CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

1) Subjects and general selection criteria

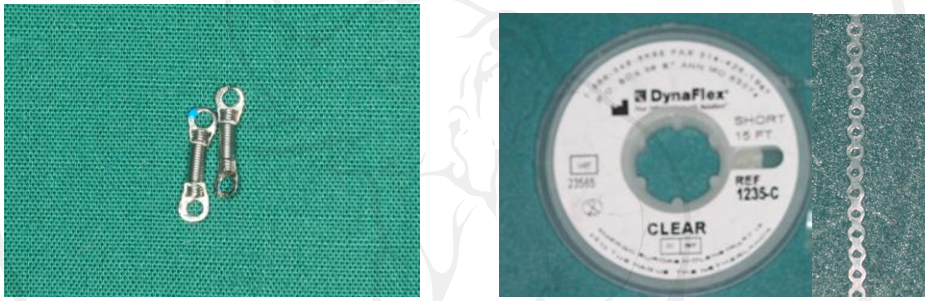
A total fifteen orthodontic patients with Class I malocclusion requiring first premolar extractions and distal canine movement were included in the study. The patients met the following criteria:

- 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.
- Lack of antibiotic therapy during the previous six months
- Absence of anti-inflammatory drug administration in the month prior to the study
- No pregnancy (women)

2) Orthodontic appliances

- Pre-adjusted edgewise appliances (Roth's prescription) with 0.018 x 0.025 inch slots.

- The main archwires : 0.016 x 0.016 inch stainless steel plain
- Sentalloy® closed coil springs (100 gm) (GAC, Central Islip, NY, USA) (Figure 3.1a)
- Dynaflex® elastomeric chains short type (Dynaflex company, St. Louis, MO, USA) (Figure 3.1b)

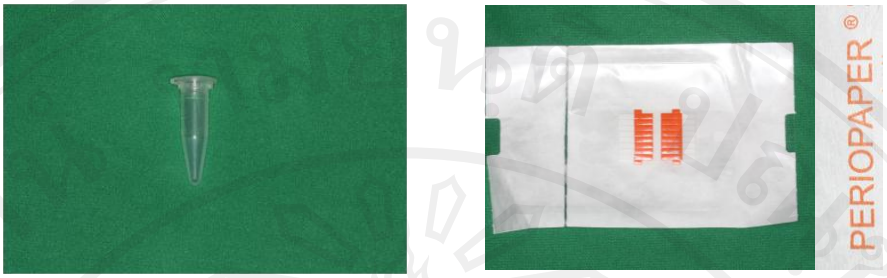


(a) (b)

Figure 3.1 Orthodontic appliances (force-generating devices): Sentalloy® closed coil springs (100 gm) (a) and Dynaflex® elastomeric chains, short type (b).

3) Sample collection instruments

- 1.5 ml Eppendorf tube (Figure 3.2a)
- Scissors
- Periopaper strip (ORAFLOW, NY, USA) (Figure 3.2b)



(a) (b)

Figure 3.2 Sample collection instruments: 1.5 ml Eppendorf tube (a) and Periopaper® strip (b)**.**

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 immunoglobin
- WF6 mAb PBS-tween
- 1%w/v BSA
- Peroxidase substrate
- 4M H2SO4

5) Study models

- Initial study model
- Progressive study models after applied retraction force at the end of the 4th, 8th and 12nd weeks during the loaded period.

6) Bracket Positioning Device

7) Push and pull force gauge (Figure 3.3)

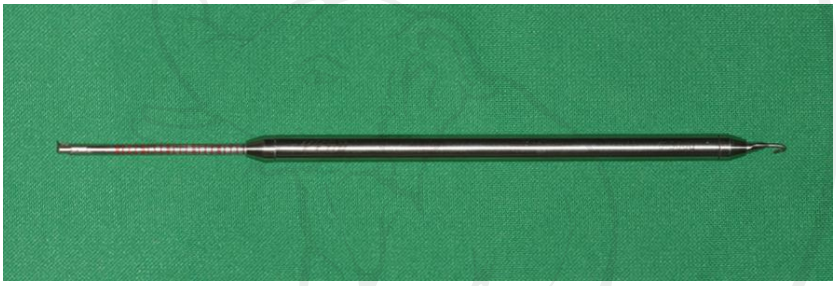


Figure 3.3 Push and pull force gauge.

