CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

1) Orthodontic appliances (Figure 1)

- Orthodontic pre-adjusted brackets (Roth prescription 0.018 x 0.025 inch bracket slots) (3M Unitek Inc., Monrovia, California, USA)
- Orthodontic bands (0.018 x 0.025 inch bracket slots)
- Orthodontic 0.016 x 0.016 inch stainless steel wires
- Transpalatal arches (0.9 stainless steel wire) (Figure 1a)
- Stainless steel ligature wires
- Banding cements
- Adhesive bonding
- 32 miniscrew implants (2.0 mm diameter, 10.0 mm length) and a driver (Bio-Ray, Syntec Scientific corporation, Chang Hua, Taiwan) (Figure 1b, 1c)
- Sentalloy® closed coil springs (Tomy, Tokyo, Japan): extra light force, light force (Figure 1d, 1e)
- Bracket positioning devices
- Force strain gauges (Figure 1f)

2) Sample collection instruments

- 1.5 ml Microcentrifuge tubes (Figure 1g)

- Gingival fluid collection strips or Periopaper® (Oraflow Inc., Smithtown, New York, New York, USA) (Figure 1h)

3) Chemical reagents and supplies for ELISA technique (Competitive Inhibition ELISA with WF6 mAb)
   - Microtiter plates (Maxisorp®, Nunc, Roskilde, Denmark)
   - Blue, yellow tips
   - Auto pipettes
   - Trays
   - Shaker, vortex
   - IgM-specific peroxidase conjugated anti-mouse immunoglobin
   - WF6 mAb
   - Tris-IB
   - 1% (w/v) Bovine serum albumin (BSA)
   - Peroxidase substrate
   - 4M H_2SO_4
4) Study casts
   - Initial study casts
   - After canine movement 4, 8 and 12 weeks
5) ABSOLUTE digimatic caliper (Mitutoyo Corporation, Kawasaki, Japan) (Figure 1i)
6) Informed consent

The experiments were approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. Before the collection of GCF samples,

the patients were informed of the experimental procedures. Then, informed consent was obtained from all patients.

(a)

(b)

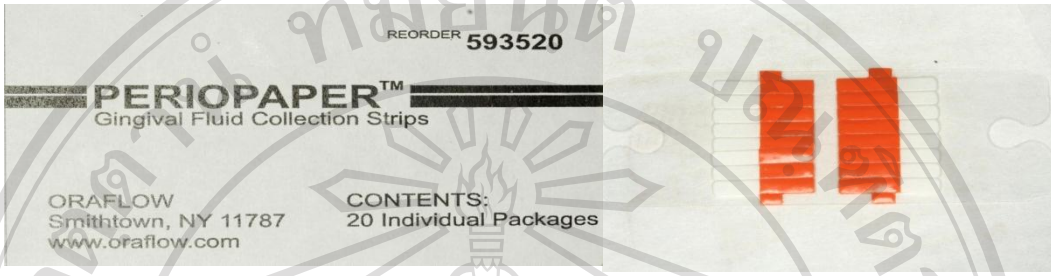
(c)

(d)

(e)

(g)

(f)



(h)

(i)

Figure 1 Orthodontic appliances and sample collection instruments; Transpalatal arch (a), Miniscrew implants (b), Miniscrew driver (c), Sentalloy® closed coil springs: extra light force (d), light force (e), Force strain gauges (f), Microcentrifuge tube (g), Gingival fluid collection strips (Periopaper®) (h) and Digital caliper (i)

3.2 Methods

Thirty-two maxillary canines (in sixteen adult patients) undergoing distal movement were used as the experimental teeth. Thirty-two mandibular canines not

needing orthodontic tooth movement were used as the control teeth. The patients met the following criteria: (1) good general and oral health with a healthy periodontium, no radiograghic evidence of bone loss, no gingival inflammation, and a probing depth of 3 mm or less at all teeth; (2) lack of antibiotic therapy during the previous six months; (3) absence of anti-inflammatory drug administration in the month preceding the study; (4) no pregnancy (women); and (5) Class I malocclusion that required orthodontic treatment with first premolar extractions and canine retraction. All patients received repeated oral hygiene instruction and the gingival health was controlled throughout the study. Thirty-two miniscrew implants (2.0 mm diameter, 10.0 mm length) were placed, two in each patients, buccally and bilaterally into the infrazygomatic bone between the maxillary first permanent molar and the maxillary second molar teeth. Transpalatal arches were also placed in all patients to secure the anchorage in case of miniscrew failure.

