

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Materials

##### 1) Subjects and general selection criteria

- A total of ten adult patients with open skeletal configuration and with anterior open bite, and who required orthodontic molar intrusion were included in the study. The patients met the 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 the previous six months
  - Absence of anti-inflammatory drug administration in the month preceding the study
  - No pregnancy (women)
  - Required maxillary molar intrusion

##### 2) Orthodontic appliances

- Transpalatal arch with soldered hook (Figure 3.1a)
- Banding cement
- 10 miniscrew implants (1.6 mm in diameter and 6.0-9.0 mm in length) (Renew Biocare Corp., San Bruno, USA) (Figure 3.1b)

- Sentalloy® closed coil springs 100 g (Tomy, Tokyo, Japan)

(Figure 3.1c)

### 3) Sample collection instruments

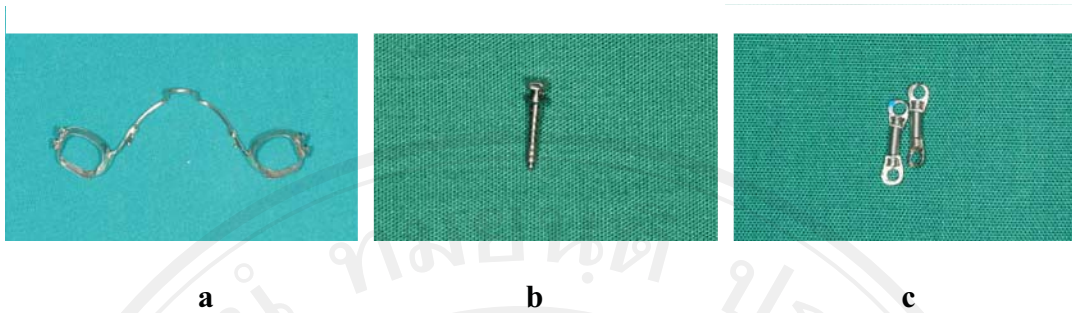
- 1.5 ml Eppendorf tube
- Scissors
- 10.0x1.0 mm Whatman® no.1 filter paper strip (Whatman International Ltd, Maidstone, Kent, UK)

### 4) Chemical reagents and supplies for ELISA technique

- Microtiter plates (Maxisorp®, Nunc, Roskilde, Denmark)
- Blue, yellow tips
- Auto pipette
- Tray
- Shaker, vortex
- IgM-specific peroxidase conjugated anti-mouse immunoglobulin
- WF6 mAb
- PBS-tween
- 1%w/v BSA
- Peroxidase substrate
- 4M H<sub>2</sub>SO<sub>4</sub>

### 5) Informed consent

The experiments were approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. Before the collection of GCF and PMICF samples, the patients were informed of the experimental procedures. Then, the informed consents were obtained.



**Figure 3.1** Orthodontic appliances; Transpalatal arch with soldered hooks (a), Miniscrew implant (1.6 mm in diameter and 6.0-9.0 mm in length) (b) and Sentalloy closed coil springs (100 g) (c)

### 3.2 Methods

#### Experimental design

The experimental design was divided into two phases.

#### Phase I: The unloaded period

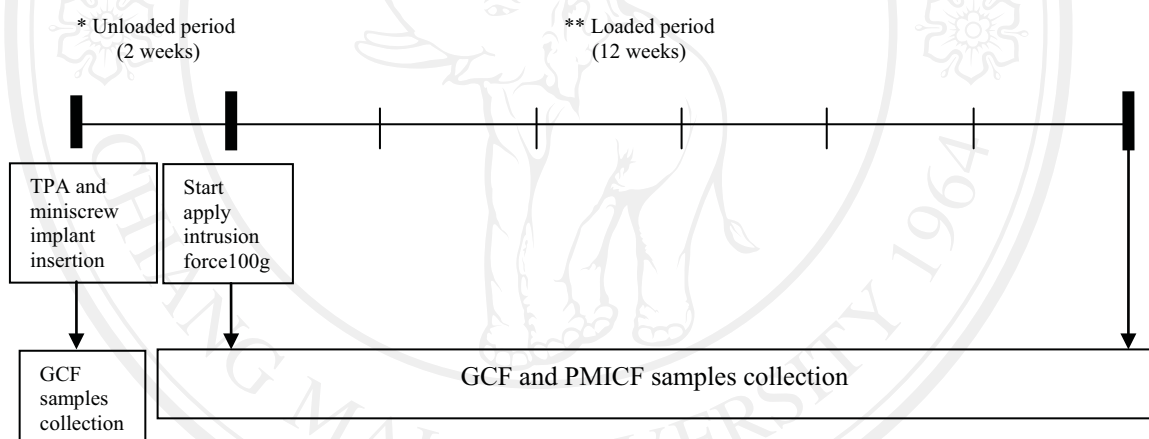
In the first week after assessment of general status and informed consent.

