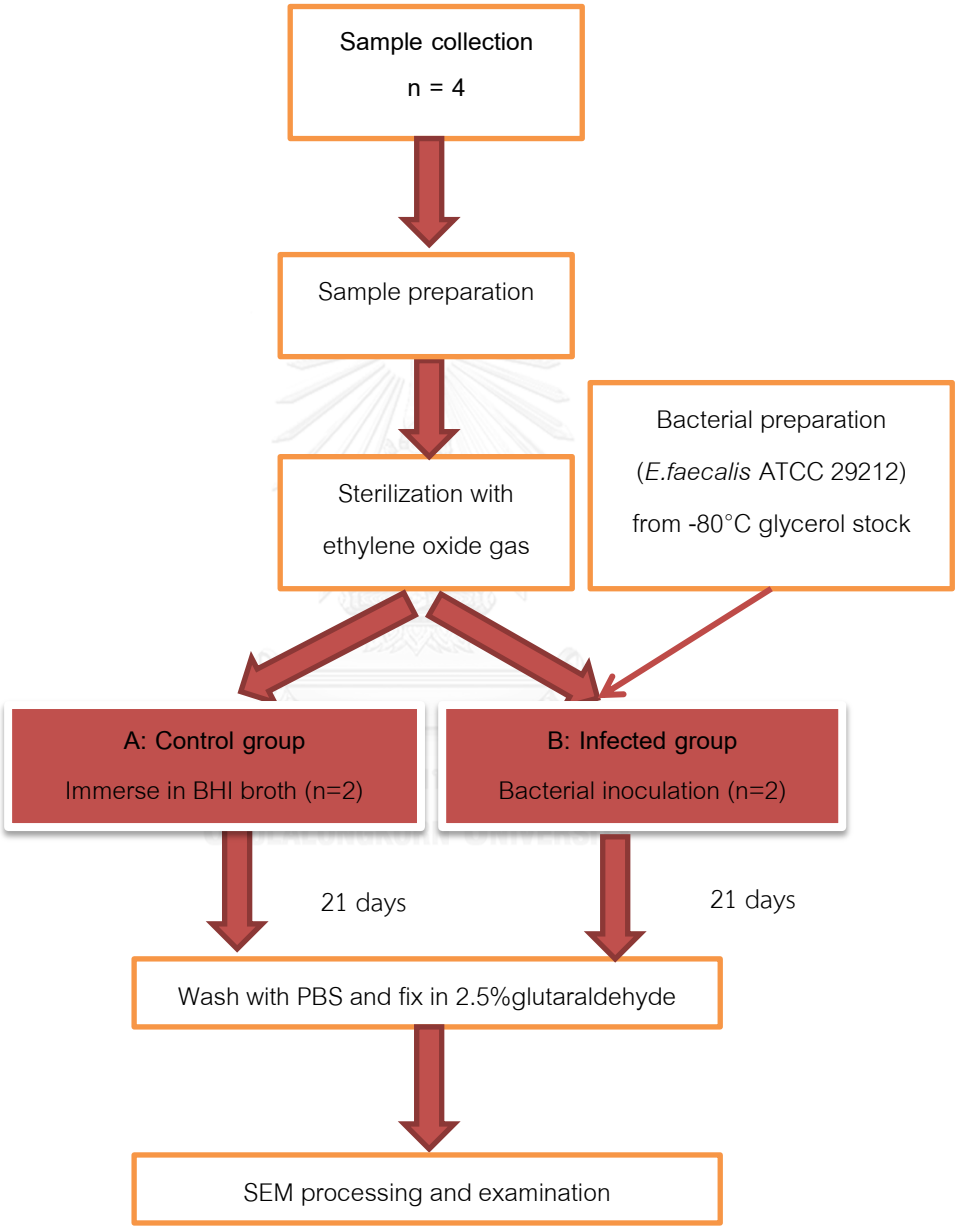


Figure 15: Flowchart summarizing the study of biofilm verification.

C. Experimental Procedure

Forty-eight roots were inoculated with *E. faecalis* as described above. After 21 days, the roots were gently flushed with 15 ml of 1%PBS and re-fixed in the silicone block. The root specimens were randomly assigned into 5 groups as follows:

Group 1-Mechanical instrumentation (MI) (n=12): Root canals were MI at a 13 mm working length (WL) using #70, 80, and 90 K-files, (Dentsply Maillefer, Ballaigues Switzerland) using a circumferential filing action. During MI, the root canals were irrigated with 5 mL of 2.5% NaOCl, after each file. After MI, the smear layer was removed by irrigating with 5 ml of 17% EDTA follow by 5 ml of 2.5% NaOCl.

Group 2-Irrigation with NaOCl (IRN) (n=12): Root canals were irrigated with 15 mL of 2.5% NaOCl.

Group 3-Passive ultrasonic irrigation (PUI) (n=12): PUI was performed using an intermittent flush technique adapted from van der Sluis et al. (90). Briefly, root canals were rinsed with 5 mL of 2.5% NaOCl. An ultrasonic tip with a non-cutting end (Irrisafe tip K20/21mm, Acteon, USA) mounted in a piezoelectric ultrasonic device (P5 power setting, 4-Satelec, Acteon, France) was inserted to 1 mm less than the WL and activated for 20 seconds. The rinsing and ultrasonic activation procedures were repeated for 3 cycles (90).

Group 4-Irrigation with normal saline (IRS) (n=6): Root canals were irrigated with 15 mL of 0.9% normal saline solution.

Group 5-Initial group (Initial) (n=6): This group served as baseline for initial bacterial count. The root canals were untreated and root specimens were further processed for microbiological sampling.

To verify that there was no contamination during the experiment, 3 sterile control roots were prepared and treated similar to those in the IRN group, except that the roots were immersed in sterile BHI broth instead of bacterial suspension.

An open-ended needle gauge 25 (Nipro, Osaka, Japan) was used to deliver root canal irrigants into the canals. The needle was inserted to 1 mm less than the WL and operated at a 3.75 mL/min flow rate. After the disinfection protocols, the root canals in groups 1–4 and sterile control were gently flushed with 5 mL of 5% sodium thiosulphate. Irrigation time was controlled to 4 minutes in each group. The irrigation volume of all groups were equally control to 20 ml except there was an additional volume of irrigation for smear layer removal in MI group as shown in table 3. The details of irrigation protocol were described in table 3 and the algorithm of the experimental design was shown in following flow chart (figure 16).