Experimental design

The experimental design was divided in two phases (**Figure 2**).

Phase I: The unloaded period

After general status assessment and informed consent, GCF samples around maxillary left and right canines were collected as baseline data, and those from mandibular canines as controls. The teeth were gently washed, and isolated with a cotton roll. Then supragingival plaque was removed without touching the marginal gingiva, and the crevicular site was then gently dried with an air syringe. GCF sample was collected with Periopaper® strips (1 mm wide) placed into the distal gingival sulcus of the canine until light resistance was felt, and left in the sulcus for

30 seconds. Care was taken to avoid mechanical injury to periodontal tissue. Strips contaminated with blood were discarded. Immediately after collection, the strips, wet with GCF, were cut two millimeters from their tips and individually transferred to microcentrifuge tubes. All strips were stored at -80ºC until further processing. The GCF was recovered from the strips by addition of 200 µl of phosphate-buffered saline, pH 7.4, and the tube was then vigorously shaken for a few minutes. The recovery rate (approximately 104.07%) from each strip was determined by a dye-binding assay, using known concentrations of sulphated GAGs as standards.

Maxillary first permanent molar teeth were separated and the patients were referred for extractions of bilateral maxillary first premolars. In the week following separation, orthodontic bands were fitted, and impressions were taken for fabrication of transpalatal arches. The left-to-right maxillary first permanent molar transpalatal arch (0.9 stainless steel wire) was fixed for stabilizing anchorage. Two miniscrew implants were placed in each patient, buccally and bilaterally into the infrazygomatic bone between the maxillary first permanent molar and second molar. Orthodontic pre-adjusted brackets (Roth prescription 0.018 x 0.025 inch bracket slots) were bonded on all teeth.

Phase II: The loaded period

After miniscrew implant placement for two weeks, impressions were taken for fabrication of study casts. The canines were moved by Sentalloy® closed coil springs on 0.016 x 0.016 inch stainless steel wires. The Sentalloy® closed coil springs were connected to the miniscrew implant heads and to the canine brackets in order to move the maxillary canine distally. The orthodontic force magnitudes were controlled at 70 and 120 grams on the right and the left sides, respectively (**Figure 3**).

