CHAPTER III

MATERIALS AND METHODS

3.1 Materials

1) Tetric® Flow (Ivorclar Vivadent, Liechtenstein)

2) Filtek™ Flow (3M ESPE, USA)

3) Aeliteflo™ (Bisco, USA)

4) MTA ProRoot™ (Dentsply, USA)

5) L.E.D. Curing Light (Smartlite™, Dentsply USA)

6) Optilux radiometer model 100 (Kerr, USA)

7) Glass slab

8) Metal spatula

9) Scapel blade

10) Blade No.15

11) 60-mm. tissue culture dish (Nunc, Denmark)

12) 12-well-flat-bottom plate (Nunc, Denmark)

13) 96-well-flat-bottom plate (Costar, USA)

14) 25 cm^2 cell culture flask (T25)

15) CO_2 incubator

16) Laminar flow hood

17) Pasteur pipette

18) Pipette tip 200, 1000 µl

19) Pipette 10, 25 ml

20) Hemocytometer

21) Phase contrast light microscope

22) Microplate reader

23) Dulbecco's Modified Eagle Medium; DMEM (Gibco BRL, USA)

24) DMEM without phenol red

25) 0.25% trypsin-EDTA

26) MTT (3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, USA)

27) Dimethyl sulfoxide (DMSO)

28) Fetal Calf Serum (FCS) (Gibco BRL, USA)

29) Penicillin G (Gibco BRL, USA)

30) Streptomycin (Gibco BRL, USA)

31) Amphotericin B (Gibco BRL, USA)

32) L-glutamine (Gibco BRL, USA)

33) Phosphate Buffer Saline (PBS)

34) 0.1 M phosphate buffer pH 7.2

35) 2.5% glutaraldehyde in 0.1 M phosphate buffer

36) Methylene blue

3.2 Medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) and 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY) were used throughout the study.

3.3 Preparation for cell culture

Human periodontal ligament cells were obtained from caries-free lower third molars extracted for clinical reason at the Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University with the patients' informed consent. The method to obtain human periodontal ligament cells from the periodontal tissues was that described by Pattamapun et al (2003). The teeth were extensively washed with sterile phosphate-buffered saline solution (PBS) and the PDL tissues were scraped out from the middle third of the roots under sterile condition with a sharp No. 15 blade and seeded in 12-well-plates (Nunc, Naperville, IL, USA). The explants were cultured in DMEM supplement with 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml) and 10% heat-inactivated FCS and incubated at the condition of 5% CO_2, 37 °C. The medium and supplements were from Gibco BRL (Carlsbad, CA, USA). Culture medium was changed twice a week. After the outgrowth of human periodontal ligament cells had reached the confluence, they were trypsinized, washed twice and then sub-cultured to new tissue culture flasks. The cells from 3^{th} to 8^{th} passages of five different donors were used in this study.

3.4 Preparation of flowable resin composites and MTA

The test materials are Tetric® flow (Ivorclar Vivadent, Liechtenstein), Filtek™ Flow (3M ESPE, USA), Aeliteflo™ (Bisco, USA) and MTA ProRoot™ (Dentsply, Tulsa, OK, USA). Ten standard cylinder discs of 6 mm in diameter and 1.5 mm in height for each tested material were prepared under aseptic conditions according to the manufacturer's directions. For flowable resin

composites preparation, one side was in contact with the glass slide while the other side was exposed to the air. Both sides of resin composite samples were polymerized for 20 seconds. Oxygen inhibited layer was removed by swabbing with 70% alcohol and distilled water soaked cotton pellet as recommended by Rud et al. (1991a). After sample preparation, each side of the samples was exposed to ultraviolet light for 30 minutes for surface disinfection. Samples were then placed into the bottom of 96-well tissue culture plate (Nunc, Naperville, IL, USA) with swabbed side facing up (in resin composite groups).