3.2 Methods

1) Inform consent

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University. Informed consent was obtained from all patients or from their parents if under 18 years old before the gingival crevicular fluid collection.

2) Experimental design

The diagram of this experimental design is shown in Figure 3.4. The experiment was divided into two periods;

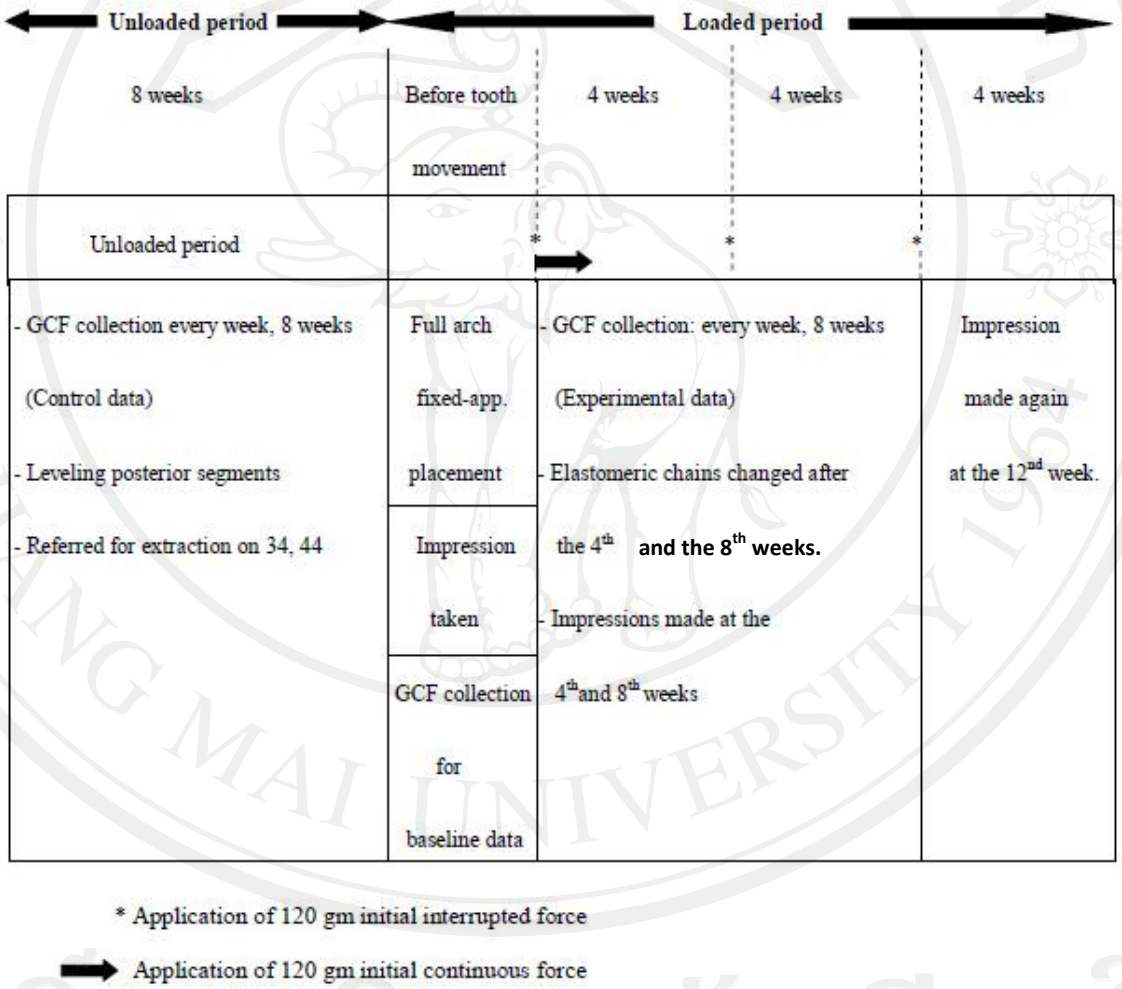


Figure 3.4 Experimental diagram.