- Gingival crevicular fluid was collected from around the maxillary first molars as base-line, and from around the right mandibular first molar and right maxillary second molar as controls.
- Teeth were separated, bands were tried and impressions were made.
- The transpalatal arch with the soldered hook was inserted.
- One miniscrew implant was placed in the midpalatal area of each patient.
- Gingival crevicular fluid and peri-miniscrew implant crevicular fluid were collected on Days 1, 4, 7 and 14 after miniscrew implant placement.

#### Phase II: The loaded period

Two weeks after the miniscrew implant was inserted

- Lateral cephalometric and periapical radiographs were made.
- Impressions were made to fabricate study model.
- Intrusion force was applied to the molars with a Sentalloy closed coil spring, 100 g/side. (Figure 3.1c) The Sentalloy closed coil springs were attached to the miniscrew implant head by using an adapted Suzuki reef knot (Figure 3.6).<sup>56</sup>
- Then gingival crevicular fluid and peri-miniscrew implant crevicular fluid samples were collected from patients every week for twelve weeks.



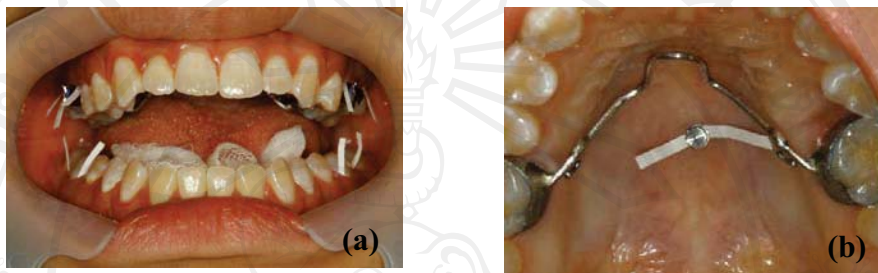
**\*Unloaded period:** sample collection on Days 1, 4, 7 and 14 after miniscrew implant placement

**\*\* Loaded period:** sample collection on Days 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91 and 98

**Figure 3.2** Diagram of the experimental design

The experimental and control sites were isolated from saliva and gently air dried and the samples were collected by using Whatman® No.1 filter paper strips (10.0x1.0 mm) inserted into the mesial gingival sulcus and peri-miniscrew implant sulcus. Care

was taken to avoid mechanical injuries. Samples containing blood were discarded. The last 2.0 mm of the filter paper strips containing GCF and PMICF (0.1  $\mu$ l of fluid, determined using the Periotron 8000<sup>TM</sup>) were cut off and put into 1.5 ml Eppendorf tube. The tubes were labeled and were stored at -80° C until the immunoassay was performed.



**Figure 3.3** GCF (a) and PMICF (b) collection.

The miniscrew implant mobility assessment was performed, after the PMICF sample was collected, by using cotton forceps.<sup>57</sup> Extremely light force was laterally applied to the miniscrew implant head. Mobility was assessed as either ‘yes’ (mobile) or ‘no’ (not mobile). If there was any mobility, the miniscrew implant was categorized as mobile, whereas the miniscrew implants that remained in the bone until the end of the study period were considered to be successful.



