

CHAPTER 2

MATERIALS AND METHODS

Subjects and Treatment

Twenty orthodontic patients, 14 females and 6 males, age range 13-23 year, from the postgraduate orthodontic clinic at Prince of Songkla University were included in the study. The inclusion criteria for all subjects were: the maxillary first premolar extraction with fixed appliance therapy, good general health, no use of any medication for at least 6 months prior to the study and healthy periodontal status (selected periapical radiographs using a standardized long cone paralleling technique with no bone resorption, probing depth values less than or equal to 3 mm in the whole dentition, no bleeding on probing, attachment loss is less than or equal to 1 mm and the percentage of the full-mouth tooth surfaces with the presence of supragingival plaque was not exceeding 20%). All patients were under control good oral hygiene by repeated oral hygiene instructions for the use of toothbrush and dental floss. The patients were informed about the experimental design. Each patient who agreed was signed in consent form to participate on a voluntary basis in the project.

All patients were treated with upper and lower preadjusted edgewise appliance (Roth's prescription). The brackets were 0.018X0.025 inch slot. The first stage of treatment was leveling. The goals of this phase were to bring the teeth into alignment and correct vertical discrepancies. So, all teeth in the maxillary arch and the mandibular arch were in good alignment before phase of canine retraction. The arch wire was left passive for one visit (minimum 1 month). In the movement phase (the second stage of treatment), space closure was carried out on passive 0.018 inch stainless steel arch wire, with the anchorage balanced between the canine and

the second premolar and first molar. Before canine retraction, GCF was collected. The beginning of space closure, the NiTi closed coil spring (Extension spring, Ormco) was placed in one quadrant of maxillary arch (NT group) and the elastic c-chain (Alastik, Unitec/3M) in the other quadrant of the same arch (CH group). The space closure mechanics were randomly allocated to the left or the right side (Fig.4). The NiTi closed coil spring and elastic c-chain were used to distalize canines, attached to the hook on the first molar at one end and the bracket on the canine at the other end. NiTi closed coil spring and elastic c-chain were activated by stretching and measured the force about 6 ounces (170 cN) as measured by a strain gauge. Supplementary intermaxillary elastic traction was not used during canine retraction because it might influence the results. After 24 hours, 1 month and 2 months of force application, GCF was collected. Moreover, plaque index (modified O'Leary Plaque Index)⁽⁶⁴⁾ and gingival index (Loe and Silness)⁽⁶⁵⁾ were measured before the GCF collection. In this study, one observer collected the samples and measured the result for unbiased study.

Nickel titanium closed coil spring

The NiTi closed coil spring was placed directly between the hook on the first molar band and the bracket on the canine (Fig.5). Then elastic module was used to tie the canine bracket and the arch wire. The initial force delivered was about 6 ounces which measured by a strain gauge, calibrated in 1 ounce (28.35 cN) increment. The NiTi closed coil spring was not renewed at subsequent visits, unless showing signs of distortion. However we measured the remained force of the NiTi closed coil spring after one month of activation to confirm providing continuous force throughout the study.

Elastic c-chain

Elastic c-chain was placed in the opposing quadrant, stretched to provide 6 ounces which measured by a strain gauge. This force was similar to the initial force of NiTi closed coil spring. Elastic c-chain was attached between the hook on the first molar band and the

distal wing of the canine bracket (Fig.6). Then elastic module was used to tie the mesial wing of the canine bracket and the arch wire. The elastic c-chain was renewed at each visit.



Fig.4: Two space closure mechanics: the nickel-titanium closed coil spring and the elastic c-chain