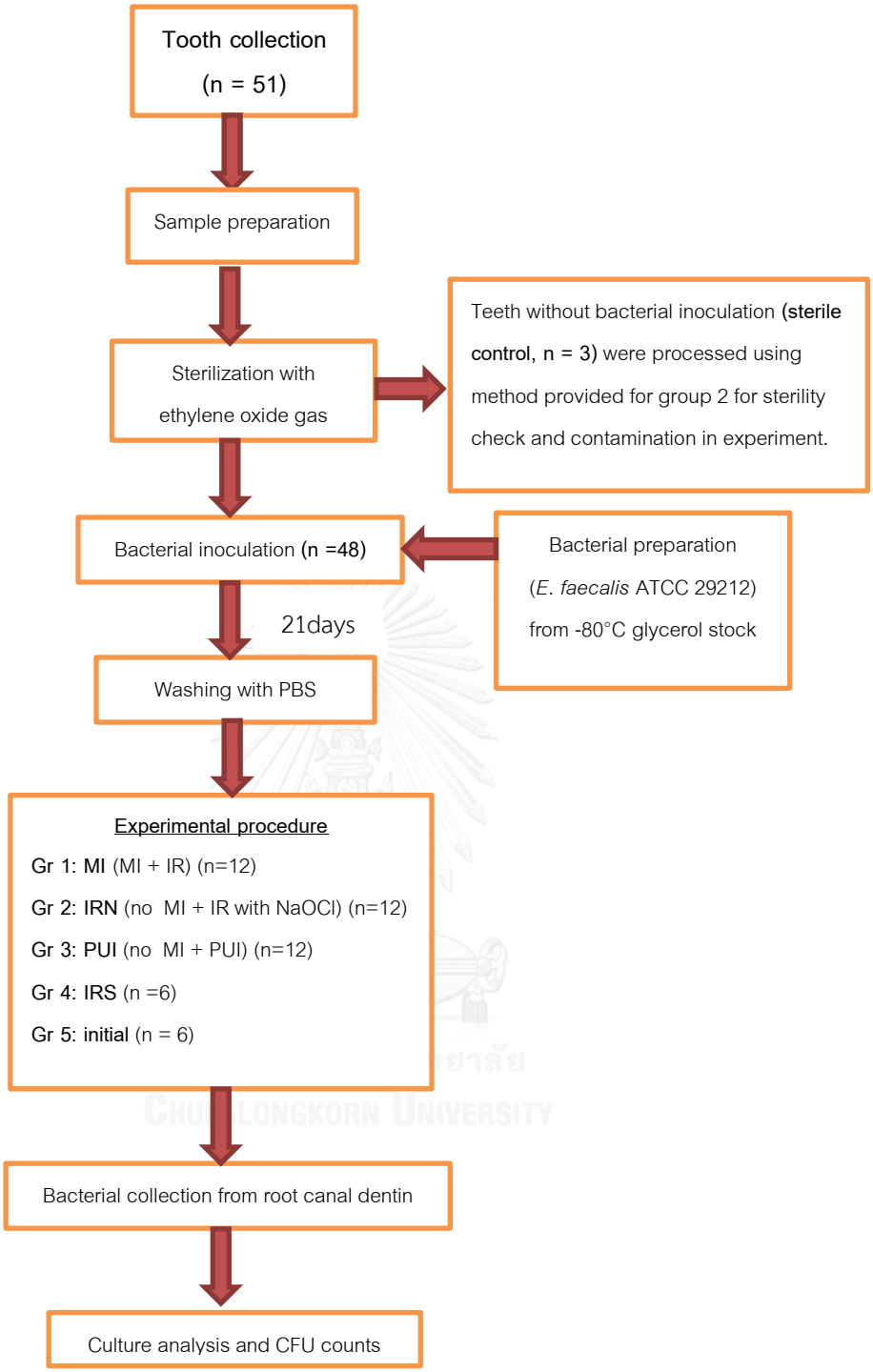


Figure 16: Flowchart summarizing the study design

Table 3: Summary of Irrigation protocol and sequence of each experimental groups.

Group	Canal instrumentation	Smear layer removal	Irrigation (ml)		
			2.5% NaOCl	0.9% NSS	10% Na thiosulphate
MI	MI size 60-90	17% EDTA 2.5%NaOCl	15	-	5
IRN	-	-	15	-	5
PUI	-	-	NaOCl 5 ml → PUI 20 s in 3 cycles		5
IRS	-	-	-	15	5
Initial	-	-	-	-	-

D. Microbiological Analysis

250 µm in depth of root canal wall dentin was grounded along the whole length of root canal using a #3 Peeso reamer. To maximize microbial collection, dentin chip attached to the flute of Peeso reamer was dislodged by spinning the working end into 1.5 ml Eppendorf tube containing 1 ml of PBS. Five sterile paper points were sequentially inserted to absorb residual fluid in root canal and transferred into the same Eppendorf tube. The specimens were then sonicated by sonicator (MicrosonTM ultrasonic cell disruption, Heat system, New York, USA) at 22.5 kHz and 20% intensity for 30 seconds to break up bacterial clumps and to disperse bacteria in the suspension. Ten-fold serial dilutions with PBS were performed before spreading 100 µl of suspension onto blood agar plates and incubated at 37

°C with 5% CO_2 for 24 hours. Then, colony-forming units per ml (CFU/ml) were count and microbiological analysis was performed in technical duplication.

E. Statistical Analysis

The data were analyzed using Statistical Package for Social Science (SPSS) software (Version17; SPSS Inc., Chicago. IL). One-way ANOVA was used to examine the differences in bacterial reduction between the 5 groups. The CFU count values were set as a dependent variable and were $\log_{10}$ transformed prior to analysis. The Tukey multiple comparison test was performed to identify any significant differences between groups. Significance was set at *P* values <.05.

The effectiveness of each disinfection protocol were calculated and reported in terms of “log10 reduction”, “magnitude of bacterial reduction” and “percentage of bacterial reduction”.

While $\log_{10}$ reduction values refer to the mean difference of $\log_{10}$ (CFU/mL), the magnitude of bacterial reduction (A/B) was calculated by taking the exponential of the mean difference of $\log_{10}$ (CFU/mL) between groups, derived from the following equation:

Log_{10} reduction = Mean difference of $\log_{10}$ (CFU/mL)

$= \log_{10}(A) - \log_{10}(B)$

$= \log_{10}(A/B)$

Where A and B are the mean CFU counts of each group.

The percentage of bacterial reduction was subsequently calculated from magnitude of bacterial reduction in each group compare to initial group, using following the equation:

Percentage of bacterial reduction

$$= \frac{(\text{Magnitude of bacterial reduction} - 1)}{\text{Magnitude of bacterial reduction}} \times 100$$

CHAPTER IV

RESEARCH RESULTS

SEM Biofilm Verification

In order to confirm the biofilms formation on root canal wall, specimen infected with *E. faecalis* for 21 days were subjected to examine by SEM. The sterile control demonstrated patent dentinal tubules without bacteria on the root canal wall (figure 18A). In contrast, bacterial clumps and their extracellular matrix were observed on the root canal walls of infected specimens (figure 18B, 18C), indicated the *E. faecalis* biofilms developed on the root canal surface. Furthermore, some dentinal tubules were invaded by bacteria (figure 18D).

