CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Subjects and general selection criteria

A total of ten orthodontic patients were included in this study. The patients met the following criteria:

- Good general health
- Lack of antibiotic therapy during previous 6 months
- Absence of anti-inflammatory drug administration in the month preceding the study
- Healthy periodontal tissue and no radiographic evidence of periodontal bone loss
- Requirements of four premolar extractions, distal canine movement and maximum anchorage control.

3.1.2 Orthodontic appliances

- Pre-adjusted brackets (Roth prescription) 0.018 x 0.025 inch slot (3M, Unitek)
- 0.016 inch nickel titanium wire (Sentalloy, Tomy, Tokyo, Japan)
- 0.016 x 0.022 inch nickel titanium wire (L&H Titan, Tomy, Tokyo, Japan)
- Twenty miniscrew implants (8.0 mm in length, 1.6 mm in diameter), (SIN, São Paulo, Brazil)
- 50g closed-coil spring (Sentalloy, Tomy, Tokyo, Japan)
- Bonding adhesive

3.1.3 PMICF sample collection instruments

- 1.5 ml Eppendorf tube
- Scissors
- 10.0x1.0 mm filter paper strip (Whatman filter paper No.1)

3.1.4 Chemical reagents and supplies for ELISA technique

- Microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark)
- Blue, yellow tips
- 1.5 ml Eppendrof tubes
- Multichannel pipete
- Auto pipette
- Tray
- Shaker, vortex
- IgM-specific peroxidase conjugated anti-mouse immunoglobulin
- Monoclonal antibody (mAb) WF6
- PBS-tween
- 1% w/v BSA
- Peroxidase substrate
- 4M H_2SO_4

3.1.5 Informed consent

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

3.2 Methods

Experimental design

This experiment consisted of two parts as follows:

Part I: Five-week period

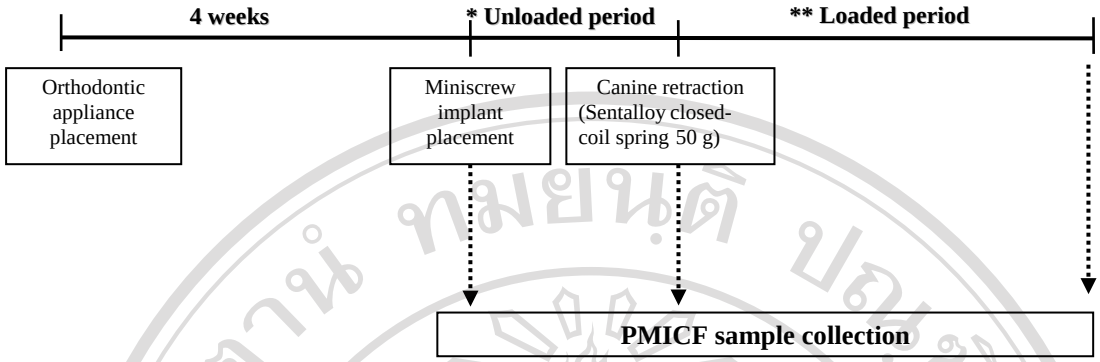
Part I of the experiment was directed to investigate CS epitope (WF6 epitope) levels in PMICF samples. This part was divided into the unloaded (one week) and the loaded (four weeks) periods. Six miniscrew implants were placed buccally, between the roots of the maxillary second premolar and of the first permanent molar. Four miniscrew implants were placed buccal and mesial to the root of the maxillary second premolar.

During the unloaded period, one PMICF sample for each miniscrew implant was collected by using a 10.0x1.0 mm Whatman No.1 filter paper strip on days 1, 3, 5 and 7. On day 7, after PMICF sample collection, a 50g closed-coil spring (Sentalloy, Tomy, Tokyo, Japan) was used to connect the miniscrew implant head and the canine bracket in order to move the maxillary canine distally. During the loaded period, the PMICF sample for each miniscrew implant was collected on days 14, 21, 28, and 35 (Figure 3.1).

Part II: Ten-week period

This part was also divided into the unloaded (one week) and the loaded (nine weeks) period. Ten miniscrew implants were placed buccally, between the roots of the maxillary second premolar and of the first permanent molar.

During the unloaded period, the PMICF sample for each miniscrew implant was collected on days 1, 3, 5 and 7. On day 7, after the PMICF sample collection, a (50g) closed-coil spring (Sentalloy, Tomy, Tokyo, Japan) was used to connect the miniscrew implant head and the canine bracket in order to move the maxillary canine distally. During the loaded period, one PMICF sample for each miniscrew implant was collected on days 14, 21, 28, 35, 42, 49, 56, 63, and 70 (Figure 3.1).



*** Unloaded period:** sample collection on days 1, 3, 5, 7 after miniscrew implant placement.

**** Loaded period:** sample collection on days 14, 21, 28, and 35 (Part I), or on days14, 21, 28, 35, 42, 49, 56, 63, and 70 (Part II).

