

CHAPTER III

MATERIALS AND METHODS

MATERIALS

The following materials were used in this study:

1) Samples

The samples in this study were 10 teeth (7 upper canines and 3 upper central incisors) from 4 adult orthodontic female patients in the Department of Orthodontics, Faculty of Dentistry, Chiang Mai University. The ages of these volunteers ranged from 20 to 21 years at the beginning of treatment.

1.1 The selection criteria for orthodontic patients were as follows:

- a. good general health
- b. lack of antibiotic therapy in the previous 6 months
- c. absence of anti-inflammatory drug administration in the month preceding the study
- d. healthy periodontal tissue and no radiographic evidence of periodontal bone loss
- e. requirement of first premolar extraction and canine movement as part of their orthodontic treatment plan
- f. and requirement of fixed appliances.

Before the study began, a signed consent form approved by an Ethical Committee of Faculty of Dentistry, Chiang Mai University was obtained from the volunteer. The volunteers participating in the study were fully informed about the nature of the whole study.

1.2 Experimental design

This study was designed as a longitudinal study. For each patient, the first premolar teeth distal to the experimental canine were extracted. The maxillary canines undergoing distal movement were used as the experimental teeth, and the maxillary central incisors were used as the control teeth.

Orthodontic direct bonded pre-adjusted brackets 0.018"x0.025" slot (3M[®] Unitex) edgewise technique were used. The canines were retracted with stainless steel closed coil springs on a 0.016"x0.016" rectangular wire. The magnitude of initial force was around 125-140 grams. For all patients, as part of their orthodontic treatment plan, anchorage of upper first molars was enhanced by a transpalatal arch.

1.3 Records of clinical parameters

The gingival health was controlled by a periodontist throughout the study. All patients received repeated oral hygiene instructions for the use of toothbrush, dental floss, and inter-dental brush. The clinical data, including gingival index (GI) and plaque index (PI), were assessed and recorded before orthodontic treatment, every 3 months during the treatment, and post-treatment, by the same periodontist every time.

Gingival Index (GI) and Plaque Index (PI) were scored at six sites per tooth as follows:

1. Disto-buccal site
2. Mid-buccal site
3. Mesio-buccal site
4. Disto-lingual site
5. Mid lingual site
6. Mesio-lingual site

The clinical measurements made during this study were:

1. Gingival index (GI) was assessed by using a method that was described by Loe & Silness (1967). The scores used to evaluate gingival soft tissue status were shown in Table 3.1.

Table 3.1 A score used to evaluate Gingival Index (GI).

Score	Gingival soft tissue status
0	Normal gingiva
1	Mild inflammation: slight change in color, slightly edema, no bleeding on probing
2	Moderate inflammation: redness, edema and glazing, bleeding on probing
3	Severe inflammation: marked redness and edema, ulceration, tendency to spontaneous bleeding

2. Plaque index (PI) was assessed by using a method that was described by Turesky-Glimore (1970) and modification of the Quigley & Hein (1962). The scores used to evaluate plaque that was revealed by a disclosing agent (basic fuchsin solution) were shown in Table 3.2.

Table 3.2 A score used to evaluate Plaque Index (PI).

Score	Plaque level
0	No plaque
1	Separate flecks of plaque at cervical margin of the tooth
2	A thin continuous band of plaque (up to 1 mm) at the cervical margin
3	A band of plaque wider than 1 mm but covering less than 1/3 of the crown of the tooth
4	Plaque covering at least 1/3 but less than 2/3 of the crown of the tooth
5	Plaque covering 2/3 or more of the crown of the tooth

2) Materials for GCF collection

- 2.1 1.5 ml microcentrifuge tubes (Eppendorf®)
- 2.2 2x10 mm filter paper strips (Whatman®)
- 2.3 Scissor
- 2.4 A deep freezer

3) Materials for the ELISA

Chemical reagents

3.1 0.1 M NaHCO₃ (pH 9.6) (coating buffer)

3.2 Phosphate buffer saline (PBS) 10X, 1l

- NaCl 80 gram
- NaH₂PO₄·H₂O 2.59 gram
- Na₂HPO₄·12H₂O 29 gram
- KCl 2 gram

3.3 PBS-Tween (pH 7.4)

- 500 ml of 1X PBS + 250 µl of Tween-20

3.4 1% BSA (bovine serum albumin)

- 0.6 gram BSA + 60 ml of 1X PBS

3.5 Citric phosphate buffer (pH 5.0) * OPD soluble 500 ml

- Citric acid monohydrate (C₆H₈O₇·H₂O) 10.30 gram
- Na₂HPO₄·3H₂O 18.16 gram

3.6 0.05 M Tris phosphate buffer (pH 8.6) * B-HABP soluble

- Tris-HCl 6.0550 gram (3.0275 gram) + (250 µl Tween-20)