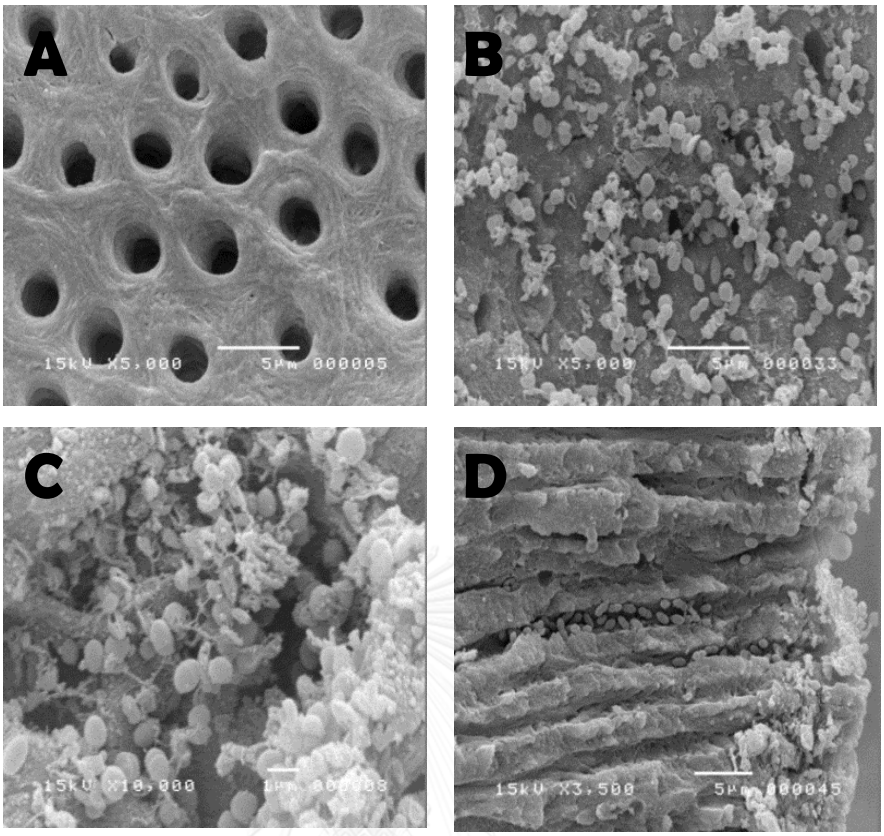


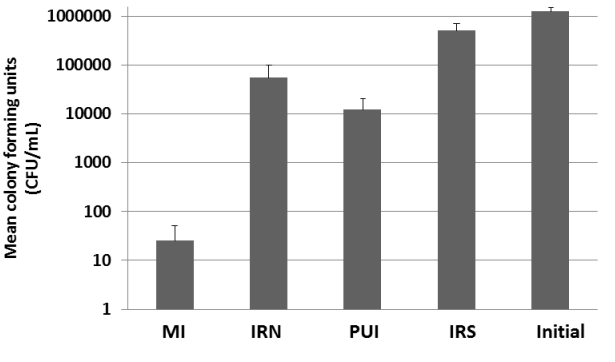
Figure 17: Scanning electron microscope images show that the root canal wall of non-infected roots (A) exhibited open dentinal tubules without bacterial cells. In infected roots, clumps of bacteria colonized on the root canal wall are observed at 5,000X (B), and 10,000X magnification (C). Bacteria are also present in the dentinal tubules of infected root at 3,500X magnification (D).

SEM results demonstrated that our bacterial inoculation protocol were able to create bacterial biofilms on the root canal wall.

Microbiological Analysis

The quantitative data of the remaining intracanal bacteria in each group is shown in figure 18. There was no bacterial observe in sterile control group. The highest mean bacterial count was observed in the initial group,

followed by the IRS, IRN, PUIN, and MIN groups. The $\log_{10}$ reduction value between pairs of experimental group was present in table 4.



	MI	IRN	PUI	IRS	Initial
n	12	12	12	6	6
Mean	25.4	5.61×10^4	1.25×10^4	5.20×10^5	1.28×10^6
SD	26.6	4.29×10^4	7.41×10^3	1.69×10^5	2.58×10^5
Minimum	0	2.05×10^4	2.20×10^3	2.30×10^5	9.05×10^5
Maximum	85	1.62×10^5	2.38×10^4	6.50×10^5	1.61×10^6

Figure 18: The mean, standard deviation, minimum, and maximum bacterial plate counts (CFU/mL) in each experimental group. MI, mechanical instrumentation; IRN, conventional irrigation with 2.5% NaOCl; PUI, passive ultrasonic irrigation; IRS, conventional irrigation with 0.9% normal saline; initial, no intervention.

Analysis of variance showed a significant difference, ($P < .01$, R^2=0.96), between the different protocols. The Tukey HSD post hoc test (table 4) indicated that the $\log_{10}$ CFU/mL of remaining bacteria was significantly higher in the IRS and initial groups, compared with the other groups (P<.05). The number of remaining bacterial cell of IRS and initial group was not significantly different (P>.05). The MI group had significantly less intracanal bacteria, compared with the IRN and PUI groups (P<.05).

Table 4: Tukey HSD post hoc analysis from One-way ANOVA demonstrates the log_{10} reduction value (mean differences), *P* value, and 95% confidence interval of log_{10} CFU/mL data between each pair of experimental groups.

Group (A)	Group (B)	Mean Difference (A-B)	*P* value	95% Confidence Interval	
				Lower Bound	Upper Bound
IRN	**MI**	3.27	<.001	2.87	3.67
	PUI	0.66	<.001	0.28	1.03
	IRS	-1.04	<.001	-1.50	-0.58
	initial	-1.50	<.001	-1.91	-0.99
PUI	**MI**	2.61	<.001	2.21	3.02
	IRS	-1.70	<.001	-2.16	-1.24
	initial	-2.11	<.001	-2.57	-1.65
IRS	**MI**	4.31	<.001	3.83	4.80
	initial	-0.41	.2000	-0.94	0.12
initial	**MI**	4.72	<.001	4.24	5.20

The magnitude of bacterial reduction indicated that the remaining bacteria in the MI group was $exp^{(2.611)}$=408 folds less than that of the PUI group and 1,862 folds less than that of the IRN group. Although the number of bacteria in the IRN group was 11 folds less compared with the IRS group, it was 4.5 folds more than that of the PUI group. The percentage of bacterial reduction of MIN, IRN, PUIN and IRS group were 99.99%, 96.83%, 99.22% and 60.93% (4.72, 1.50, 2.11 and 0.41 log10 reduction), respectively.

CHAPTER V

DISCUSSION

Our study compared the effectiveness of different disinfection protocols on bacterial reduction in teeth with large root canals. We found that MI, chemo-mechanical preparation, was the most effective method. There was no significant difference in bacterial number between the IRS and initial groups. Although PUI significantly improved the effectiveness of conventional NaOCl irrigation, it was much less effective than MI.

Persistent apical periodontitis is associated with residual bacteria, mainly in the form of biofilms (1). Therefore, to evaluate the effectiveness of disinfection protocols, we simulated biofilm formation on the root canal wall. *E. faecalis* was selected as the test microorganism because it can resist the chemo-mechanical procedure and withstand harsh environments (34, 36). Similar to previous studies, our SEM images showed clumps of aggregated bacterial cells in an extracellular matrix on the root canal wall (18, 47).

Our data suggests that the effect of antibacterial irrigation on root canal bacteria was the result of antibacterial properties of the irrigant rather than its flushing effect. This was demonstrated by a significant difference in number of remaining bacteria between the initial and IRN groups, however,

no significant difference was detected between the initial and IRS groups. The antibacterial effect of irrigation is also influenced by the irrigant concentration, flow-rate, and contact time (105).