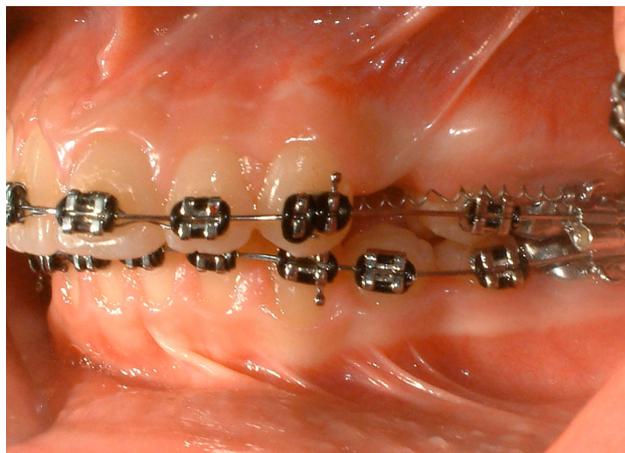


Fig.5: Canine retraction with nickel-titanium closed coil spring



Fig.6: Canine retraction with elastic c-chain

GCF Collection and Volume Measurement

To avoid salivary contamination, only maxillary arches were included in the study. The mouth of each subject was divided into quadrants: distolabial aspect of maxillary canine of each quadrant was used for the GCF sample collection. After isolating the tooth with cotton roll, supragingival plaque was removed without touching the marginal gingiva and the crevicular site was gently dried with an air syringe. The GCF from each individual was collected five times; before bracket placement (pre-tx), before canine retraction (0h) and after force application 24 hours (24h), 1 month (1mo) and 2 months (2mo). The GCF was collected with No.30 standardized sterile paper point. It was inserted into the gingival crevice until slight resistance was felt and left in situ for 60 seconds (Fig.7). Care was taken to avoid mechanical injury to the gingival crevice. After GCF collection, paper points were transferred to sterile tubes. The adsorbed GCF volume in each single paper point was weighted after GCF collection immediately. And before the sampling procedure, each single paper point was weighted by an analytical balance (Sartorius model MC 210S); considering GCF density value = 1, the difference in weight was showed the adsorbed GCF volume.⁽⁶⁶⁾

The difference in weight = adsorbed GCF volume

Then GCF was kept at -80°C until being assayed. At the time of analyses, each paper point was eluted by 250 μl of sterilized phosphate buffered saline (PBS, pH 7.4) and was subjected to centrifugation (13,000 g, 4°C , 15 min). Supernatant was taken into a new tube. The eluted solution was immediately used for interleukin analysis (Fig.8).



Fig.7: The collection of GCF with No.30 standardized sterile paper point.