Period I: Unloaded period

Gingival crevicular fluid from the distal sulcus of bilateral mandibular canines was collected at the end of each week during the 8-week unloaded period. One to two months before mandibular canine retraction, fixed orthodontic appliances were placed bilaterally in the posterior segment of the mandibular dental arch (mandibular second premolars, first molars, and second molars) to level the teeth, using 0.016-inch Nickel-Titanium alloy sectional arch-wires. The patients were referred for extraction of bilateral mandibular first premolars after GCF collection at the end of the 8th week of this period.

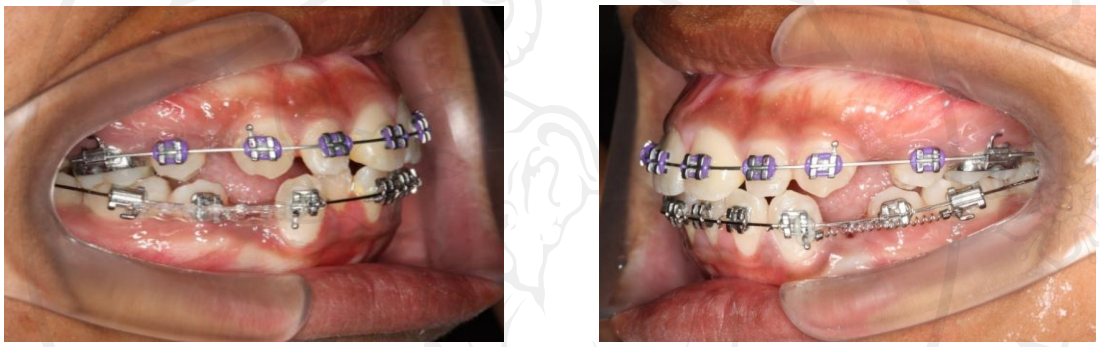
Period II: Loaded period

Before mandibular canine movement

Impressions were made to fabricate study models before mandibular canine retraction. The fixed orthodontic appliances were fixed on all remaining teeth in the mandibular arch (the brackets were placed on central and lateral incisors on both sides in the ideal position but the brackets were placed on canines on both sides in passive positions). The main arch-wire was placed around and ligated to the brackets with ligature wires. The gingival crevicular fluid was collected from the distal sulcus of the mandibular canines before applying the force.

During mandibular canine movement

The retraction force was applied with elastomeric chains calibrated at 120 gm initial force from the right molars to the right canine (Figure 3.5a) and with Nickel-Titanium closed coil springs (100 gm), stretched to provide 120 gm initial force, from the left molars to the left canine (Figure 3.5b), respectively.

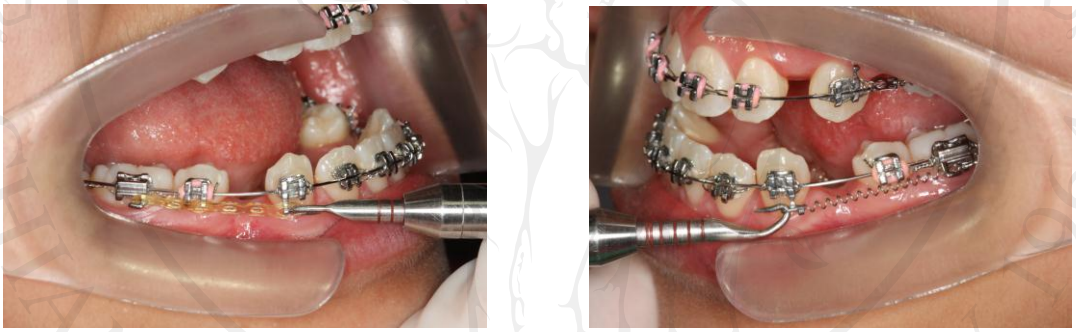


(a) (b)