In small root canals, MI removes infected dentin and provides space allowing irrigant penetration to the apical root canal (6, 7). In our study, the apical root canals were standardized to 0.6 mm in diameter, which readily providing apical irrigant access. Moreover, 13 mm root segment with predetermined root canal size to standardize the initial volume of root canal space which essential CFU count. Although direct exposure of a biofilm to potent root canal irrigants such as 6% NaOCl leads to biofilm elimination and marked bacterial reduction (5, 60, 63, 64), the exposure to lower concentrations of NaOCl resulted in higher survival rate of stem cell (106). Therefore, high concentration of irrigants was not used in our study. The difference in NaOCl concentrations, exposure times and method of sample evaluation may explain the discrepancy between our results and those of other study.

According to the irrigation sequence in this study, total irrigation volume was controlled in all groups, except MI group. As a result of mechanical instrumentation, the smear layer was created on the root canal wall and may reduce bacterial penetration into the dentinal tubules. It is recommended to remove smear layer prior to root canal obturation because

it consists of dentin debris, pulp tissue remnant and bacteria (107). Since rinsing with 17% EDTA and 2.5% NaOCl was reported to be an effective method to remove both inorganic and organic component of smear layer (108), extra volume of irrigants for smear layer removal was added into the MI group.

PUI induces two phenomena to improve mechanical cleansing in the root canals. The acoustic streaming leads to shear stress on bacterial cells. Furthermore, cavitation causes the collapse of gas bubbles, which creates a pressure-vacuum effect to clean the root canal wall and destroys bacterial cells (80). Moreover, the increase in temperature by PUI enhances the bactericidal effect of NaOCl (109).

There are 2 types of ultrasonic irrigation technique (87). The first type is the technique that applied ultrasonic instrumentation and irrigation (I-PUI) into the root canal seperately. The second type is the continuous ultrasonic irrigation (C-PUI), which allows simultaneous continuous irrigant delivery and ultrasonic activation at the same time. Although both C-PUI and I-PUI could introduce irrigant into the apical third of root canal (87, 96), C-PUI could introduce more irrigant extrusion out of the root apex than IPUI (92). Clinically, the apical extrusion of the irrigant into the periapical area is undesirable. Therefore, I-PUI was chosen to be one of the tested techniques in our large root canal model. According to the efficacy in bacterial reduction,

Carver et al. (2007) demonstrated the efficacy of 1 minute C-PUI in reduced bacterial-positive culture (8). Recently, Guerreiro-Tanomaru et al. (2015) exhibited that intermittent flush with three cycles of ultrasonic activation and irrigant refreshment (I-PUI) could reduce intraradicular bacteria effectively (91).

Our findings conformed to previous studies that supplementary irrigation with PUI could enhance the reduction of bacteria in dentinal tubules and biofilm (9, 18, 102). However, a supplementation with PUI did not reduce bacterial levels comparable to those obtained by MI to three size larger. This finding emphasizes the importance of the infected dentine removal, even in the case when irrigant access was initially provided. Although there was suggestion in preparing canal to one size larger than the initial one (16), our study using standard root canal enlargement with three sized larger file instead. Further study needs to compare the efficacy of minimal MI such as one or two increasing file size and routine root canal preparation. However, a favorable outcome achieved after endodontic treatment without MI in revascularization procedures (14, 15, 23, 24), suggests that the combination of the antibacterial effect of irrigants, intracanal medicaments, and host immune response play an important role in periapical healing (110).

Because of difficulty in collecting the naturally large root canals, we prepared the specimens to create the root canal models that have not been mechanical instrumented to meet condition of naturally large root canal root

canal wall. The apical end of the root section was then capped with resin composite to facilitate the retention of the irrigant in root canal teeth with open apex without apical barrier. Moreover, we also controlled the volume of irrigant, flow rate of irrigation and irrigation time during experiment. Previous study demonstrated that *E. faecalis* was able to invade into dentinal tubule in range of 193.9 ± 15.3 μm (111). In microbiological analysis, a #3 peeso reamer was used for collecting dentin chip up to the depth of 250 μm. This method allowed us to collected bacteria in deep dentin better than the use of an H-file or paper point alone. With this method, we could collect both of bacterial biofilm on root canal surface and invading bacterial cell in dentinal tubules. Because the aggressive dentin collection did not allow us to compare number of bacteria in before-after manner, the initial group was used to calculate the initial bacterial count and was compared to other groups in this study.

The non-invasive protocols used in our study were far less effective than MI. However, in teeth with large root canals (apical size of 50–60) with thin dentin walls or in regenerative endodontics where MI is avoided to preserve the vitality of stem cell, dentin removal by MI might negatively affect root strength (10-12). In this clinical situation, it was suggested that the bacterial elimination protocol should not primarily rely on routine MI (18, 102). Therefore, further study designed to evaluate the effectiveness of

alternative minimal MI or other non-invasive disinfection protocols in teeth with large root canals will be useful. Moreover, the additional effect of root canal medication after non-invasive disinfection protocols should also be evaluated.

In conclusion, to disinfect a large root canal where irrigant access to the apical portion was initially available, chemo-mechanical preparation was the most reliable disinfection protocol. Utilizing an antibacterial agent supplemented with PUI improved the effectiveness of conventional irrigation; however, none of the non-invasive protocols was as effective as MI.

Limitations

This study is an in *vitro* experimental study which may not be the best evidence to be applied to clinical work. *E. faecalis* used in this study represents single species biofilm in root canal infection which different from naturally occur multispecies bacterial biofilm. The specimen model in this study might not imitate the real large root canal teeth. In term of apical end of specimens, there were also different from what presents in clinical situation. Three millimeters from root apex was cut off in order to eradicate apical ramification and reduced anatomical variation. In this study, apical end of tooth section will be sealed with composite resin to maintain irrigant within root canal space. The results of our study may provide some valuable information that can be adapted for clinical application.

Conclusion

Under the condition of this study, chemo-mechanical preparation was the most effective disinfection protocol in teeth with large root canal where irrigant access to the apical portion was initially available. PUI combined with antibacterial irrigant could significantly eliminate more bacterial biofilm on root canal wall, compared with sole antibacterial irrigation alone. However, none of the non-invasive protocols was as effective as MI.

REFERENCES

1. Ricucci D, Siqueira JF, Jr., Bate AL, Pitt Ford TR. Histologic investigation of root canal-treated teeth with apical periodontitis: a retrospective study from twenty-four patients. J Endod. 2009 Apr;35(4):493-502.
2. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001 Jan;9(1):34-9.
3. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001 Jul 14;358(9276):135-8.
4. Jose F. Siqueira JR INRDR. Biofilms in endodontic infection. Endodontic Topics. 2012;22:33-49.
5. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against Enterococcus faecalis biofilms. J Endod. 2006 Jun;32(6):527-31.
6. Bystrom A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res. 1981 Aug;89(4):321-8.
7. Shuping GB, Ørstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. J Endod. 2000 Dec;26(12):751-5.
8. Carver K, Nusstein J, Reader A, Beck M. In vivo antibacterial efficacy of ultrasound after hand and rotary instrumentation in human mandibular molars. J Endod. 2007 Sep;33(9):1038-43.
9. Harrison AJ, Chivatxaranukul P, Parashos P, Messer HH. The effect of ultrasonically activated irrigation on reduction of Enterococcus faecalis in experimentally infected root canals. Int Endod J. 2010 Nov;43(11):968-77.
10. Er K, Tasdemir T, Siso SH, Celik D, Cora S. Fracture resistance of retreated roots using different retreatment systems. Eur J Dent. 2011 Aug;5(4):387-92.

