CHAPTER III

MATERIALS AND METHODS

Cell culture

Human dental pulp cells were obtained from caries-free lower third molars extracted for orthodontic reason at the department of oral surgery, Faculty of Dentistry, Chulalongkorn University with patient's informed consent. The teeth were extensively washed with sterile phosphate-buffered saline solution (PBS) and cracked open along the longitudinal axis. The pulps were gently removed by forceps, minced into small pieces (1x1x1 mm^3) and seeded in 35-mm plastic tissue culture dishes (Nunc, Naperville, IL, USA). The explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B and incubated at the condition of 5% CO_2, 37 °C. The medium and supplements were purchased from Gibco BRL (Carlsbad, CA, USA). After the outgrowth of human cultured dental pulp cells reached confluence, they were subcultured into new culture dishes. The 3^{rd} to 5^{th} passages of three different donors were used in this study.

Fluocinolone aceonide preparation

Fluocinolone acetonide (FA) was purchased from FARMABIOS S.R.I. (Stabilimento e Direzione, Gropello Cairoli, Italy). To prepare stock solution, 4.52 mg fluocinone acetonide was dissolved in dimethyl sulfoxide (DMSO) solution to obtain 10 mM concentration. When the experiment was done, the stock solution was then serial diluted into 10 to 0.1 (10, 1, 0.1) µM concentrations by mixing with culture medium. The sterilization technique in all procedures was strictly controlled and new prepared solution was used in each experiment.

Colorimetric (MTT) assay for cell proliferation

Cultured human dental pulp cells were seeded at 20,000 cells/ml in 24 well plates for 24 hours. After the overnight incubation, the medium was replaced twice by serum free medium at 3 hours intervals in order to wash out the serum. Cells were then treated with the dilutions of various concentrations (50, 10, 1, 0.1 µM) of fluocinolone acetonide for 24, 48 and 72 hours. Cells cultured in serum-free medium were used as control. At the end of the experiment, the media without phenol red containing 4.5 mg of MTT formazan powder (3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co., St. Louis, MO, USA) was added. After 30 minute incubation, 900 µl of dimethyl sulfoxide and 125 µl of glycine buffer (0.1 M NaCl + 0.1 M Glycine, pH 10.5) were added into each well to dissolve the formazan crystal. The survival or proliferation

rates of the cells were calculated from spectrophotometer measurement at 570 nm wavelength. Data obtained from the MTT assay was shown as relative cell number, by comparing with control. This experiment was done as a pilot study to select the suitable concentrations of fluocinolone acetonide for further experiments.

Type I collagen synthesis

The cells were cultured to 6-well plates at a density of 3 x 10^5 cells/well/ml in 2 ml of DMEM. After the overnight incubation, the cells were then treated with the selected concentrations of fluocinolone acetonide (10, 1, 0.1 μM), resulted from MTT experiment. The medium was changed every 48 hours and the cells are incubated for at least 5 days. Culture medium supplemented with ascorbic acid and DMSO solution was used as control. At the end of the experiment, the cells were washed three times with PBS. The 500 μl of 1 M acetic acid was then added to each well. The cells were scraped and transferred to 1.5 ml tubes. The tubes were rotated at 4°C overnight and centrifuged. The supernatant was collected and subjected to lyophilization.

The amount of total protein from each extract was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of protein from each sample was mixed with running buffer and subjected to electrophoresis on 7.5% polyacrylamide gel along with pre-stained high molecular weight standards (Bio-Rad, Hercules, CA, USA).

The proteins were transferred to a nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) using a trans-blot cell (Gibco BRL, Carlsbad, CA, USA) at

15 V for 1 hour 45 minutes. The nitrocellulose membrane was incubated overnight in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at 4°C.

The nitrocellulose membrane was stained for 1 hour with primary antibody for type I collagen (L-19) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) which was diluted to 1:200 with blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water). The membrane was then washed with PBS and incubated with biotinylated-secondary antibody (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 minutes, respectively. The protein bands were detected using a SuperSignal® West Pigo Trial Kit (Pierce, Rockford, IL, USA) and were exposed on CL-X Posture film (Pierce, Rockford, IL, USA). The molecular weight of protein was obtained by determining the distance of bands driven onto the nitrocellulose membrane. The data was then compared with the protein marker to indicate the type of protein. The band intensity was determined by scion image analysis software and optical density was adjusted to percentage of expression, by comparing with control. The intensity of protein bands indicated the relative amounts of protein in the samples. The experiments were performed from three different donors.

Reverse-transcription polymerase chain reaction (RT-PCR)

The cells were cultured to 6-well plates at a density of 3×10^5 cells/well/ml in 2 ml of DMEM. After the overnight incubation, the cells were then treated with the

selected concentrations of fluocinolone acetonide (1 μM) and culture medium. Culture medium mixed with DMSO solution was used as control. After 24 hours, total cellular RNA was extracted with Trizol (Gibco BRL) according to manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by reverse transcription using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) for 60 minutes at 42°C. Subsequent to the reverse transcription, polymerase-chain reaction (PCR) was performed for detection of type I collagen cDNA. The primers specific to type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared by GENSET (Singapore) using reported sequences from GenBank (type I collagen; GI: 34193787, GAPDH; GI:83641890). The oligonucleotide sequences of type I collagen and GAPDH primers were:

Type I collagen	sense	5' CTGGCAAAGAAGGCGGCAAA 3'
	antisense	5' CTCACCACGATCACCACTCT 3'
GAPDH	sense	5' TGAAGGTCGGAGTCAACGGAT 3'
	antisense	5' TCACACCCATGACGAACATGG 3'

The PCR was performed using Taq polymerase (Qiagen, Hilden, Germany) with PCR volume 25 μl. The reaction mixtures contained 25 pM of primers and 1 μl of RT reaction. The PCR working condition was set at denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C, and chain elongation for 1.45 min at 72°C on DNA thermal cycler (Omn-EThermal cycler, Hybaid, Middlesex, UK). The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide

fluorostaining. The band intensity was determined by scion image analysis software and the optical density was adjusted to percentage of expression, by comparing with the control condition. The intensity of bands indicated the relative amounts of the mRNA in the samples.

In vitro calcification

The cells were cultured to 12-well plates at a density of 3 x 10^4 cells/well/ml in 1 ml of DMEM. At a 90% confluence, the cells were treated with the selected concentrations of fluocinolone acetonide (10, 1, 0.1 μM) and medium supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The media for all groups was changed every 2 days for up to 28 days. Culture medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and DMSO solution was used as control. At the end of the experiment, the cells were washed with PBS and fixed with ice-cold methanol for 10 minutes. The cells were washed twice with deionized water and then stained with Alizarin red dye solution (0.1% NH_4OH, pH 6.5) for 5 minutes. The excess dye was washed several times with deionized water. Calcified nodules with red color were then observed on culture plates. Morphology of the cells and calcified nodules at various stages were recorded and photographed with a phase contrast microscope (Olympus CK2, Olympus America Inc., Mel-. ville, NY, USA).

Statistical analysis

The relative amounts of the collagen determined by scion image analysis software were evaluated by using SPSS version 11.5. At the 95% confidence interval, the data from Western blot analysis and RT-PCR was statistically analyzed by using One-way ANOVA followed by post hoc multiple comparison (Scheffe) test, and t test respectively.