CHAPTER 3

Materials and Methods

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. In order to simulate dynamic irrigation in an immature tooth, plastic, enlarged-root-canal models, mimicking the closed environment of the tooth, were customized.

3.1 Preparation of *ex-vivo* immature root canal models

The plastic tooth models (Nissin, Kyoto, Japan) were modified by cutting off the crown and root to achieve a root length of 10 mm. The root canals were enlarged to a 2.5 mm-diameter and split into two halves. A 2-mm-wide longitudinal slot was created along the internal surface of one half in order to fit a sample slice of human dentin.

One hundred and eight human premolar teeth without carious lesions were used to prepare the dentin slices. The internal root portion was trimmed using a low-speed diamond saw (Isomet, Buehler Ltd., Evanston, IL, USA) into rectangular sections, 2 mm (width) x 9 mm (length) x 0.5 mm (thickness) (Figure 3.1*a*), and sequentially ground with silicon carbide papers to achieve a smooth surface. Then, the samples were sonically immersed in 17% EDTA and 1.25% NaOCl for one minute each to remove the smear layer. Lastly, the samples were washed with PBS solution and stored in PBS until used. One week before the experiments, the dentin slices were separately covered with 1,000-mg/mL CH paste (Univar; Ajax Finechem, Sydney, Australia) and incubated at 37°C in a humidified atmosphere in order to imitate the clinical medication step. To remove the medicament, the dentin slices were roughly rinsed with PBS before use.

On the day of the experiment, the CH-medicated dentin samples were carefully inserted into the slot inside the plastic tooth models (Figure 3.1*b*). Then the other half of each model was reassembled using light-cured resin (Tetric-flow; Ivoclar-Vivadent, Schaan, Liechtenstein).

A composite resin cuff was created 1mm beyond the apical end of the tooth model in order to imitate the closed environment of the root canal system. During the irrigation experiment, the models were stabilized on plastic wells.

3.2 Irrigation protocols

The tooth models with their inserted-dentin slices were randomly assigned according to the different dynamic irrigation techniques showed in table 3.1, as follows:

- **Group A: Needle irrigation (NI).** A 30-gauge, side-vented needle (Kerr, Orange, CA, USA) with a 10-mL syringe was used by placing the tip 1 mm short of the apical limit, delivering the irrigant at a rate of 2 mL per minute.
- **Group B: NI supplemented with Sonic irrigation (NI+EA).** An EndoActivator (Dentsply, Oklahoma City, OK, USA) was used as an irrigating supplement during NI. A yellow-labeled plastic tip was placed 3 mm from the coronal limit, and activated at a frequency of 190 Hz for 30 seconds after each five minutes of irrigation.
- **Group C: NI supplemented with Ultrasonic irrigation (NI+PUI).** An Irrisafe® tip (Acteon, Merignac, France) connected to a piezo-electric ultrasonic unit (P5 Newtron; Acteon) was used as an irrigating supplement during NI. The irrisafe 25/21 tip was placed 3 mm from the coronal limit, and activated using power level 9 for 30 seconds after each five minutes of irrigation.
- **Group D: Control group (Non-dynamic).** The irrigant delivery was through a side-vented needle, but without the dynamic movement of irrigant. The volume of irrigant was limited to the capacity of the root canal without replenishment.

The total irrigation time was 16 minutes in every group. In each experimental group, three different irrigating protocols were applied; **1)** NSS for 16 minutes, **2)** 17% EDTA for 15 minutes + NSS for one minute, **3)** 0.12% CHX for five minutes + 17% EDTA for 10 minutes + NSS for one minute. All experiments were performed in triplicate.

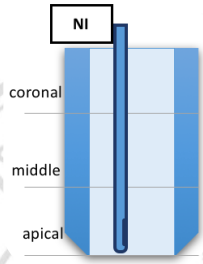
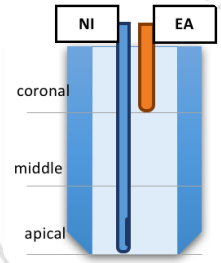
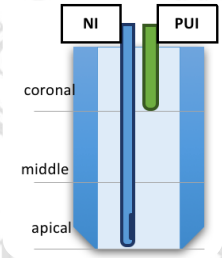
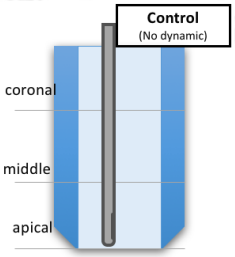
Groups	Irrigating techniques		Irrigation regimens
A.	NI		NSS EDTA CHX/EDTA
B.	NI+EA		NSS EDTA CHX/EDTA
C.	NI+PUI		NSS EDTA CHX/EDTA
D.	Control		NSS EDTA CHX/EDTA

Table 3.1 Summary of irrigation protocols.

3.3 Investigation of APC attachment to the conditioned root canal dentin

After irrigation, dentin samples from each experiment were aseptically removed from the tooth models and prepared for cell attachment assay. Evaluation of APC attachment ability was carried out on the dentin slice, focusing on the three different levels of the root canal: coronal, middle, and apical (Figure 3.1*c, d,* and *e*).