11. Trope M, Ray HL, Jr. Resistance to fracture of endodontically treated roots. Oral Surg Oral Med Oral Pathol. 1992 Jan;73(1):99-102.

12. Wilcox LR, Roskelley C, Sutton T. The relationship of root canal enlargement to finger-spreader induced vertical root fracture. J Endod. 1997 Aug;23(8):533-4.

13. Weine. Endodontic Tgerapy. St Louis: C.V. Mosby; 1972.

14. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod. 2004 Apr;30(4):196-200.

15. Iwaya SI, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. Dent Traumatol. 2001 Aug;17(4):185-7.

16. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006 Feb;32(2):93-8.

17. Card SJ, Sigurdsson A, Orstavik D, Trope M. The effectiveness of increased apical enlargement in reducing intracanal bacteria. J Endod. 2002 Nov;28(11):779-83.

18. Bhuva B, Patel S, Wilson R, Niazi S, Beighton D, Mannocci F. The effectiveness of passive ultrasonic irrigation on intraradicular Enterococcus faecalis biofilms in extracted single-rooted human teeth. Int Endod J. 2010 Mar;43(3):241-50.

19. Zou L, Shen Y, Li W, Haapasalo M. Penetration of sodium hypochlorite into dentin. J Endod. 2010 May;36(5):793-6.

20. Chow TW. Mechanical effectiveness of root canal irrigation. J Endod. 1983 Nov;9(11):475-9.

21. van der Sluis LW, Versluis M, Wu MK, Wesselink PR. Passive ultrasonic irrigation of the root canal: a review of the literature. Int Endod J. 2007 Jun;40(6):415-26.

22. Fouad AF. The microbial challenge to pulp regeneration. Adv Dent Res. 2011 Jul;23(3):285-9.

23. Lei L, Chen Y, Zhou R, Huang X, Cai Z. Histologic and Immunohistochemical Findings of a Human Immature Permanent Tooth with Apical Periodontitis after Regenerative Endodontic Treatment. J Endod. 2015 Jul;41(7):1172-9.

24. Wang Y, Zhu X, Zhang C. Pulp Revascularization on Permanent Teeth with Open Apices in a Middle-aged Patient. J Endod. 2015 Sep;41(9):1571-5.

25. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000. 2006;42:80-7.

26. Figdor GSaD. Life as an ebdodontic pathogen.Ecological differences between the untreated and root-filled root canals. Endodontic Topics. 2003;6:3-28.

27. Fabricius L, Dahlen G, Holm SE, Moller AJ. Influence of combinations of oral bacteria on periapical tissues of monkeys. Scand J Dent Res. 1982 Jun;90(3):200-6.

28. Siqueira JF, Jr., Rocas IN, Souto R, de Uzeda M, Colombo AP. Actinomyces species, streptococci, and Enterococcus faecalis in primary root canal infections. J Endod. 2002 Mar;28(3):168-72.

29. Markus Haapasalo Tu, Unni endal. Persistent, recurrent, and acquired infection of the root canal system post-treatment. Endodontic Topics. 2003;6:29-56.

30. Love RM. Regional variation in root dentinal tubule infection by Streptococcus gordonii. J Endod. 1996 Jun;22(6):290-3.

31. Matsuo T, Shirakami T, Ozaki K, Nakanishi T, Yumoto H, Ebisu S. An immunohistological study of the localization of bacteria invading root pulpal walls of teeth with periapical lesions. J Endod. 2003 Mar;29(3):194-200.

32. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med. 2002;13(2):171-83.

33. Haapasalo M. Eradication of endodontic infection by instrumentation and irrigation solutions. Endodontic Topics. 2005;10:77-102.

34. Chavez De Paz LE, Dahlen G, Molander A, Moller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J. 2003 Jul;36(7):500-8.

35. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998 Jan;85(1):86-93.

36. Distel JW, Hatton JF, Gillespie MJ. Biofilm formation in medicated root canals. J Endod. 2002 Oct;28(10):689-93.

37. Zoletti GO, Siqueira JF, Jr., Santos KR. Identification of Enterococcus faecalis in root-filled teeth with or without periradicular lesions by culture-dependent and-independent approaches. J Endod. 2006 Aug;32(8):722-6.

38. Sakamoto M, Siqueira JF, Jr., Rocas IN, Benno Y. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. Oral Microbiol Immunol. 2008 Aug;23(4):275-81.

39. Bergenholtz GSG. Biofilms in endodontic infections. Endodontic Topics. 2004;9:27-36.

40. Narayanan LL, Vaishnavi C. Endodontic microbiology. J Conserv Dent. 2010 Oct;13(4):233-9.

41. Larsen T. Susceptibility of Porphyromonas gingivalis in biofilms to amoxicillin, doxycycline and metronidazole. Oral Microbiol Immunol. 2002 Oct;17(5):267-71.

42. Shani S, Friedman M, Steinberg D. The anticariogenic effect of amine fluorides on Streptococcus sobrinus and glucosyltransferase in biofilms. Caries Res. 2000 May-Jun;34(3):260-7.

43. Chavez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod. 2007 Jun;33(6):652-62.

44. Hubble TS, Hatton JF, Nallapareddy SR, Murray BE, Gillespie MJ. Influence of Enterococcus faecalis proteases and the collagen-binding protein, Ace, on adhesion to dentin. Oral Microbiol Immunol. 2003 Apr;18(2):121-6.

45. Ricucci D, Siqueira JF, Jr. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. J Endod. 2010 Aug;36(8):1277-88.

46. Ramachandran Nair PN. Light and electron microscopic studies of root canal flora and periapical lesions. J Endod. 1987 Jan;13(1):29-39.

47. George S, Kishen A, Song KP. The role of environmental changes on monospecies biofilm formation on root canal wall by Enterococcus faecalis. J Endod. 2005 Dec;31(12):867-72.

48. Mohammadi Z, Palazzi F, Giardino L, Shalavi S. Microbial biofilms in endodontic infections: an update review. Biomed J. 2013 Mar-Apr;36(2):59-70.

49. Haapasalo M. Bacteroides buccae and related taxa in necrotic root canal infections. J Clin Microbiol. 1986 Dec;24(6):940-4.

50. Bergmans L, Moisiadis P, Van Meerbeek B, Quirynen M, Lambrechts P. Microscopic observation of bacteria: review highlighting the use of environmental SEM. Int Endod J. 2005 Nov;38(11):775-88.

51. Wright CJ, Shah MK, Powell LC, Armstrong I. Application of AFM from microbial cell to biofilm. Scanning. 2010 May-Jun;32(3):134-49.