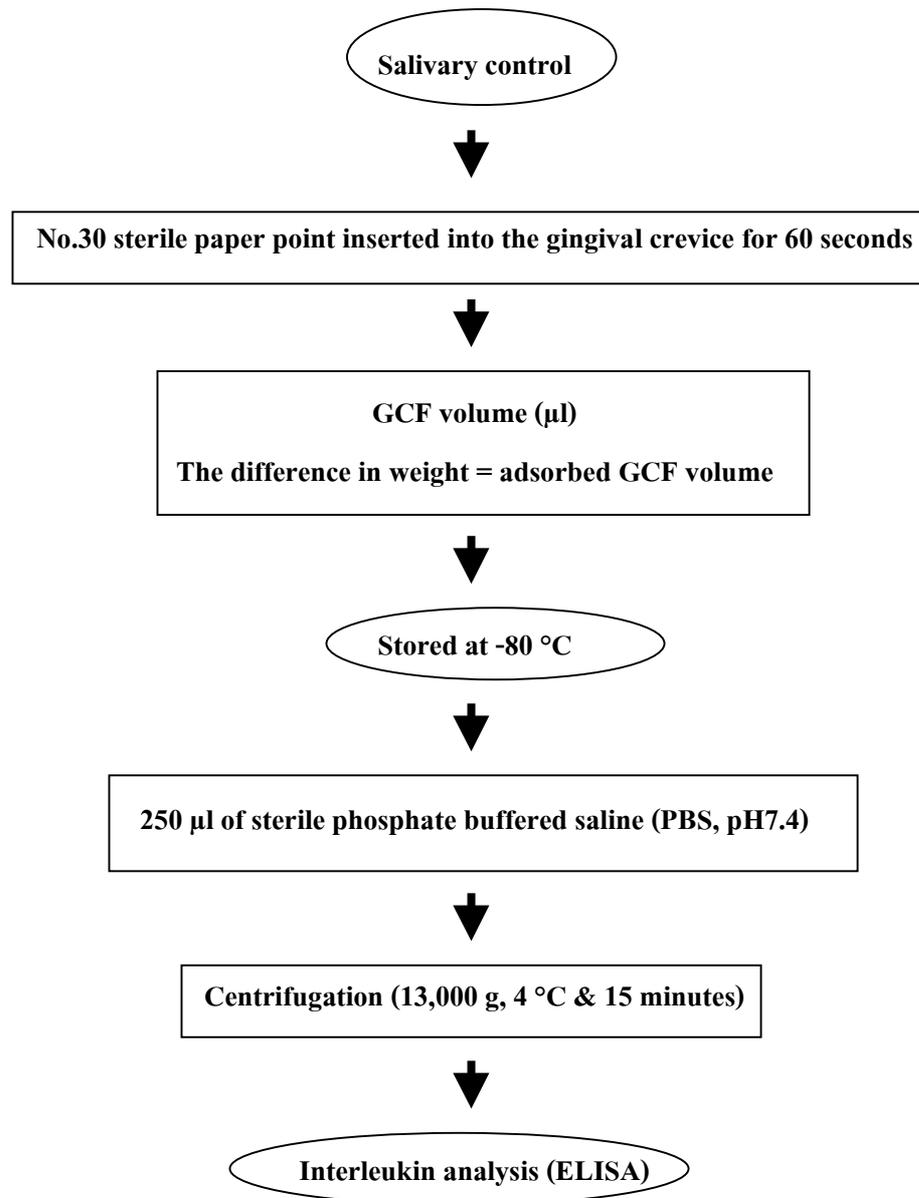
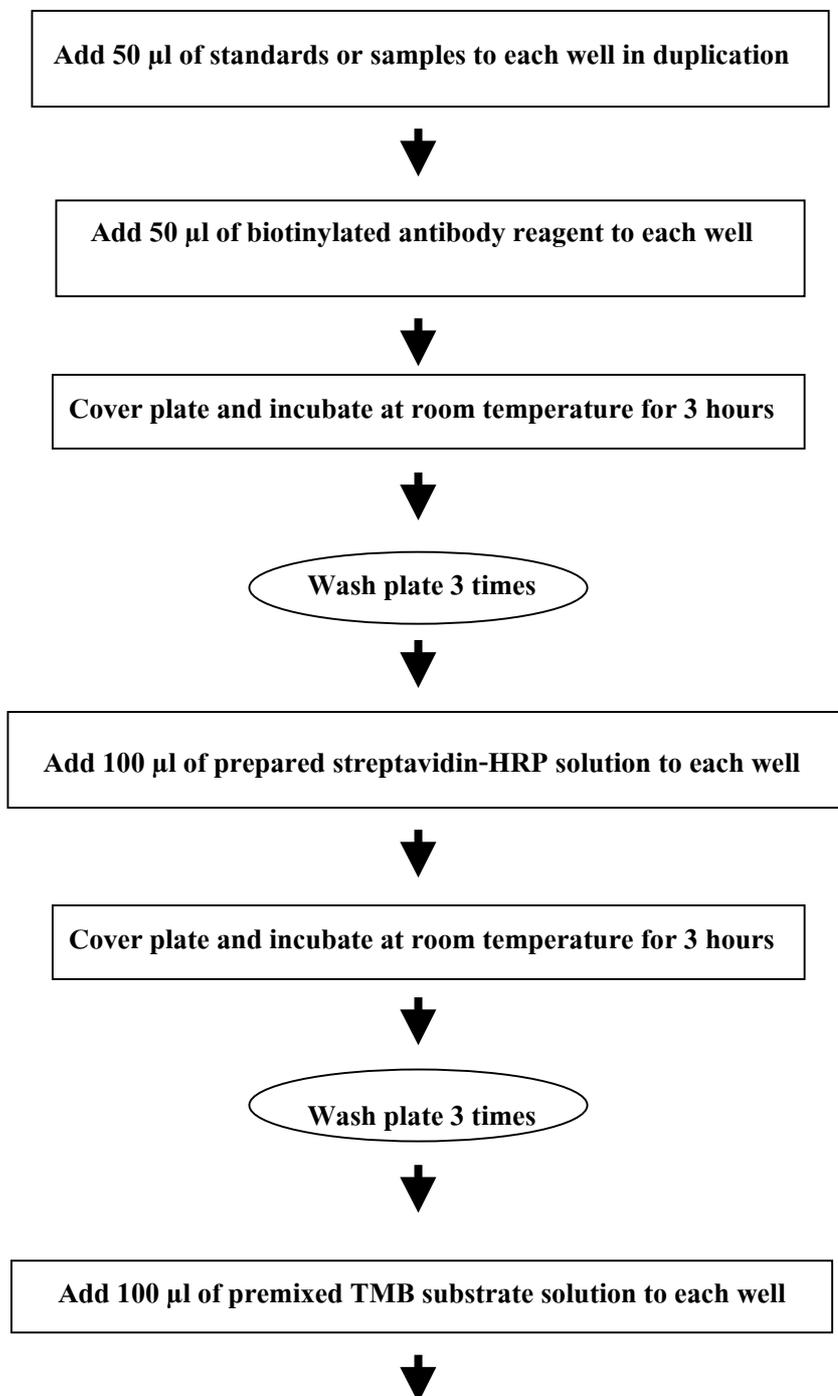


Fig.8: Diagram presents the steps of GCF collection, volume and elution.

