CHAPTER III

MATERIALS AND METHODS

3.1 Materials

1. Orthodontic appliances

- Transpalatal archs with soldering hooks (Figure 1a)
- Banding cement
- 10 miniscrew implants (Figure 1b)
    - 1.6 mm diameter, 9.0 mm length
    - 1.6 mm diameter, 8.0 mm length
    - 2.0 mm diameter, 6.0 mm length
- Sentalloy® closed coil spring 100 g (Figure 1c)

2. Sample collection instruments

- 1.5 ml Microcentrifuge tubes
- 10.0x1.0 mm filter paper strips (Whatman® filter paper No.1, Whatman International Ltd., Maidstone, UK)

3. Alkaline Phosphatase Detection Kit, Fluorescence® (Sigma-Aldrich Inc., Saint Louis, USA)

4. Bio-Rad Protein Assay Kit II® (Bio-Rad, Hercules, USA)

Figure 1 Orthodontic appliances used in this study; Transpalatal arch with soldering hooks soldered to maxillary first molar bands (a), Miniscrew implant (1.5 mm diameter, 9.0 mm length) (b) and Sentalloy® closed coil springs (100 g) (c)

3.2 Methods

1. Informed consent: prior to the collection of gingival crevicular fluid and peri-miniscrew implant crevicular fluid

2. Experimental subjects

10 miniscrew implants were placed in midpalatal area of 10 adult patients with skeletal open configuration and with anterior open bite as anchorage for molar intrusion. Transpalatal arch with soldering hooks were placed in all patients to prevent lingual tipping of the molars. The patients met these following criteria:

- Good general and oral health with a healthy periodontium, no radiographic evidence of bone loss, no gingival inflammation and a probing depth of 3 mm or less at all teeth.
- Lack of antibiotic therapy during previous 6 months
- Absence of anti-inflammatory drug administration preceding the study
- No pregnancy (women)

- Need maxillary molar intrusion

Experimental design

The experimental design was divided in two periods.

Phase I: The unloaded phase:

In the first week, gingival crevicular fluid around maxillary first molars was collected as base line and right mandibular first molars and right maxillary second molars as control. After isolating the tooth with a cotton roll, supragingival plaque was removed without touching the marginal gingiva, and the crevicular site were then gently dried with an air syringe. Gingival crevicular fluid was collected with 10.0x1.0 mm filter paper strips, which were inserted into the crevice until light resistance was felt. Strips contaminated with blood were discarded. Immediately after collection, two millimeter of wetting part of strips (containing 0.1 µl of fluid, determined using the Periotron 8000™) were cut and transferred to microcentrifuge tubes. All strips were stored at -70ºC until further processing. Transpalatal archs with soldering hooks were fabricated and inserted to patients. A miniscrew implant was placed in midpalatal area for each patient. Then gingival crevicular fluid and peri-miniscrew implant crevicular fluid were collected in day 1, 4, 7 and 14 after miniscrew implant placement.

Phase II: The loaded phase

Two weeks after miniscrew implant placement, periapical radiographs were performed at the intruded maxillary first molars. Intrusion force using Sentalloy® closed coil springs (100 g/side) were applied to maxillary first molars. All springs

were attached to the miniscrew implant head with ligature wire (Figure 2). Gingival crevicular fluid and peri-miniscrew implant crevicular fluid samples were collected from patients every week for twelve weeks.

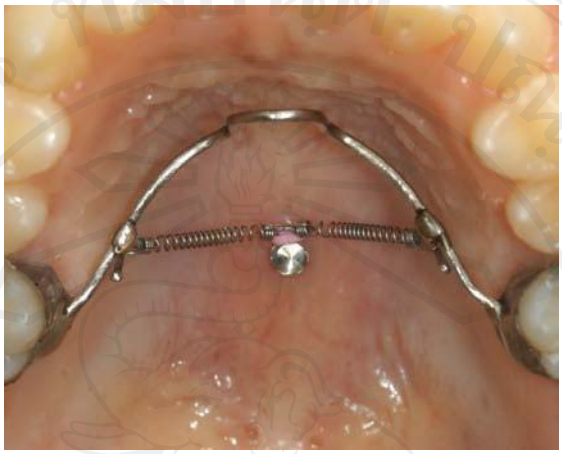

Figure 2 Sentalloy® closed coil springs attached transpalatal arch and miniscrew implant

Levels of alkaline phosphatase and total protein content were detected in all samples. The values of alkaline phosphatase were normalized by the amount of total protein content in each sample.

Alkaline phosphatase assay:

Phosphate buffer saline solution (200 µl) was added to microcentrifuge tubes and samples were centrifuged for 1 minute. The papers were then removed. Samples were analyzed for alkaline phosphatase using Alkaline Phosphatase Detection Kit, Fluorescence® (Sigma-Aldrich Inc., Saint Louis, USA). Alkaline phosphatase standard (0-500 ng/ml) and samples were added to the microtitre plate (20 µL/well) in duplicate and incubated at 65°C for 30 minute. The samples were cooled to room temperature and fluorescence assay buffer (dilution buffer and fluorescence assay

buffer at 1:8 ratio) 180 µL were added to each well. Then the 4 µl of diluted substrate (10 mM of 4-methylumbelliferyl phosphate disodium) were added to each well. The plates were incubated in the dark at room temperature for 30 min and fluorescence were measured at 340 nm excitation and 460 nm emission. Alkaline phosphatase concentrations were determined from a standard curve.

Protein assay:

Total protein concentration was determined by using the Bio-Rad Protein Assay Kit II® (Bio-Rad, Hercules, USA), based on the Bradford dye-binding procedure. It was a simple colorimetric assay for measuring total protein concentration. Bio-Rad's protein assay was based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye bound to primarily basic (especially arginine) and aromatic amino acid residues. Bovine serum albumin (BSA) standards (0-1,000 µl/well) and samples were added to the microtiter plates (10 µl/well) in triplicate. Dye Reagent Concentration and deionizes distilled water were mixed together (1:4) and added to each well (200 µl/well). The plates were incubated at room temperature for 5 min and the absorbance is measured at 620 nm. Protein concentrations were determined from a standard curve.

Protocol for miniscrew implant placement:

Miniscrew implants were placed with pre-drilling technique in midpalatal area, correspond to maxillary first molar position, under local anesthesia. The patient rinsed with 0.02% chlorhexidine mouthwash. Miniscrew implant drilling was

performed under saline cooling. The miniscrew implant status was monitored for two weeks before force application. Sentalloy® closed coil springs (100g/side) were connected between miniscrew implant heads and soldering hooks on transpalatal arch, which were attached to first molar bands, by wire ligation to create intrusion force.

Statistical analysis

The data were analyzed using the statistical SPSS *v*17 for Windows. The Kolmogorov-Smirnov test was used to test the distribution of ALP levels from the unloaded and loaded periods. In each phase, the differences between the ALP levels of the unloaded and loaded periods were determined by the Wilcoxon signed ranks test. Results were considered to be statistically significant at $P < .05$.