52. Wang Z, Shen Y, Haapasalo M. Dentin extends the antibacterial effect of endodontic sealers against Enterococcus faecalis biofilms. J Endod. 2014 Apr;40(4):505-8.

53. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002 Apr;15(2):167-93.

54. Atila-Pektas B, Yurdakul P, Gulmez D, Gorduysus O. Antimicrobial effects of root canal medicaments against Enterococcus faecalis and Streptococcus mutans. Int Endod J. 2013 May;46(5):413-8.

55. Lee JK, Park YJ, Kum KY, Han SH, Chang SW, Kaufman B, et al. Antimicrobial efficacy of a human beta-defensin-3 peptide using an Enterococcus faecalis dentine infection model. Int Endod J. 2013 May;46(5):406-12.

56. Chavez de Paz LE, Bergenholtz G, Svensater G. The effects of antimicrobials on endodontic biofilm bacteria. J Endod. 2010 Jan;36(1):70-7.

57. Duggan JM, Sedgley CM. Biofilm formation of oral and endodontic Enterococcus faecalis. J Endod. 2007 Jul;33(7):815-8.

58. Giardino L, Ambu E, Savoldi E, Rimondini R, Cassanelli C, Debbia EA. Comparative evaluation of antimicrobial efficacy of sodium hypochlorite, MTAD, and Tetraclean against Enterococcus faecalis biofilm. J Endod. 2007 Jul;33(7):852-5.

59. Spratt DA, Pratten J, Wilson M, Gulabivala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. Int Endod J. 2001 Jun;34(4):300-7.

60. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod. 2006 May;32(5):434-7.

61. Ozdemir HO, Buzoglu HD, Calt S, Stabholz A, Steinberg D. Effect of ethylenediaminetetraacetic acid and sodium hypochlorite irrigation on Enterococcus faecalis biofilm colonization in young and old human root canal dentin: in vitro study. J Endod. 2010 May;36(5):842-6.

62. Soares JA, Roque de Carvalho MA, Cunha Santos SM, Mendonca RM, Ribeiro-Sobrinho AP, Brito-Junior M, et al. Effectiveness of chemomechanical preparation with alternating use of sodium hypochlorite and EDTA in eliminating intracanal Enterococcus faecalis biofilm. J Endod. 2010 May;36(5):894-8.

63. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. J Endod. 2014 Apr;40(4):509-14.

64. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod. 2012 Oct;38(10):1376-9.

65. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MS, Gomes de Moraes I, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod J. 2012 Feb;45(2):162-8.

66. Stojicic S, Shen Y, Haapasalo M. Effect of the source of biofilm bacteria, level of biofilm maturation, and type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. J Endod. 2013 Apr;39(4):473-7.

67. Dalton BC, Ørstavik D, Phillips C, Pettiette M, Trope M. Bacterial reduction with nickel-titanium rotary instrumentation. J Endod. 1998 Nov;24(11):763-7.

68. Falk KW, Sedgley CM. The influence of preparation size on the mechanical efficacy of root canal irrigation in vitro. J Endod. 2005 Oct;31(10):742-5.

69. Torneck CD, Smith JS, Grindall P. Biologic effects of endodontic procedures on developing incisor teeth. 3. Effect of debridement and disinfection procedures in the treatment of experimentally induced pulp and periapical disease. Oral Surg Oral Med Oral Pathol. 1973 Apr;35(4):532-40.

70. Diogenes AR, Ruparel NB, Teixeira FB, Hargreaves KM. Translational science in disinfection for regenerative endodontics. J Endod. 2014 Apr;40(4 Suppl):S52-7.

71. Anibal Diogenes MAH, Fabricio B. Teixeira &, Hargreaves KM. An update clinical regenerative endodontics. Endodontic Topics. 2013;8:2-123.

72. Cohenca N, Heilborn C, Johnson JD, Flores DS, Ito IY, da Silva LA. Apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing on root canal disinfection in dog teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010 Jan;109(1):e42-6.

73. Ricks-Williamson LJ, Fotos PG, Goel VK, Spivey JD, Rivera EM, Khera SC. A three-dimensional finite-element stress analysis of an endodontically prepared maxillary central incisor. J Endod. 1995 Jul;21(7):362-7.

74. Wu MK, van der Sluis LW, Wesselink PR. Comparison of mandibular premolars and canines with respect to their resistance to vertical root fracture. J Dent. 2004 May;32(4):265-8.

75. Lertchirakarn V, Palamara JE, Messer HH. Patterns of vertical root fracture: factors affecting stress distribution in the root canal. J Endod. 2003 Aug;29(8):523-8.

76. Zandbiglari T, Davids H, Schafer E. Influence of instrument taper on the resistance to fracture of endodontically treated roots. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006 Jan;101(1):126-31.

77. AAE. AAE Clinical Considerations for a Regenerative Procedure2013.

78. Huang GT. A paradigm shift in endodontic management of immature teeth: conservation of stem cells for regeneration. J Dent. 2008 Jun;36(6):379-86.

79. Trope M. Treatment of immature teeth with non-vital pulps and apical periodontitis. Endodontic Topics. 2003;14:51-9.

80. Martin H, Cunningham W. Endosonics--the ultrasonic synergistic system of endodontics. Endod Dent Traumatol. 1985 Dec;1(6):201-6.

81. Martin H. Ultrasonic disinfection of the root canal. Oral Surg Oral Med Oral Pathol. 1976 Jul;42(1):92-9.

82. Walmsley AD, Lumley PJ, Laird WR. Oscillatory pattern of sonically powered endodontic files. Int Endod J. 1989 May;22(3):125-32.

83. Walmsley AD. Ultrasound and root canal treatment: the need for scientific evaluation. Int Endod J. 1987 May;20(3):105-11.

84. Ahmad M, Pitt Ford TJ, Crum LA. Ultrasonic debridement of root canals: acoustic streaming and its possible role. J Endod. 1987 Oct;13(10):490-9.

85. Roy RA, Ahmad M, Crum LA. Physical mechanisms governing the hydrodynamic response of an oscillating ultrasonic file. Int Endod J. 1994 Jul;27(4):197-207.

86. Ahmad M, Pitt Ford TR, Crum LA, Walton AJ. Ultrasonic debridement of root canals: acoustic cavitation and its relevance. J Endod. 1988 Oct;14(10):486-93.

87. Castelo-Baz P, Martin-Biedma B, Cantatore G, Ruiz-Pinon M, Bahillo J, Rivas-Mundina B, et al. In vitro comparison of passive and continuous ultrasonic irrigation in simulated lateral canals of extracted teeth. J Endod. 2012 May;38(5):688-91.

88. van der Sluis LWM WM, Wesselink PR. A comparison of two flushing methods used during passive ultrasonic irrigation of the root canal. Quint Int. 2009;40: 875-9.

89. Yoo YJ, Lee W, Kim HC, Shon WJ, Baek SH. Multivariate analysis of the cleaning efficacy of different final irrigation techniques in the canal and isthmus of mandibular posterior teeth. Restor Dent Endod. 2013 Aug;38(3):154-9.