Quantity of IL-1 β and IL-8

Commercial IL-1 β and IL-8 enzyme-linked immunosorbent assay (ELISA) kits (Pierce Endogen) were used to quantify the level of IL-1 β and IL-8 in the GCF samples. The levels (pg/ml) of IL-1 β and IL-8 were calculated according to the reference calibration curves of standards. Then the GCF volume of each sample was used to divide the level of IL-1 β and IL-8 for the total concentration (pg/ μ l) of IL-1 β and IL-8 in the sample.

IL-1 β assay: 50 μ l of each sample and standards were added to each well precoated with anti-human IL-1 β antibodies in duplicate. 50 μ l of biotinylated antibody reagent containing 0.01% thimerosal was then added, and the plate was covered and incubated at room temperature (20 $^{\circ}$ C-25 $^{\circ}$ C) for 3 hours. After washing 3 times with wash buffer, streptavidin-horseradish peroxidase (S-HRP) solution, diluted 1:400 with S-HRP dilution buffer, was applied and the plate was incubated at room temperature for 30 minutes. After washing 3 times, 100 μ l of premixed tetramethylbenzidine substrate was added and allowed to react at room temperature for 30 minutes in the dark. 100 μ l of stop solution (0.18 mol/l sulfuric acid) was added to terminate the reaction and the optical density was measured at 450 nm. The total protein level (pg/ml) of each sample was calculated from the constructed linear standard curve (Fig.9-12).