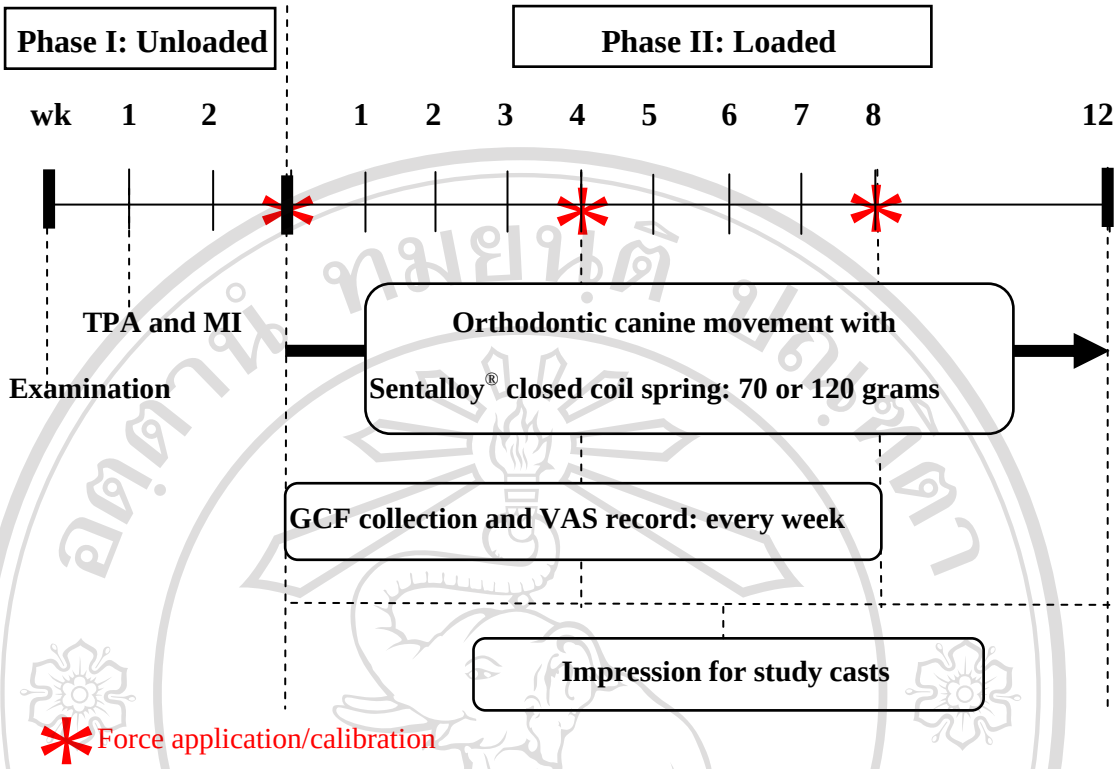


Figure 2 Diagram of the experimental design. (TPA = Transpalatal arch, MI = Miniscrew Implant, GCF = Gingival crevicular fluid, VAS = Visual Analog Scale)

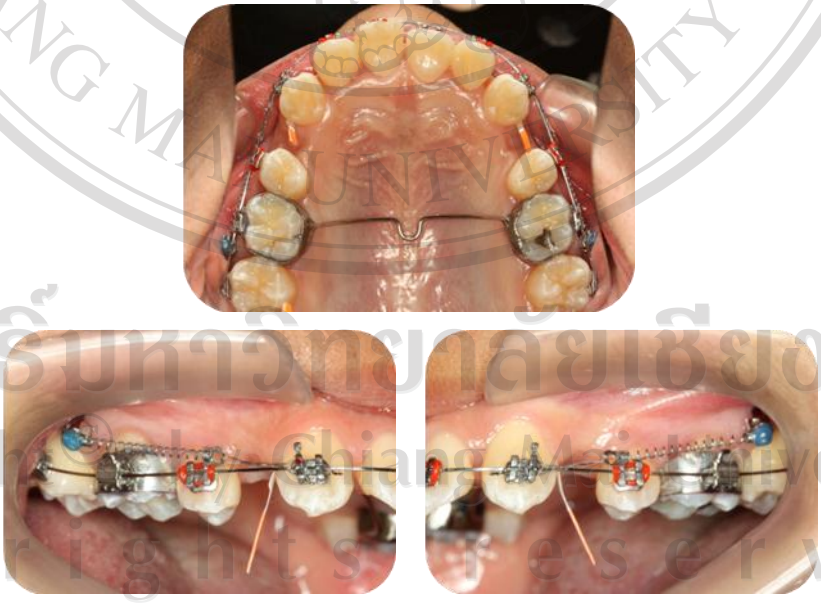


Figure 3 The right and the left experimental maxillary canines were controlled at 70 and 120 grams retraction force, respectively. Periopaper® strips were placed into the distal gingival sulcus of the canines for GCF collection.

The GCF samples were collected from patients every week for eight consecutive weeks. The CS (WF6 epitope) levels were analyzed by competitive ELISA with WF6 monoclonal antibody. The study casts were made after orthodontic canine movement every four weeks until the 12th week. The distance of maxillary canine movement was measured by using an ABSOLUTE digimatic caliper and the rate of canine movement was calculated.

The VAS was used to evaluate the patient's sensation of pain during orthodontic canine retraction. The patients reported their pain experience, separately for each force magnitude, using the VAS, every week (1st to 8th week) for eight consecutive weeks. **Figure 4** shows the linear scale properties which ranged from 0 (Absence of pain) to 10 (Worst possible or unbearable pain). From now on, the VAS scores in this study represented the amount of pain that the patients felt.