**Figure 3.4** Clinical mobility assessment of miniscrew implant.

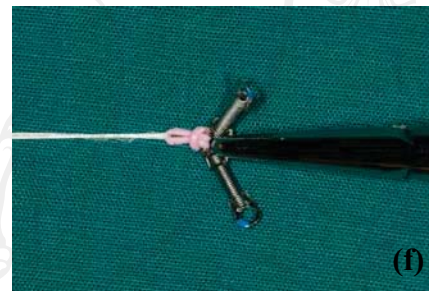
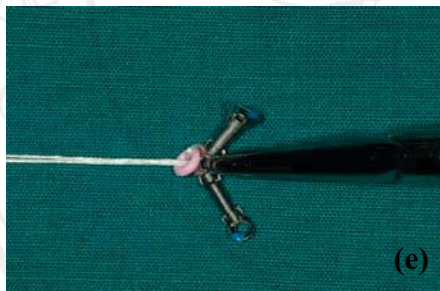
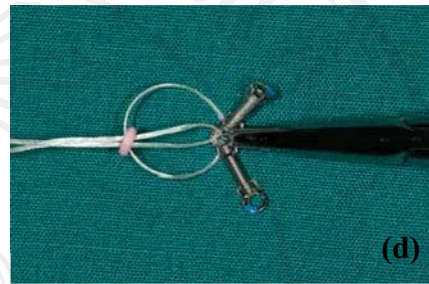
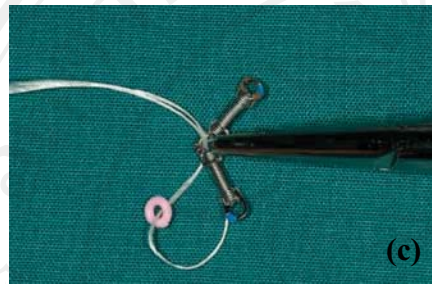
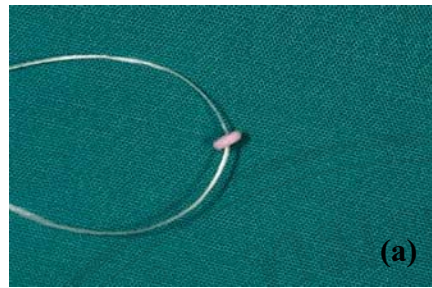
All samples were analyzed by competitive ELISA with WF6 monoclonal antibody. The study casts were made at weeks 4, 8 and 12 after the application of intrusion force. The lateral cephalometric radiograph and periapical radiographs were made before and twelve weeks after intrusion force application. The lateral cephalometric radiograph was made with the Frankfort horizontal plane parallel to the floor, and with the teeth in centric occlusion, using the same cephalostat for each patient with standardized settings.

#### **Protocol for miniscrew implant placement**

The miniscrew implant was placed in a pre-drilled hole in the midpalatal area, corresponding to the maxillary first molar position, under local anesthesia. The patient then rinsed the mouth with 0.02% chlorhexidine mouthwash. The miniscrew implant drilling was performed under saline cooling. The miniscrew implant was monitored for two weeks before force application. Two Sentalloy closed coil springs (100 g) were connected between the miniscrew implant head and the soldering hooks on the transpalatal arch, which was attached to the first molar band, by a modified Suzuki Reef Knot to create the intrusion force.



**Figure 3.5** Miniscrew implant placement procedure. Predrilling (a), miniscrew implant insertion(b), after miniscrew implant placement(c).



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**Figure 3.6** Procedures for connecting two Sentalloy closed coil springs to miniscrew implant head. Adapted from Suzuki Reef Knot; Dental floss inserted into elastomeric ligature (a), two sentalloy closed coil springs held together with a Mathieu needle holder (b), dental floss passed through the aperture of the Sentalloy closed coil spring (c), the end of the dental floss inserted through the aperture of the elastomeric ligature (d), the dental floss pulled (e), elastomeric ligature passing through the hole of Sentalloy closed coil spring (f), elastomeric ligature placement on the miniscrew implant head (g), dental floss removal by stretching of one end of the dental floss (h), two Sentalloy closed coil springs connected to miniscrew implant head (i), intra-oral picture during application of intrusion forces (j).

#### **A competitive Inhibition ELISA with WF6 mAb**

- The ELISA method was performed as follows:

1. The microtiter plates (maxisorp®, Nunc) were coated overnight at room temperature with 10 µg/ml shark PG-A1 fraction (100µl/well) in the coating buffer.
2. The plates were rinsed three times with PBS-tween, 150 µl/well, and dried.
3. Bovine serum albumin (BSA) 1% (w/v) 150 µl/well was added to all plates in the incubating buffer for 60 minutes at 37°C to block non-specific adsorption of other proteins to the plate. After washing, 100 µl of the mixture, sample or standard competitor (Shark PG-A1D1 fraction: range 39.06-10,000 ng/ml) in mAb WF6 (1:100), were added.
4. After incubation for 60 minutes at 37°C, the plates were washed and then the IgM-specific peroxidase conjugated anti-mouse immunoglobulin was added (100



µl/well; 1:2,000) and incubated for 60 minutes at 37°C. Then the plates were washed again.