Figure 3.5 An elastomeric chain calibrated at 120 gm initial force was attached between the hook of the right mandibular first molar bracket and that of the right canine bracket (a). A Nickel-Titanium closed coil spring calibrated at 120 gm initial force was attached between the hook of the left mandibular first molar bracket and that of the left canine bracket (b).

Both types of force-generating devices were calibrated before use with a push and pull force gauge. GCF of retracted mandibular canines was collected every week for eight weeks. At the end of the 1st to the 8th weeks, force generated by elastomeric chains was measured (Figure 3.6a). At the end of the 4th and the 8th weeks, the elastomeric chains were replaced new ones, calibrated

for an initial force of 120 gm. Force generated by Nickel-Titanium closed coil springs was measured every week (Figure 3.6b) and recalibrated for an initial force of 120 gm. Impressions for progressive models were made at the end of the 4th, 8th and 12th weeks of this period. Patients' pain and discomfort during orthodontic canine movement were evaluated using visual analog scales (VAS), with a score range of 0 to 10. This assessment was performed at the end of the 1st and 5th weeks during canine movement.



(a) (b)

Figure 3.6 Measuring the force generated by an elastomeric chain at the end of the 1st to the 8th weeks during the loaded period (a). Measuring the force generated by a Nickel-Titanium closed coil spring at the end of the 1st to the 8th weeks during the loaded period (b). A push and pull force gauge was used to measure the force generated by these force-generating devices.

GCF collection

GCF was collected from the distal sulcus of the experimental teeth at the end of every week during the 8-week unloaded period for control data, before mandibular canine retraction (the beginning of the 1st week of the loaded period) for baseline data, and at the end of every week during the 8-week loaded period for experimental data. Briefly, experimental teeth were gently washed, gently air-dried and then isolated by cotton roll. GCF was collected by Periopaper® strip, placed in the sulcus until light resistance was felt (Figure 3.7). Strips contaminated with blood were discarded. The last 2.0 mm of each Periopaper® strip containing the GCF sample was cut off and individually frozen at -80°C in a microcentrifuge tube for further analysis.

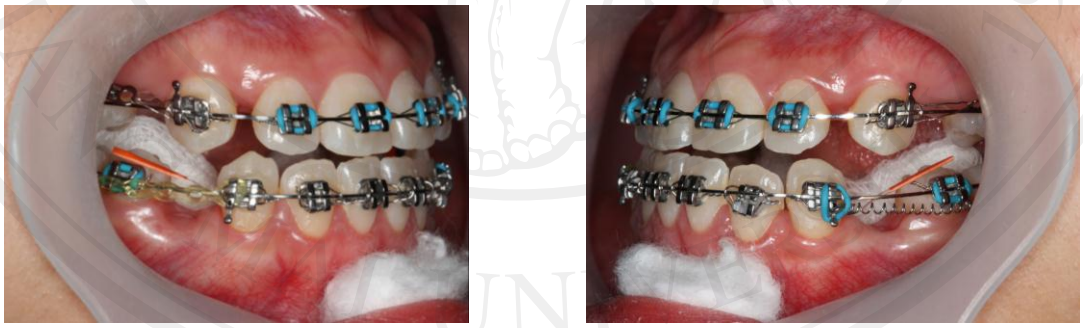


Figure 3.7 GCF collection from experimental mandibular canines on both sides during the loaded period.