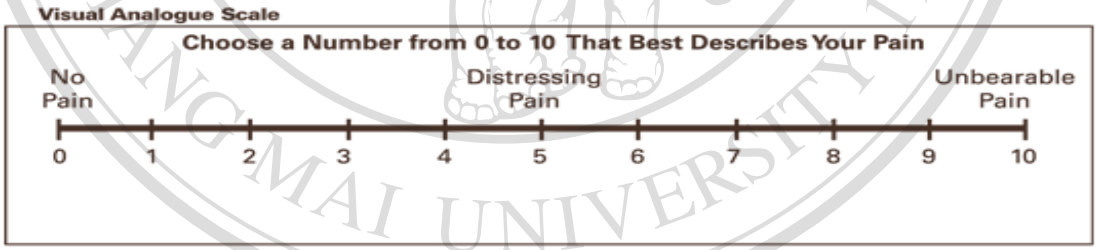


Figure 4 Visual Analogue Scales (VAS).

Protocol for miniscrew implant placement

The miniscrew implants were placed into the infrazygomatic bone between the maxillary first permanent molar and second molar under local anesthesia; after that, the patients rinsed with 0.02% chlorhexidine mouthwash. The miniscrew implant drilling was performed under saline cooling. The miniscrew implants were monitored for two weeks before force application.

Competitive Inhibition ELISA with WF6 mAb

Microtiter plates were coated overnight at room temperature with 10 µg/ml shark PG-A_1 fraction (100µl/well) in a coating buffer (20 mM sodium carbonate buffer, pH 9.6). The following morning, the plates were washed three times with Tris-IB 150 µl/well and dried. Bovine serum albumin (BSA) 1% (w/v) 150 µl/well in an incubating buffer (Tris-IB), was added to all plates. The plates were incubated at 37˚C for 60 minutes to block non-specific adsorption of other proteins to the plates, and washed once again. After this washing, 100 µl/well of the mixture, sample or standard competitor (Shark PG-A_1D_1 fraction: range 39.06-10,000 ng/ml) in mAb WF6 (1:100), were added. After a second incubation at 37˚C for 60 minutes, the plates were washed once more and IgM-specific peroxidase conjugated anti-mouse immunoglobulin (100 µl/well; 1:2,000) was added and incubated a third time at 37˚C for 60 minutes. Then, the plates were washed again. After that, the peroxidase substrate (100 µl/well) was added and incubated at 37˚C for 20 minutes to allow the color to develop. The reactions were stopped by the addition of 50 µl/well of 4M H_2SO_4. Eventually, the absorbance ratio at 492:690 nm was measured using a Titertek Multiskan® MCC/340 multiplate reader (ICN/Flow Laboratories, Costa Mesa, California, USA).

Protein assay

Total protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad office, USA), based on the Bradford dye-binding procedure,[88] a simple colorimetric assay for measuring total protein concentration. The Bio-Rad protein assay was based on the color change of Coomassie Brilliant Blue G-250 dye

in response to various concentrations of protein. The dye binds to primarily basic (especially arginine), and to aromatic, amino acid residues. The BSA standards (0-1,000 µl/well) and samples were added to the microtiter plates (10 µl/well) in triplicate. A dye reagent and de-ionized 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

The data were analyzed using the Statistical Package for Social Sciences version 17 for Windows (SPSS Inc., Chicago, Illinois, USA). The Kolmogorov-Smirnov test was used to determine the distribution of CS (WF6 epitope) levels, rate of canine movement and VAS score. The differences between the CS (WF6 epitope) levels, during the unloaded and the loaded periods were determined by the Wilcoxon signed-rank test; the differences between the levels of force magnitude (70 and 120 grams) during each experimental period were determined by the Mann-Whitney U-test. The differences between the rates of canine movement with 70 and that with 120 grams of orthodontic force were determined by the Independent-T-test. The differences in VAS scores between the levels of force magnitude (70 and 120 grams) during each experimental period were determined by the Mann-Whitney U-test. The results were considered statistically significant at $P<0.05$