90. van der Sluis LW, Vogels MP, Verhaagen B, Macedo R, Wesselink PR. Study on the influence of refreshment/activation cycles and irrigants on mechanical cleaning efficiency during ultrasonic activation of the irrigant. J Endod. 2010 Apr;36(4):737-40.

91. Guerreiro-Tanomaru JM, Chavez-Andrade GM, de Faria-Junior NB, Watanabe E, Tanomaru-Filho M. Effect of Passive Ultrasonic Irrigation on Enterococcus faecalis from Root Canals: An Ex Vivo Study. Braz Dent J. 2015 Jul-Aug;26(4):342-6.

92. Tasdemir T, Er K, Celik D, Yildirim T. Effect of passive ultrasonic irrigation on apical extrusion of irrigating solution. Eur J Dent. 2008 Jul;2(3):198-203.

93. Alves FR, Almeida BM, Neves MA, Moreno JO, Rocas IN, Siqueira JF, Jr. Disinfecting oval-shaped root canals: effectiveness of different supplementary approaches. J Endod. 2011 Apr;37(4):496-501.

94. Paiva SS, Siqueira JF, Jr., Rocas IN, Carmo FL, Ferreira DC, Curvelo JA, et al. Supplementing the antimicrobial effects of chemomechanical debridement with either passive ultrasonic irrigation or a final rinse with chlorhexidine: a clinical study. J Endod. 2012 Sep;38(9):1202-6.

95. Paiva SS, Siqueira JF, Jr., Rocas IN, Carmo FL, Leite DC, Ferreira DC, et al. Molecular microbiological evaluation of passive ultrasonic activation as a supplementary disinfecting step: a clinical study. J Endod. 2013 Feb;39(2):190-4.

96. de Gregorio C, Estevez R, Cisneros R, Paranjpe A, Cohenca N. Efficacy of different irrigation and activation systems on the penetration of sodium hypochlorite into simulated lateral canals and up to working length: an in vitro study. J Endod. 2010 Jul;36(7):1216-21.

97. Gutarts R, Nusstein J, Reader A, Beck M. In vivo debridement efficacy of ultrasonic irrigation following hand-rotary instrumentation in human mandibular molars. J Endod. 2005 Mar;31(3):166-70.

98. van der Sluis LW, Wu MK, Wesselink PR. The efficacy of ultrasonic irrigation to remove artificially placed dentine debris from human root canals prepared using instruments of varying taper. Int Endod J. 2005 Oct;38(10):764-8.

99. Cameron JA. The use of ultrasonics in the removal of the smear layer: a scanning electron microscope study. J Endod. 1983 Jul;9(7):289-92.

100. Cheung GS, Stock CJ. In vitro cleaning ability of root canal irrigants with and without endosonics. Int Endod J. 1993 Nov;26(6):334-43.

101. Spoleti P, Siragusa M, Spoleti MJ. Bacteriological evaluation of passive ultrasonic activation. J Endod. 2003 Jan;29(1):12-4.

102. Grundling GL, Zechin JG, Jardim WM, de Oliveira SD, de Figueiredo JA. Effect of ultrasonics on Enterococcus faecalis biofilm in a bovine tooth model. J Endod. 2011 Aug;37(8):1128-33.

103. Cvek M, Nord CE, Hollender L. Antimicrobial effect of root canal debridement in teeth with immature root. A clinical and microbiologic study. Odontol Revy. 1976;27(1):1-10.

104. Schneider SW. A comparison of canal preparations in straight and curved root canals. Oral Surg Oral Med Oral Pathol. 1971 Aug;32(2):271-5.

105. Haapasalo M, Shen Y, Wang Z, Gao Y. Irrigation in endodontics. Br Dent J. 2014 Mar;216(6):299-303.

106. Martin DE, De Almeida JF, Henry MA, Khaing ZZ, Schmidt CE, Teixeira FB, et al. Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. J Endod. 2014 Jan;40(1):51-5.

107. Violich DR, Chandler NP. The smear layer in endodontics - a review. Int Endod J. 2010 Jan;43(1):2-15.

108. Yamada RS, Armas A, Goldman M, Lin PS. A scanning electron microscopic comparison of a high volume final flush with several irrigating solutions: Part 3. J Endod. 1983 Apr;9(4):137-42.

109. van der Sluis LW, Gambarini G, Wu MK, Wesselink PR. The influence of volume, type of irrigant and flushing method on removing artificially placed dentine debris from the apical root canal during passive ultrasonic irrigation. Int Endod J. 2006 Jun;39(6):472-6.

110. Franklin Garcia-Godoy PEM. Recommendations for using regenerative endodontic procedures in permanent immature traumatized teeth. Dental Traumatology. 2011:1-8.

111. Chivatxaranukul P, Dashper SG, Messer HH. Dentinal tubule invasion and adherence by Enterococcus faecalis. Int Endod J. 2008 Oct;41(10):873-82.

APPENDIX

APPENDIX A

Enterococcus faecalis ATCC 29212 was used in this study. Growth curve of bacterial culture was twice observed at optical density 600 nm as shown in figure 19. At log phase of 0.5 optical density (OD) was used to adjust bacteria for tooth sample inoculation. From the preliminary study of serial dilution and plate count, the number of bacteria is approximate to 7.4 X 10^8 CFU/mL.

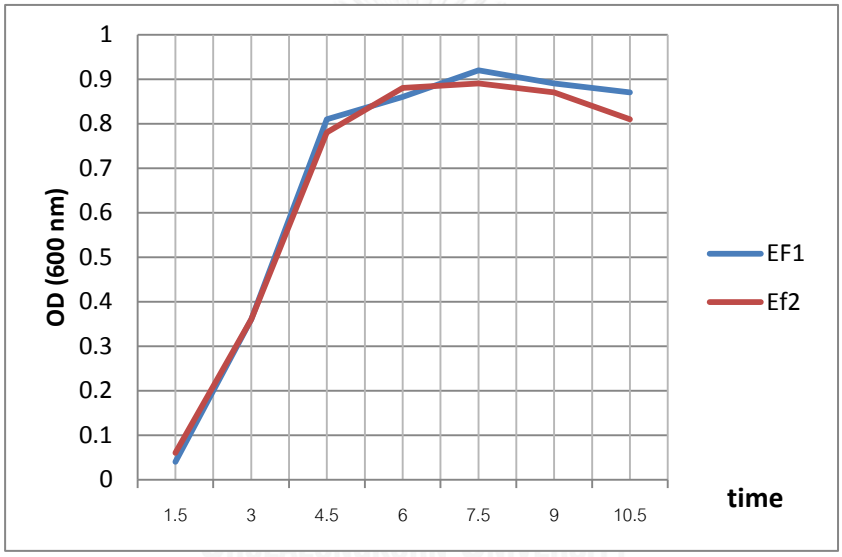


Figure 19: Growth curve of Enterococcus faecalis ATCC 2921 was twice observed. (EF1: 1st time observe, EF2: 2nd time observe)

APPENDIX B

Ultrasonic cell disruption (Heat system, New York, USA) was used to break clumps of bacteria in collected dentin samples in eppendoft tube containing 1 ml of PBS. The preliminary study was done to confirm that sonication with 20% intensity for 30 seconds was enough for disrupt and break the clumps of bacteria in dentin and had less effect on viability of bacteria. Figure 20 demonstrated the plates of bacterial colonies from non-sonicated (figure 20A) and sonicated tube (figure 20B). It was exhibited that numbers of bacterial colony were similar.

