CHAPTER 3

Methodology

This study was designed to evaluate the ability of APCs to attach to dentin surfaces that were treated with recommended concentration of 3Mix and $Ca(OH)_2$. The samples (dentin slices) were randomly assigned to six experimental groups (24 slices/ group).

Group 1: No medication

Group 2: Dentin slices treated with 3Mix at 0.39 µg/mL

Group 3: Dentin slices treated with 3Mix at 100 µg/mL

Group 4: Dentin slices treated with 3Mix using a paste-like preparation

Group 5: Dentin slices treated with $Ca(OH)_2$ at 1 mg/mL

Group 6: Dentin slices treated with $Ca(OH)_2$ at 1,000 mg/mL

The APCs were seeded on the treated dentin slice surfaces for four days. Twenty dentin slices from each group were randomly divided into three experiments: Nine for fibronectin immunocytochemistry, nine for alamarBlue® cell viability assay, and six for SEM examination. Schematic diagram of the experimental procedures is shown in Figure 13.

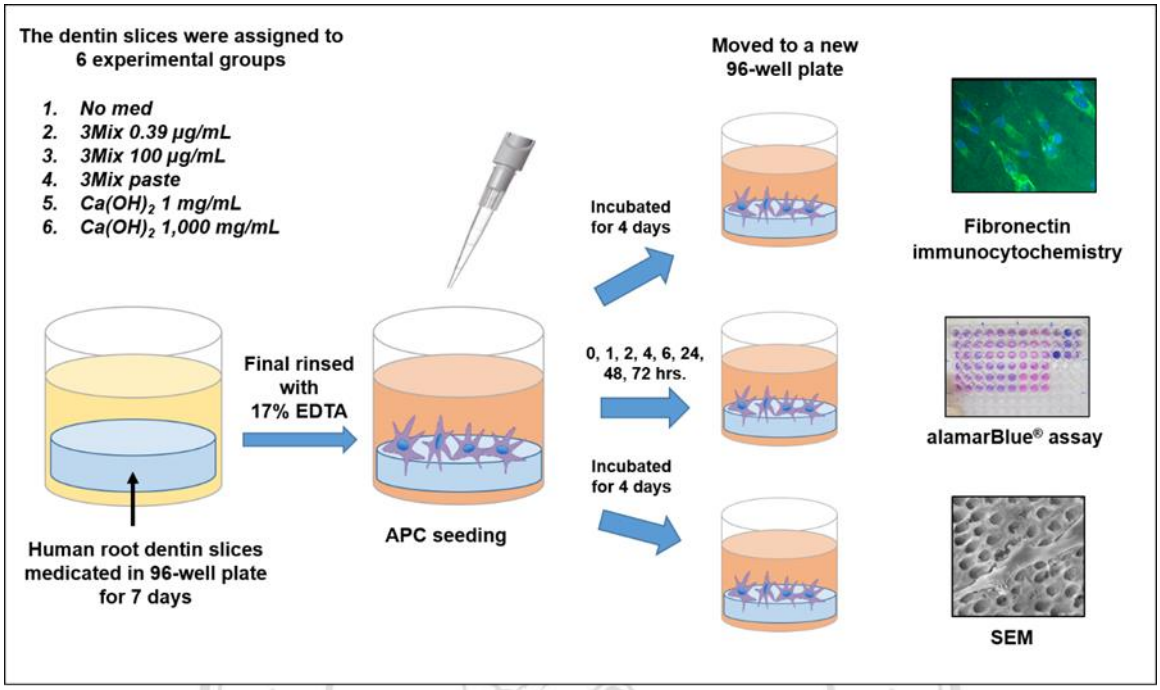

Figure 13 Schematic diagram illustrating the experimental procedures. Dentin slices were immersed in medication for seven days. Treated dentin slices were moved to a new 96-well plate containing α-MEM culture medium and primary human APCs were seeded. Fibronectin immunocytochemistry, alamarBlue® cell vitality assay and SEM were performed to evaluate cell attachment, viability and morphology.

1. Patient Recruitment

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. After informed consent was obtained, normal, immature, human third molars were collected from young healthy patients (aged 16-25) at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University (n=3).

Inclusion criteria

- Immature third molars without pathosis from young healthy patients aged 16-25 years.

Exclusion criteria

- Teeth with caries, pulpal or periapical disease.
- Volunteers who had severe underlying diseases, e.g., cancer, AIDS.