Figure 3.1 Diagram of the experimental design

The experimental procedures of both parts were similar. These are described in detail as follows:

3.2.1 Leveling period of orthodontic treatment

All patients underwent a standard pretreatment examination, including extra-oral and intra-oral photographs, dental model analysis, radiographs and cephalometric analysis. The orthodontic treatment plans required four premolar extractions and distal canine movement. Miniscrew implants were used as orthodontic anchorages. Pre-adjusted brackets (Roth prescription) 0.018 x 0.025 inch slot size (3M, Unitek), were fixed on all teeth. A 0.016 inch nickel titanium wire (Sentalloy, Tomy, Tokyo, Japan) was used for four weeks to align all teeth.

3.2.2 Miniscrew implant placement procedure

The miniscrew implants were placed into attached gingiva, buccally and bilaterally, in the alveolar bone between the upper first and second premolars or between the second premolar and first molar. After local anesthesia was applied, patients rinsed their mouths with 0.02 % chlorhexidine mouthwash. Surgical guides were tightened to a main arch wire, and were used to safely and precisely drill the pre-

drilled hole, as well as to place the miniscrew implants.[103] Dental bitewing radiographs were made to assess the precision of the surgical guide positions, both before and after miniscrew implant placement. The hole drilling and miniscrew implant placement were performed under saline cooling. The miniscrew implants were slowly and softly driven into the pre-drilled hole with a manual screwdriver. The heads of the miniscrew implants, in the horizontal dimension, were in contact with the attached gingiva (Figure 3. 2). In the vertical dimension they were coronal to the free gingival junction.

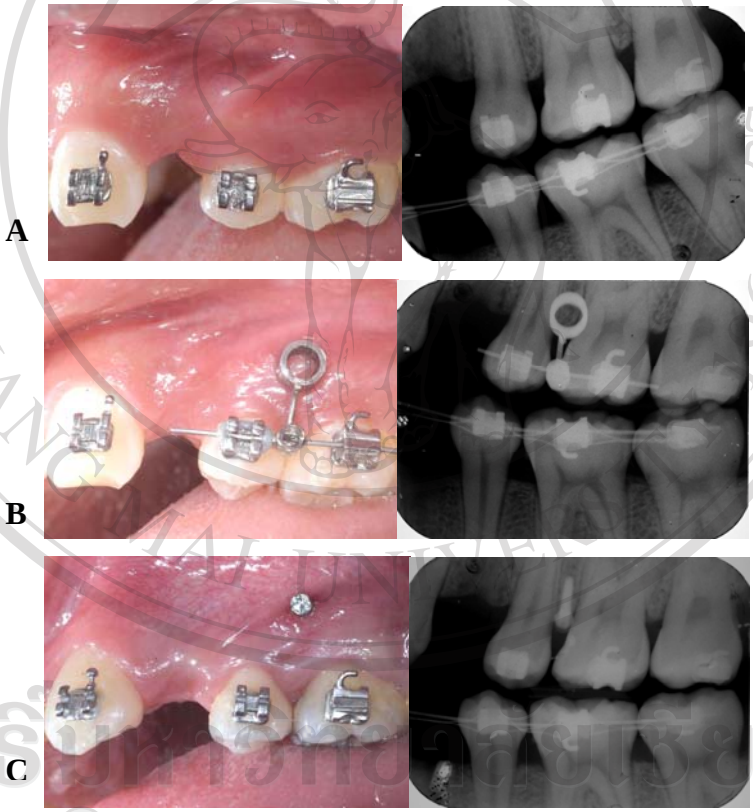


Figure 3.2 Miniscrew implant placement procedure: (A) Miniscrew placement site between second premolar and first molar, (B) placement of surgical guide, (C) Position of miniscrew implant head, lying on attached gingiva

3.2.3 Orthodontic force application

After placement, the miniscrew implant was monitored for one week in order to allow adequate wound healing. A 0.016 x 0.022 inch nickel titanium wire (Tomy L&H Titan Tomy, Tokyo, Japan) was engaged before distal movement of the canines was begun. The 50g closed-coil spring (Sentalloy, Tomy, Tokyo, Japan) was used to connect the miniscrew implant heads and the canine brackets in order to create orthodontic forces (Figure 3.3).