These ten prepared samples from each material were subdivided into 5 groups of two samples regarding to their number of days being immersed and stored in cell culture medium (DMEM without phenol red). The first group was the samples in freshly mixed state while the other groups were the groups that samples were immersed and stored for a day, two days, three days and four days, respectively.

3.5 Colorimetric (MTT) assay for cytotoxicity of extracted medium from materials (Elution)

Cell culture medium (200 µl/well) used for eluting test materials was added into the cultured wells with the material discs in the bottom (except group of freshly mixed material). The material discs along with cultured medium were incubated at the condition of 5% CO_2, 37 °C for a day, two days, three days or four days depend on the number of days assigned for materials to be immersed (figure 1, 2). Culture medium was replaced with new medium everyday until the period of elution was dued. Then the cultured medium was

no longer changed but it would be drawn from material and used to incubate with periodontal ligament cells for cytotoxicity testing.

Cultured human periodontal ligament cells were seeded at 20,000 cells/well/200 µl in a new 96-well- tissue culture plate for 24 hours. After the overnight incubation, the medium in cell culture was replaced by the extracted medium that was drawn from material and cells were incubated for another 24 hours. Cells cultured in fresh medium (not extracted medium) were used as control.

After 24 hours incubation, colorimetric (MTT) assay for cytotoxicity of extracted medium from materials was performed. The medium was removed from cells and replaced with 100 µl of media without phenol red containing 0.5 mg/ml of MTT formazan powder (3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co., St. Louis, MO, USA). After 210 minutes incubation, 200 µl of dimethyl sulfoxide was added into each well to dissolve the formazan crystal. The survival rate of the cells was calculated from spectrophotometer measurement at 570 nm wavelength. Data obtained from the MTT assay was shown as relative percent, by comparing with the control.

3.6 Colorimetric (MTT) assay for cytotoxicity of material (Direct contact)

Cultured human periodontal ligament cells supplement with 1% L-glutamine and 5% FCS were seeded on material in either group of freshly mixed state or groups that materials had been eluted and cultured medium was removed (after experiment 3.5) at 20,000 cells/well/200 µl and incubated

for 24 hours (figure 1, 2). Cells cultured in fresh medium (without material) were used as control.

At the end of the experiment, the colorimetric (MTT) assay for cytotoxicity of material was performed in the same manner as mentioned in experiment 3.5.

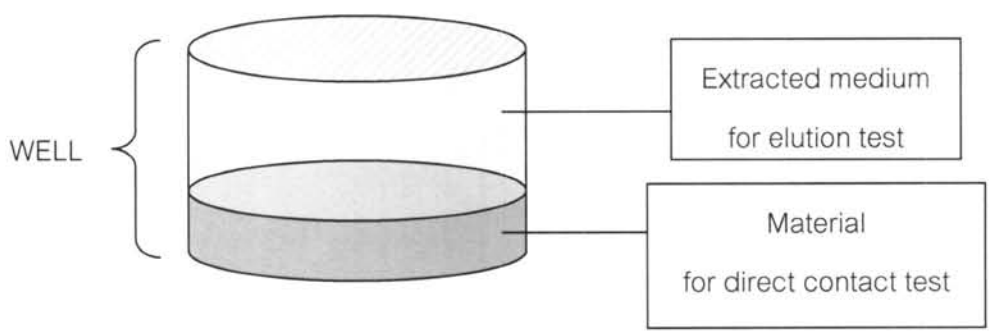

Figure 1 Diagram illustrated cultured well plate with material (for direct contact test) and extracted medium (for elution test)