2. Culture of primary human APCs

The outer tooth surface was carefully rinsed with sterile normal saline, then apical papilla tissue was gently dissected from the apical part of the roots and immediately transferred into α-MEM transport medium (Sigma-Aldrich, St Louis, MO, USA) (Figure 14). The tissue was minced and digested in a solution of 3 mg/mL collagenase type I (Gibco/Invitrogen; Gaithersburg, MD, USA) and 4 mg/mL dispase (Sigma-Aldrich), for 45 minutes at 37°C. Afterwards, the cell suspension was centrifuged at 1,500 rpm for five minutes. Cells were cultured in α-MEM (Sigma-Aldrich) with 10% FBS (Gibco/Invitrogen), 1% penicillin and streptomycin (Sigma-Aldrich) (100.00 U/mL penicillin, 100.00 µg/mL streptomycin) and 100.00 µM L-ascorbic acids (Sigma-Aldrich) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were monitored daily under an inverted-light microscope in order to observe their morphology. The culture medium was replaced every three days. Cells at the 3rd passage were used in the experiment.

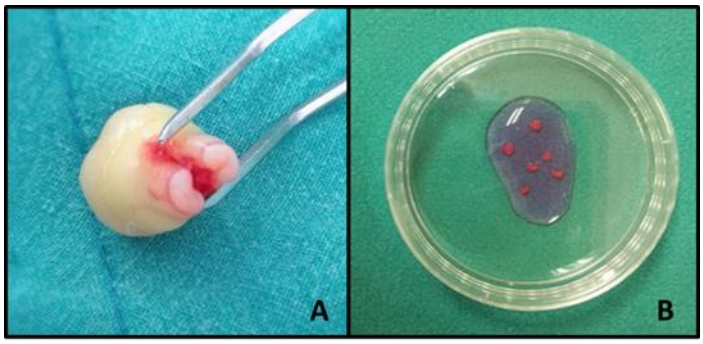

Figure 14 APC culturing. The apical papilla tissue was gently dissected from the root apex (A) and minced into small pieces (B).

3. Preparation of dentin slices

Seventy-two single-rooted, human premolar teeth extracted for orthodontic reasons were collected from 18-to-30-year-old patients. The crown of each tooth was removed using a water-cooled, low-speed handpiece with diamond discs. The root was cut longitudinally into two halves. A 1 mm-thick slice was cut from the inner, cut surface of each root half with a low-speed diamond saw (Isomet, Buehler Ltd., Evanston, IL, USA) under water cooling. After that the dentin slices were trimmed into a disc shape to fit the inner wall of a 96-well plate (Figure 15). The inner surface of the dentin disc was ground sequentially by 600-, 800-, 1,000- and 2,000-grit silicon carbide paper to achieve a smooth surface. Then all dentin slices were immersed in 17% EDTA for one minutes in an ultrasonic bath to remove the smear layer. Finally, all the dentin specimens were rinsed with sterile phosphate buffer saline (PBS) for one minute. The samples were autoclave sterilized for 20 minutes at 121 °C, and kept in sterile PBS until used.

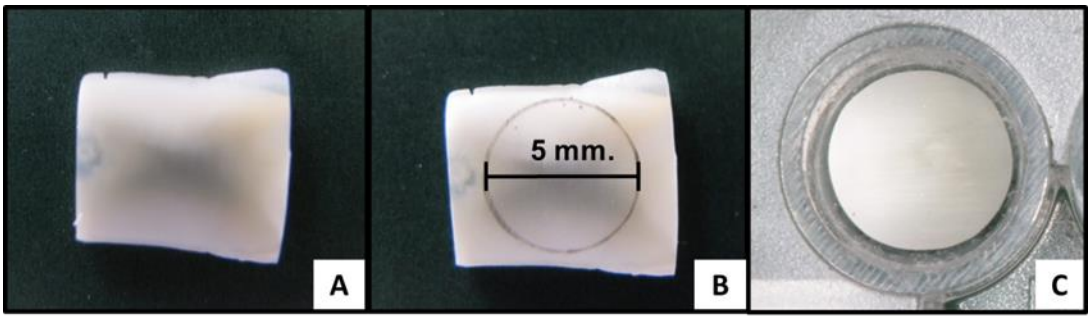

Figure 15 Preparation of dentin slices. The root was cut into a 1 mm-thick slice with a low-speed diamond saw and trimmed into a 5 mm-radius disc shape to fit the inner wall of a 96-well plate.