Rate of mandibular canine movement

The rate of experimental mandibular canine movement was derived from the entire distance of canine movement per unit of time (mm/month). The method for calculating the rate was modified from that reported by Dixon *et al.* (2002). Distances between the cusp tips of the experimental canines and the buccal grooves of the second or the first permanent molars in each quadrant, from the initial study models and from the progressive models at the end of the 12th week during the loaded period, were used for calculating the entire distance of mandibular canine movement (Figure 3.8).

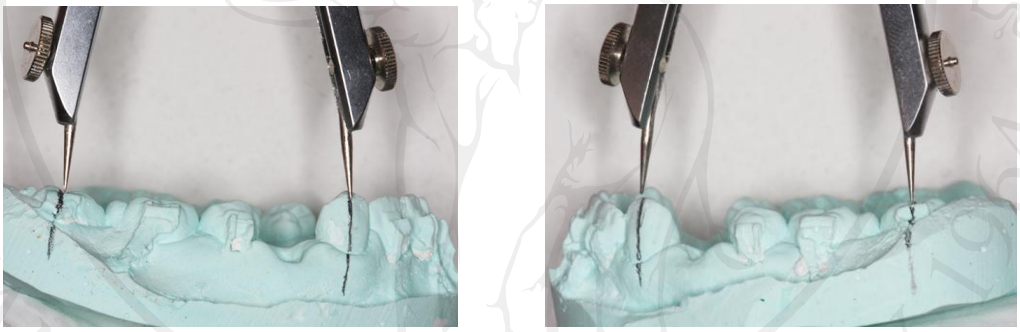


Figure 3.8 Measuring the distance between both reference points.

Competitive Inhibition ELISA with WF6 mAb

The ELISA method was performed as follows:

The microtiter plates were coated at room temperature with 10µg/ml shark PG-A1 fraction (100µl/well) in a coating buffer (20 mM sodium carbonate buffer, pH 9.6). The plates were rinsed three times with Tris-IB buffer, 150 µl/well and dried. Bovine serum albumin (BSA) 1% (w/v), 150 µl/well in the incubating buffer (Tris-IB buffer), was

added to all wells 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- AlDl fraction: range 39.06-10,000 ng/ml) in mAb WF6 (1:100), were added. After incubation for 60 minutes 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 the plates were incubated for 60 minutes at 37˚C. Then the plates were washed again. The peroxidase substrate (100 µl/well) was added and the plates were incubated at 37˚C for 20 minutes to allow the color to develop. The reaction was stopped by the 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 (Flow Laboratories, McLean, VA, USA).

Protein assay

Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), based on the Bradford dye-binding procedure (Bradford *et al.*, 1976). This assay is a simple colorimetric assay for measuring total protein concentration. Bio-Rad's protein assay is 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 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 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

In all tests, the level of significance was pre-chosen at $P = 0.05$.

Medians of CS (WF6 epitope) levels by either a continuous or an interrupted force at each week during the 8-week unloaded period (control data) and the beginning of the 1st week during the loaded period (baseline data) were compared using Friedman's test.

Medians of CS (WF6 epitope) levels by either a continuous or an interrupted force at the beginning of the loaded period (baseline data) and during the 8-week loaded period (experimental data) were compared using the Wilcoxon signed ranks test.

During the loaded period, medians of CS (WF6 epitope) levels by either a continuous or an interrupted force at the beginning of the 1st week (baseline data) and of each successive week for eight weeks were compared using the Wilcoxon signed ranks test.

During the loaded period, the medians of CS (WF6 epitope) levels between a continuous and an interrupted force patterns at each week (8 weeks) were compared using the Mann-Withney U-test.

Mean rates of mandibular canine movement by either a continuous or an interrupted force pattern were compared using the Independent T-test.

Mean VAS scores of patients' pain and discomfort during mandibular canine movement at either the end of the 1st week or the end of the 5th week during the loaded period between a continuous and an interrupted force patterns were compared using the Independent T-test.