3.7 B-HABP (biotinylated-HA binding protein)

3.8 Anti-Biotin conjugated with peroxidase

- Anti-Biotin 2.5 µl + PBS 10 ml (dilution 1 : 4,000)
- Anti-Biotin 5.0 µl + PBS 10 ml (dilution 1 : 2,000)
- Anti-Biotin 10 µl + PBS 10 ml (dilution 1 : 1,000)

3.9 Substrate

- OPD 0.0080 – 0.0100 gram
- Citrate phosphate buffer 12 ml
- 30% H₂O₂ 7 µl

3.10 4 M H₂SO₄ solution

- Concentrated H₂SO₄ 23 ml
- Sterile water 77 ml

4) Supplies

- 4.1 A microtiter plate (Maxisorp®, Nunc)
- 4.2 Blue and yellow tips
- 4.3 1.5ml microcentrifuge tubes (Eppendorf®)
- 4.4 A multichannel pipette
- 4.5 Auto pipettes
- 4.6 Tray
- 4.7 A vortex mixer
- 4.8 A hair dryer
- 4.9 Toilet paper
- 4.10 A timer
- 4.11 Plastic bags

5) Instruments

- 5.1 The incubator
- 5.2 An ELISA microplate reader

METHODS

The methods of this study were divided into two parts.

Part I: The GCF (Gingival Crevicular Fluid) collection

Part II: The ELISA (Enzyme-linked Immunosorbent Assay)

Part I: The GCF (Gingival Crevicular Fluid) collection

GCF collection

The GCF sampling was performed using the method of Offenbacher et al. 1986. The GCF was collected from the experimental and control teeth. The tooth was gently washed with water, and the sites under investigation were isolated with cotton rolls to minimize saliva contamination and gently dried with an air syringe. Paper strips were carefully inserted into the gingival crevice for 1 mm and left there for 15 sec. Then the paper strip was cut for 2 mm in length and kept in a microcentrifuge tube. After a one minute interval, a second strip was placed at the same site. Care was taken to avoid mechanical injury. The paper strips from the individual sites were stored at -80°C until further processing was carried out to prevent any enzymatic degradation of GAG components. The volume of fluid in the paper was measured by a micropipette. The weight of fluid in the paper was measured by a fine measurement OHAUS Explorer 210.

Times of collection

T 0 : Pre-orthodontic appliance insertion

L 0,4,... : Leveling phase (every 4 weeks)

M 0,1,2,3... : Movement phase (every week)

S 0,1,2,3 ... : Complete movement phase (every week)

Part II: The ELISA (Enzyme-linked Immunosorbent Assay) (Pothacharoen, 2000)

I: A competitive-based ELISA for HA

II: Protein assay

I: A competitive-based ELISA for HA

Microtiter plates (Maxisorp®), Nunc) were coated overnight at 4°C with umbilical cord HA (100 µl/well) in coating buffer. Free sites were then blocked with 150 µl/well of 1% (w/v) BSA in incubating buffer for 60 min at 25°C. After washing, 100 µl/well of the mixture, sample or standard competitor (HA Healon: range 4.53-2,000 ng/ml) in B-HABP (1:200), was added. After incubating for 60 min at 25°C, plates were washed and then the peroxidase-conjugated mouse monoclonal anti-biotin (100 µl/well; 1:4000) was added and incubated for 60 min at 25°C. 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. The reaction was stopped by addition of 50 µl of 4M H₂SO₄. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

II: Protein assay

Total Protein concentration was determined using the Bio-Rad protein assay, based on the Bradford dye-binding procedure (Farndale *et al.*, 1986), which 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. BSA standards (0-1,000 ng/µl/well) and samples were added to the microtiter plates (10 µl/well) in triplicate. Concentrated dye reagent and deionized distilled water were mixed together (at the ratio of 1 to 4) and added to each well (200 µl/well). The plates were incubated at room temperature for 5 min and

the absorbance was read at 620 nm. Total protein concentrations in the samples were determined from a standard curve.

STATISTICAL ANALYSES

1. The non-parametric statistical analysis was used to evaluate gingival index and plaque index.
2. Descriptive analysis was used to determine the changes of HA levels in GCF.
3. The non-parametric statistical analysis (Friedman Test) was used to compare the medians of the HA levels in each treatment time of seven experimental canines and three control incisors.
4. The non-parametric statistical analysis (Mann-Whitney U Test) was used to compare the medians of the HA levels between canines and incisors.