4. Drug preparation

3Mix and $Ca(OH)_2$ at different concentrations were prepared. For the 3Mix group, concentrations of 0.39 µg/mL and 100 µg/mL, and a paste-like consistency, which mimic the clinical usage dose, were prepared. A stock solution of 3Mix was made as previously described by Chuensombat *et al* (18). Briefly, a stock solution of Ciprofloxacin (250 mg; Siam Pharmaceutical, Bangkok, Thailand), metronidazole (200 mg; Siam Pharmaceutical), and minocycline (50 mg; Qualimed, Samut Prakarn, Thailand) was prepared at a concentration of 150 µg/mL. Each pill of drug was separately ground and dissolved in deionized water as shown in Table 1.

Table 1 Volume of deionized water used for preparing a stock solution of each antibiotic at a concentration of 150 µg/mL

Antibiotics	Deionized water (mL)
Ciprofloxacin	1666.67
Metronidazole	1333.33
Minocycline	333.33

Each antibiotic solution was filtered using 2.00 micron pore-size filter paper (Whatman, Maidstone, England) (Figure 16) and 0.2 micron pore size micro-filters (Corning, Oneonta, NY, USA). For the mixture of 3Mix, an equal volume of each antibiotic stock solution was mixed to obtain a solution at 150 µg/mL concentration. The stock solution of 3mix was freshly diluted in deionized water to the concentrations of 0.39 µg/mL and 100 µg/mL. For the paste-like consistency of 3Mix, 500 mg of each antibiotic was mixed with a mixture of macrogol ointment and propylene glycol (MP), following the protocol described by Hoshino *et al* (35). In brief, 500 mg of each ground antibiotic was blended into a homogeneous powder. An equal amount of macrogol ointment and propylene glycol was combined together, then the antibiotic powders and MP were incorporated in an approximate proportion of 5:1::powder:MP to obtain a paste-like consistency.

For the $Ca(OH)_2$ preparation, concentrations of 1,000 mg/mL and 1 mg/mL were freshly prepared. One gram of $Ca(OH)_2$ powder (Univar, Ajax Finechem, Sydney, Australia) was gradually mixed with 1 mL sterile distilled water to yield the $Ca(OH)_2$ paste at a 1,000 mg/mL concentration. At this concentration, $Ca(OH)_2$ has a creamy texture, as in clinical use. For 1 mg/mL $Ca(OH)_2$, 1 mg $Ca(OH)_2$ powder was gently dissolved with 1 mL of sterile distilled water.

Figure 16 Preparation of stock solution of 3Mix. The solution of each antibiotic was filtered using 2.00 micron pore-size filter paper.

5. Cell attachment and viability evaluation

In order to follow the clinical revascularization protocol currently proposed by the AAE (33), the dentin slices were separately placed in a 96-well plate and irrigated with 100 µL of 1.5% NaOCl for one minute. Afterward, the dentin slices were rinsed twice with 200 µL of sterile PBS and randomly assigned to the six experimental groups as described above. The samples were immersed in 100 µL of prepared drug (Groups 2-6) and sterile PBS (group 1). All specimens were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for seven days. At the end of the culture period, the dentin slices were irrigated again, twice with sterile PBS for five minutes, then with 100 µL of 17% EDTA for one minute. After that the dentin slices were removed and separately placed in a new 96-well plate containing 200 µL of α-MEM complete medium and incubated for 24 hours in order to equilibrate all dentin slices. Harvested APCs (15,000 cells/well) were seeded on the treated dentin slices and incubated for four hours in order to allow sufficient cell attachment. Then, complete medium was added to get the final volume at 200 µL in each well. All cells were cultured for four days.

Twenty-four dentin slices from each group were randomly divided into three experiments: Nine for fibronectin immunocytochemistry examination, nine for alamarBlue® cell vitality assay, and two for SEM examination.

5.1 Fibronectin immunocytochemistry

Cells after four days of culture on dentin slices were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) solution in PBS for ten minutes. Then, cells were treated for one hour with a blocking agent, a 3% bovine serum albumin (Sigma-Aldrich) solution in PBS, and incubated overnight at 4°C with a mixture of 1:100 diluted rabbit primary antibodies against fibronectin (Sigma-Aldrich) (Figure 17A) in 3% bovine serum albumin solution in PBS. After washing in PBS, the bound primary antibodies were visualized by incubation for two hours with 1:100 diluted fluorescein-conjugated anti-rabbit IgG (Sigma-Aldrich) (Figure 17B) in PBS. Finally, the dentin slices were counterstained using 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (Figure 17C). Cells were monitored under a fluorescence microscope (U-LH100HGAPO; Olympus, Tokyo, Japan) at 200x magnification. Nine fields were randomly captured from each dentine slice. Fibronectin positive-staining cells per microscopic field were counted using the cell counter plug-in of ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the average of positive-staining cells on each dentin slice was calculated.