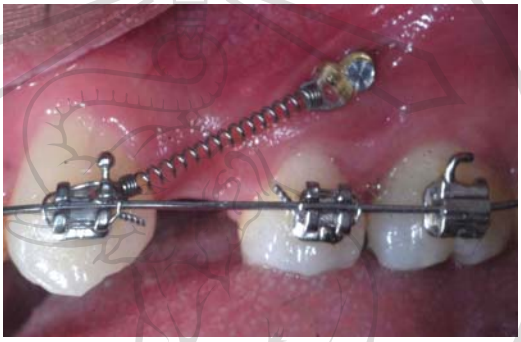


Figure 3.3 Sentalloy closed coil spring (50 g) was used to connect the miniscrew implant head and the canine bracket

3.2.4 Peri-miniscrew implant crevicular fluid (PMICF) collection

Before collecting the PMICF sample, the Sentaloy® closed coil spring was removed. The miniscrew implant placement site was isolated from saliva and gently air dried. Whatman® No.1 filter paper strip (10.0x1.0 mm.) was inserted into the peri-miniscrew implant sulcus, and left in place for 3 minutes. Care was taken to avoid mechanical injury. Samples containing blood were discarded. The last 2.0 mm of filter paper strip containing the PMICF sample was cut off, and put in a 1.5 ml Eppendorf tube. The tube was labeled and stored at minus 80^0C until the immunoassay was performed (Figure 3.4).

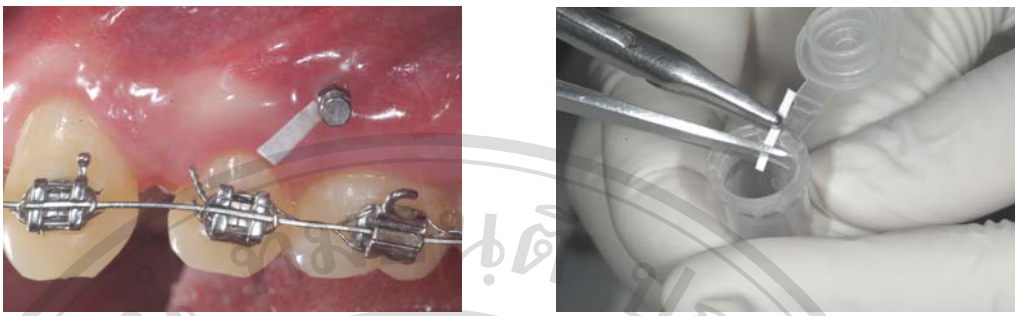


Figure 3.4 Peri-miniscrew implant crevicular fluid (PMICF) collection

3.2.5 Clinical mobility assessment of the miniscrew implant

After collecting the PMICF sample, a clinical mobility assessment of the miniscrew implants was recorded. The mobility of each miniscrew implant was assessed using cotton forceps.[22] Extremely light force was laterally applied to the miniscrew implant head (Figure 3.5). Mobility was assessed either as 'yes' (mobile), or 'no' (not mobile). If there was any discernible mobility, the miniscrew implant was categorized as mobile. The miniscrew implants that maintained in the bone until the end of study period, or until intentional removal (regardless of mobility), were considered to be successful. Any miniscrew implants that were loose and could not serve as anchorages during the study period were considered as failures.

Figure 3.5 Clinical mobility assessment of the miniscrew implant

3.2.6 Competitive inhibition ELISA with mAb WF6

A quantitative ELISA was modified from a previous study[101] for the epitopes recognized by mAb WF6. The standard agent used was shark cartilage aggrecan (Shark PG A_1D_1 fraction: range 39.06-10,000 ng/ml) at a concentration of 10 µl/ml. The coating antigen was Shark PG A_1D_1 and the competitor agent was shark PG-A1. The primary antibody was mAb WF6 and the secondary antibody was IgM-specific anti-mouse immunoglobulin with peroxidase.

A competitive inhibition ELISA with mAb WF6 was performed as follows:

1. Microtiter plates (Maxisorp®, Nunc) were coated overnight at room temperature with 10 µg/ml shark PG-A1 fraction (100 µl/well) in the coating agent.

2. The following morning, the plates were washed three times with PBS-tween (150 µl/well) and left to airdry.

3. The uncoated area was then blocked with 150 µl/well of 1% (w/v) bovine serum albumin (BSA) in the incubating buffer for 60 min at 37°C. After washing, 100 µl of the mixture, PMICF sample or standard competitor (Shark PG-A1D1 fraction: range 39.06-10,000 ng/ml) in mAb WF6 (1:100), was added.

4. After incubation for 60 min at 37°C, the plates were washed and then the IgM-specific peroxidase conjugated anti-mouse immunoglobulin (100 µl/well; 1:2,000) was added and incubated for 60 min at 37°C.

5. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 20 min to allow the color to develop.

6. The reaction was stopped by addition of 50 µl of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader (ICN, Flow, USA)

3.2.7 Statistical Methods

The data were analyzed using the statistical SPSS v13 for windows. The Komolgorov-Smirnov one-sample test was used to test the distribution of CS epitope (WF6 epitope) levels from Part I and Part II of the experiment. In each part, the differences between the CS epitope (WF6 epitope) levels of unloaded and loaded periods were determined by the Mann-Whitney U–test. Results were considered statistically significant at $P < .05$.