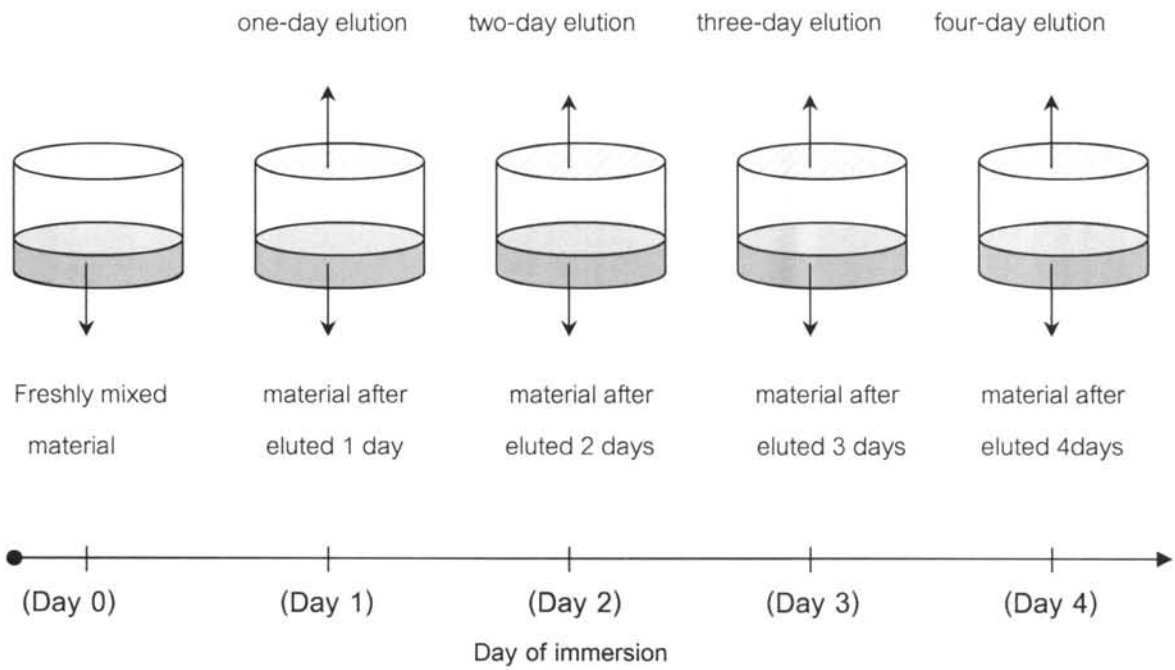

Figure 2 Diagram illustrated extracted medium and material from cultured well used for cytotoxicity testing

3.7 Cell morphology and attachment by scanning electron microscope

Two prepared samples from each material placed into the bottom of 96-well tissue culture plate were incubated with cell culture medium (DMEM without phenol red, 200 µl/well) at the condition of 5% CO_2, 37 °C for four days. The cultured medium was changed everyday. After that cultured human periodontal ligament cells were seeded on material at 20,000 cells/well/200 µl in 96-well- tissue culture plate and incubated for 24 hours. Cells cultured in fresh medium (without material) were used as control. After incubation, the disc of tested material along with the cells grown on their surface were washed three times with phosphate-buffered solution, fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer 200 µl for 120 minutes at room temperature. After

that the material discs were washed again in phosphate-buffered solution and they were removed from the bottom of wells by drilling with round bur from another side of wells. A blunt end instrument was used to gently push material out of wells. The material discs were dehydrated in ascending grades of ethanol, critical point dried with CO_2, mounted on copper stubs and sputter-coated with 15 nm gold palladium. The cells grown on material discs were examined at center and peripheral area of material by using a scanning electron microscope at 15X, 200X and 1000X (JSM-5410LV, JOEL, Japan) compared with the cells grown on bottom of well.

3.8 Statistic Analysis

Results of the data obtained from colorimetric (MTT) assay for cytotoxicity of extracted medium from materials (Elution) and from materials (Direct contact) were expressed as mean of relative percent $\pm$ standard deviation (SD).

For statistical analysis, the data was statistically analyzed by using One-way ANOVA followed by post hoc multiple comparison (Scheffe) test and t test at the 95% confidence interval. Differences with a *P* value of less than 0.05 were considered significant.