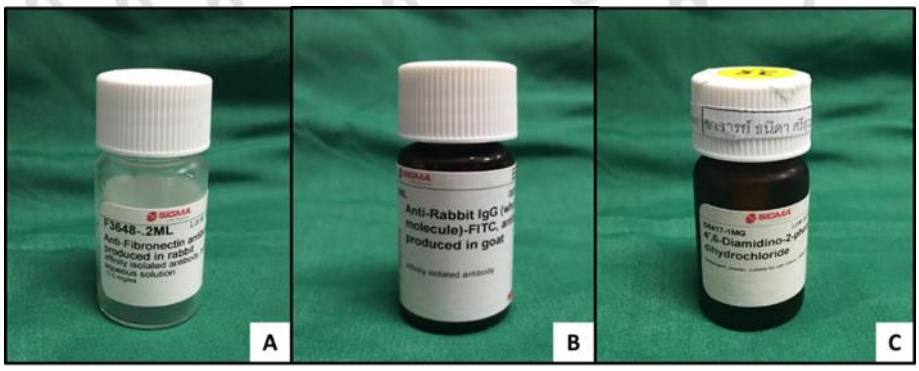

Figure 17 Fibronectin immunocytochemistry agents. Primary antibodies against fibronectin from rabbit (A), fluorescein-conjugated anti-rabbit IgG (B), and DAPI (C).

5.2 The alamarBlue® vitality assay

After four hours of cell seeding, nine dentin slices from each group containing APCs were removed to a new 96-well plate and 150 µL of α-MEM complete medium was added. Then 15 µL of alamarBlue® was added to each well (Figure 18). One hundred microliters of the mixed solution in each well was separately transferred to another new 96-well plate and the colorimetric quantitative change of alamarBlue® was monitored using a spectronanometer (Sunrise; Tecan, Mannerdorf, Switzerland) at 540 nm. The absorbance was monitored at the following specific time intervals: 0, 1, 2, 4, 6, 24, 48, and 72 hours. After each measurement, the solution was re-transferred into the first new 96-well plate containing dentin slices and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were also seeded in nine of the wells without dentin slices, as a control group.

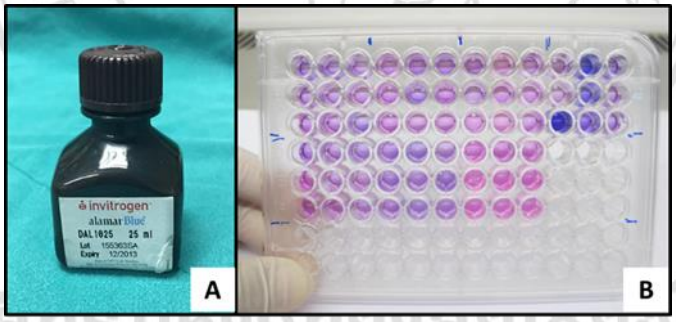

Figure 18 The alamarBlue® vitality assay. Fifteen microliters of alamarBlue® was added to each well. One hundred microliters of the mixed solution in each well was separately transferred to the second new 96-well plate and the colorimetric quantitative change of alamarBlue® was monitored using a spectronanometer.

5.3 Scanning Electron Microscopic Observation

Six dentin slices from each group were prepared for scanning electron microscopy. The specimens were fixed in 4% paraformaldehyde for 30 minutes and dehydrated using a graded dehydration series of ethanol (30%, 50%, 70%, 80%, 90%, and 100% for 10 minutes each). After that, all samples were critical point dried and coated with gold. Cellular attachment and morphology were observed and qualitatively described under a scanning electron microscope (JSM 6610-LV; JEOL, Eching, Germany).

6. Statistical Analysis

The number of fibronectin positive cells and the absorbance of alamarBlue® solution were analyzed with 1-way analysis of variance using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Multiple comparisons of the fibronectin-positive cells and the absorbance were analyzed using the Dunnett T3 and Duncan tests, respectively. The difference between experimental groups was considered to be statistically significant at $p<0.05$.