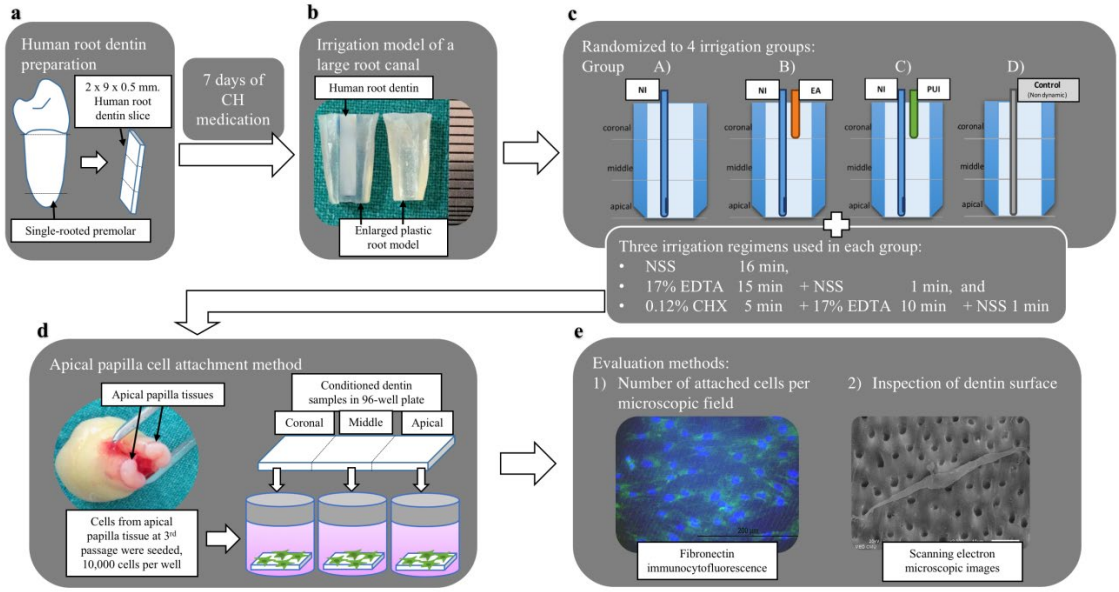


Figure 3.1 Schematic diagram representing the experimental procedures.

Dentin samples after being medicated were inserted into plastic root models and allocated to various irrigation protocols (*a-c*). The conditioned dentin samples were transferred to be used for the APC attachment process in 96-well plates containing culture medium (α-MEM) (*d*). Fibronectin immunofluorescence assay and SEM were conducted (*e*).

3.3.1 APC collection. APCs were obtained from apical papilla tissues of non-carious immature mandibular third molars. Third molar teeth from three 16-25-year-old healthy patients, who had undergone removal of impacted third molars at the Oral and Maxillofacial Surgery Clinic, Faculty of Dentistry, Chiang Mai University were selected (n = 3). The apical papilla tissues were gently detached from the root apex, and immediately digested using 3mg/mL Collagenase I (Gibco/Invitrogen, Gaithersburg, MD, USA) and 4mg/mL Dispase II (Sigma-Aldrich, St Louis, MO, USA) for 45 minutes at 37^0C. Cells were cultured in alpha modification of Eagle's medium (α-MEM) (Sigma-

Aldrich) with 10% FBS (Gibco/Invitrogen), 1% penicillin and streptomycin (Sigma-Aldrich) and 100.00 μM L-ascorbic acids (Sigma-Aldrich) at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. Cells at the 2nd to 3rd passage were used in this experiment.

3.3.2 Cell attachment and fibronectin immunofluorescent assay. The dentin slices were carefully split equally into three pieces, representing the dentin at the coronal, middle and apical levels of the root canal, and incubated in 100 μL of α-MEM for 24 hours. Then, APCs were seeded at 10,000 cells/slice and incubated for four hours to encourage initial cell attachment. Then, 200 μL of complete medium was later added. Another sample of APCs was also seeded without dentin slices, serving as positive controls. After 72 hours' incubation, the cells on dentin slices were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) solution in PBS for 10 minutes. Then, the cells were treated for two hours with a blocking agent, a 5% bovine serum albumin (BSA) solution in PBS, and subsequently incubated at 4°C with rabbit primary immunoglobulin G (IgG) against fibronectin (Sigma-Aldrich) diluted to 1:100 in PBS containing 3% BSA. After washing in PBS, the bound primary antibodies were visualized by incubation with 1:100 diluted fluorescein-conjugated anti-rabbit IgG (Sigma-Aldrich) in PBS for two hours. Finally, the slices were counterstained using 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The cells were monitored under a fluorescence microscope (U-LH100HGAPO; Olympus, Tokyo, Japan) at 200X magnification. Three fields were randomly captured from each dentin slice. Fibronectin positive-staining cells per microscopy field were counted using Image J software (National Institutes of Health, Bethesda, MD, USA), and the averages of positive-staining cells on each dentin slice were calculated. The comparison of overall results and comparison of various irrigants at each observational level were analyzed using one-way ANOVA, and the *post hoc* Tukey HSD test was used to make multiple comparisons, using SPSS version 17.0 (SPSS, Chicago, IL, USA). The level of statistical significance was set at $p < 0.05$.

3.4 Morphological evaluation using scanning electron microscopy (SEM)

Three dentin slices from each group were processed by fixing them in 4% paraformaldehyde for 30 minutes, and dehydrated with serial ethanol washes (30%, 50%, 70%, 80%, 90% and 100% for ten minutes each), followed by critical point drying and gold coating. The dentin surface morphology was observed and qualitatively described under a scanning electron microscope (JSM 6610LV; JEOL, Akishima, Japan).