(A) (B)

Figure 20: The colonies of *Enterococcus faecalis* ATCC 29212 at 10^{7} dilution of non-sonicated tube (A) and sonicate tube with ultrasonic cell disruption in 20% intensity for 30 seconds (B). The numbers of bacterial colony were similar (57 and 52 colonies respectively).

APPENDIX C

Table 5: Raw data of CFU counts and log_{10} (CFU/ml) of experimental groups.

Specimen number of "MI group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
A1	20	20	20	1.30
A2	20	40	30	1.48
A3	0	0	0	NA
A4	40	60	50	1.70
A5	90	80	85	1.93
A6	0	10	5	0.70
A7	60	30	45	1.65
A8	0	10	5	0.70
A9	10	30	20	1.30
A10	0	0	0	NA
A11	0	0	0	NA
A12	40	50	45	1.65

*NA: There was no value of log_{10} transfer as a result of no bacterial growth.

Specimen number of "IRN group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
B1	1.58E+05	1.66E+05	1.62E+05	5.21
B2	3.20E+04	2.00E+04	2.60E+04	4.41
B3	3.80E+04	2.00E+04	2.90E+04	4.46
B4	2.00E+04	2.10E+04	2.05E+04	4.31
B5	3.30E+04	1.70E+04	2.50E+04	4.40
B6	2.90E+04	1.60E+04	2.25E+04	4.35
B7	1.01E+05	8.20E+04	9.15E+04	4.96
B8	3.30E+04	2.80E+04	3.05E+04	4.48
B9	9.80E+04	7.80E+04	8.80E+04	4.94
B10	5.10E+04	1.01E+05	7.60E+04	4.88
B11	6.50E+04	7.30E+04	6.90E+04	4.84
B12	2.70E+04	4.00E+04	3.35E+04	4.53

Specimen number of "PUI group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
C1	2.23E+04	2.52E+04	2.38E+04	4.38
C2	3.70E+03	2.70E+03	3.20E+03	3.51
C3	2.30E+03	2.10E+03	2.20E+03	3.34
C4	1.23E+04	1.18E+04	1.21E+04	4.08
C5	1.16E+04	1.21E+04	1.19E+04	4.07
C6	2.70E+03	2.80E+03	2.75E+03	3.44
C7	8.60E+03	8.80E+03	8.70E+03	3.94
C8	2.27E+04	2.40E+04	2.34E+04	4.37
C9	1.04E+04	2.12E+04	1.58E+04	4.20
C10	1.94E+04	7.90E+03	1.37E+04	4.14
C11	1.61E+04	1.19E+04	1.40E+04	4.15
C12	2.27E+04	1.53E+04	1.90E+04	4.28

Specimen number of "IRS group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
D1	4.80E+05	6.10E+05	5.45E+05	5.74
D2	5.90E+05	6.10E+05	6.00E+05	5.78
D3	4.80E+05	8.70E+05	6.75E+05	5.83
D4	7.70E+05	5.30E+05	6.50E+05	5.81
D5	2.90E+05	1.70E+05	2.30E+05	5.36
D6	3.90E+05	4.50E+05	4.20E+05	5.62

Specimen number of "Initial group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
E1	1.20E+06	1.75E+06	1.48E+06	6.17
E2	1.29E+06	1.38E+06	1.34E+06	6.13
E3	1.60E+06	1.62E+06	1.61E+06	6.21
E4	9.60E+05	8.50E+05	9.05E+05	5.96
E5	1.14E+06	1.01E+06	1.08E+06	6.03
E6	9.80E+05	1.55E+06	1.27E+06	6.10

Specimen number of "sterile control group"	CFU count (CFU/ml)			Log_{10} (CFU/ml)
	1st technical duplication	2nd technical duplication	Average	
F1	0	0	0	-
F2	0	0	0	-
F3	0	0	0	-

Table 6: Normality test with SPSS program

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
residuallog	.109	48	.200*	.961	48	.110

a. Lilliefors Significance Correction

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

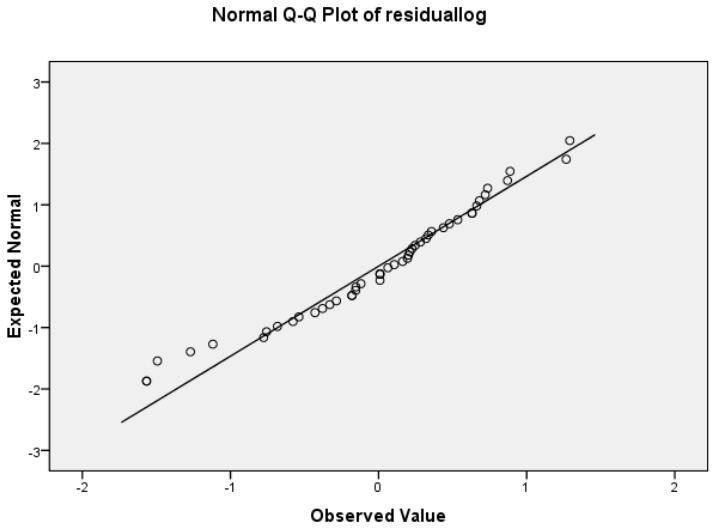


Table 7: One-way ANOVA with SPSS program

ANOVA

logcfu

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	153.598	4	38.399	196.429	.000
Within Groups	8.406	43	.195		
Total	162.003	47			

logcfu

Tukey HSD[a,,b]

group	N	Subset for alpha = 0.05			
		1	2	3	4
1	12	1.034327934			
3	12		3.990312656		
2	12			4.648639487	
4	6				5.690290335
5	6				6.098541122
Sig.		1.000	1.000	1.000	.327

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table 8: Magnitude of bacterial reduction

Group (A)	Group (B)	Mean Difference (A-B)	Magnitude of bacterial reduction
IRN	MI	3.270	1,862.09
	PUI	0.658	4.55
	IRS	-1.042	11.02
	Initial	-1.500	31.62
PUI	MI	2.611	408.32
	IRS	-1.700	50.12
	Initial	-2.108	128.23
IRS	MI	4.311	20,464.45
	Initial	-0.408	2.56
Initial	MI	4.719	52,360.04

Table 9: Log_{10} reduction and percentage of bacterial reduction

Experiment groups (compare to initial group)	Log_{10} reduction	Percentage of bacterial reduction
MI	4.72	99.99
IRN	1.50	96.83
PUI	2.11	99.22
IRS	0.41	60.93

VITA

Ms. Patinee Pladisai was born on 21th November 1996. She got bachelor degree of Doctor of Dental Surgery with first class honor from Faculty of Dentistry, Chulalongkorn University in 2010. She served the government as general dentist at Khoksamrong hospital of Lop Buri province in year 2010-2011. From 2011 to present, she is a part-time dentist at private dental clinic, Bangpakok 9 International hospital, Khasemrad hospital and Kluaynamthai hospital.