5. The peroxidase substrate (100 µl/well) was added and incubated at 37°C for 20 minutes to allow the color to develop.

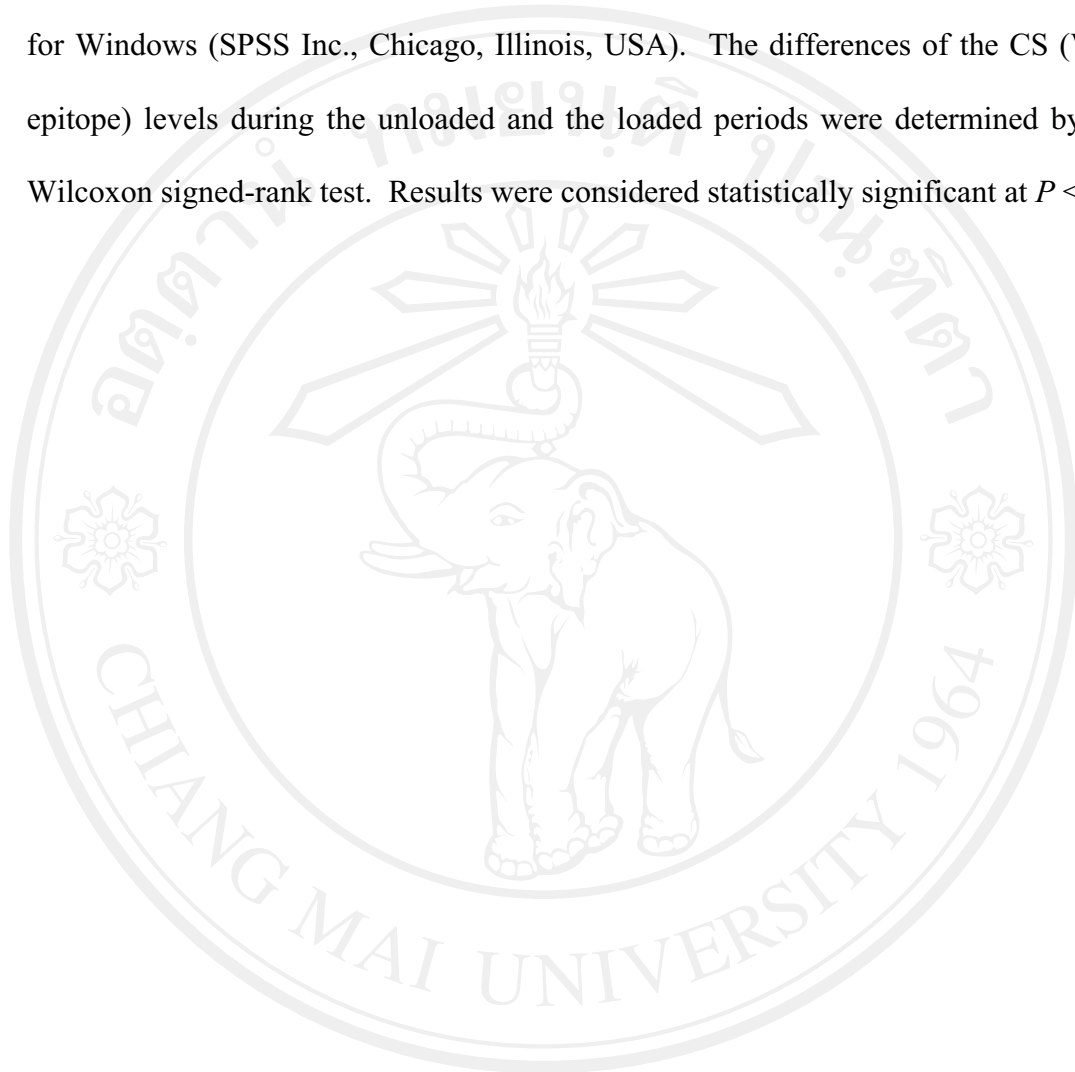
6. The reaction was stopped by the addition of 50 µl of 4M H<sub>2</sub>SO<sub>4</sub>. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, CA, USA).

### **Protein assay**

Total protein concentration was determined by using the Bio-Rad protein assay, based on the Bradford dye-binding procedure. It is a simple colorimetric assay for measuring total protein concentration. The Bio-Rad protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye primarily binds to 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 deionized distilled water were mixed together (1:4) and added to each well (200 µl/well). The plates were incubated at room temperature for five minutes and the absorbance was measured at 620 nm. Protein concentrations were determined from a standard curve.

**Statistical analysis**

Data were analyzed using the Statistical Package for Social Sciences version 17 for Windows (SPSS Inc., Chicago, Illinois, USA). The differences of the CS (WF6 epitope) levels during the unloaded and the loaded periods were determined by the Wilcoxon signed-rank test. Results were considered statistically significant at  $P < .05$



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