IL-1 β assay

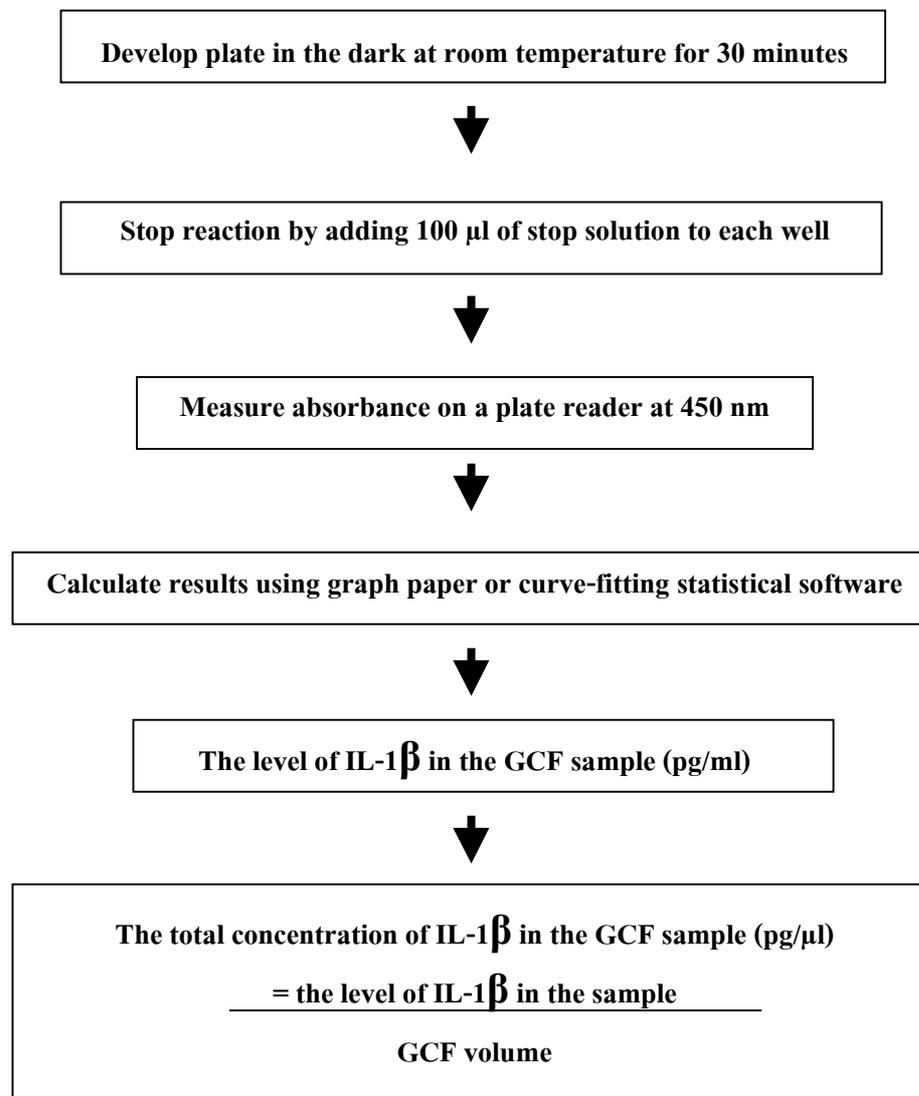


Fig.9: Diagram shows steps of interleukin-1 β analysis.

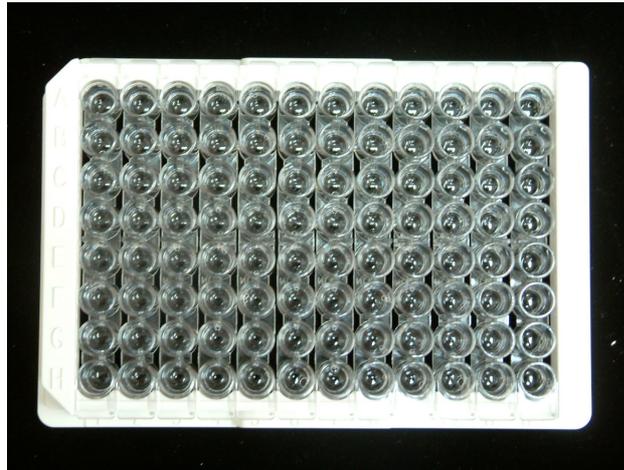


Fig.10: Step of interleukin-1 β analysis 1: each sample and standard were added to each well precoated with anti-human IL-1 β antibodies in duplicate. Biotinylated antibody reagent was then added.

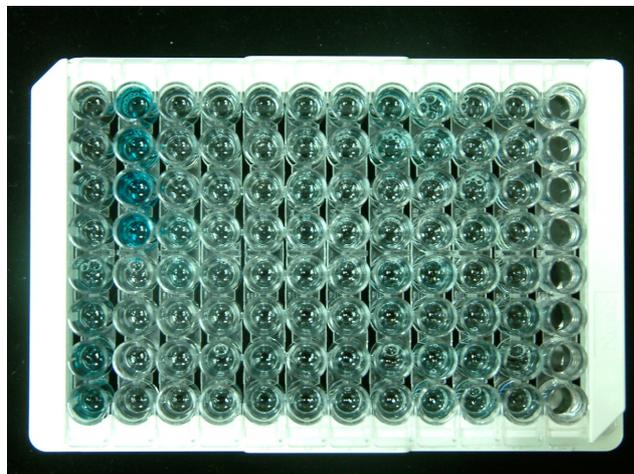


Fig.11: Step of interleukin-1 β analysis 2: after tetramethylbenzidine substrate was added and allowed to react at room temperature for 30 minutes in the dark.

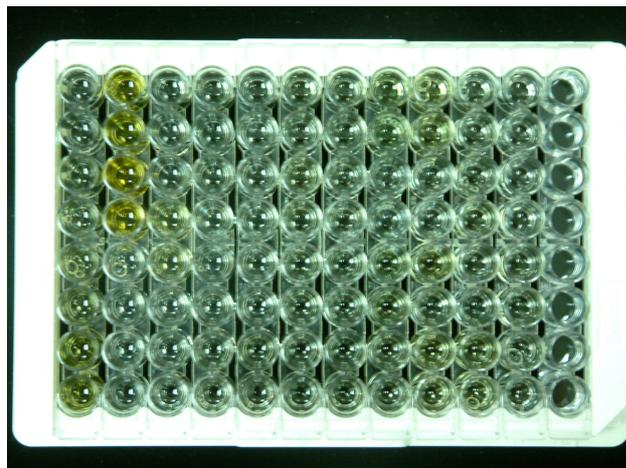
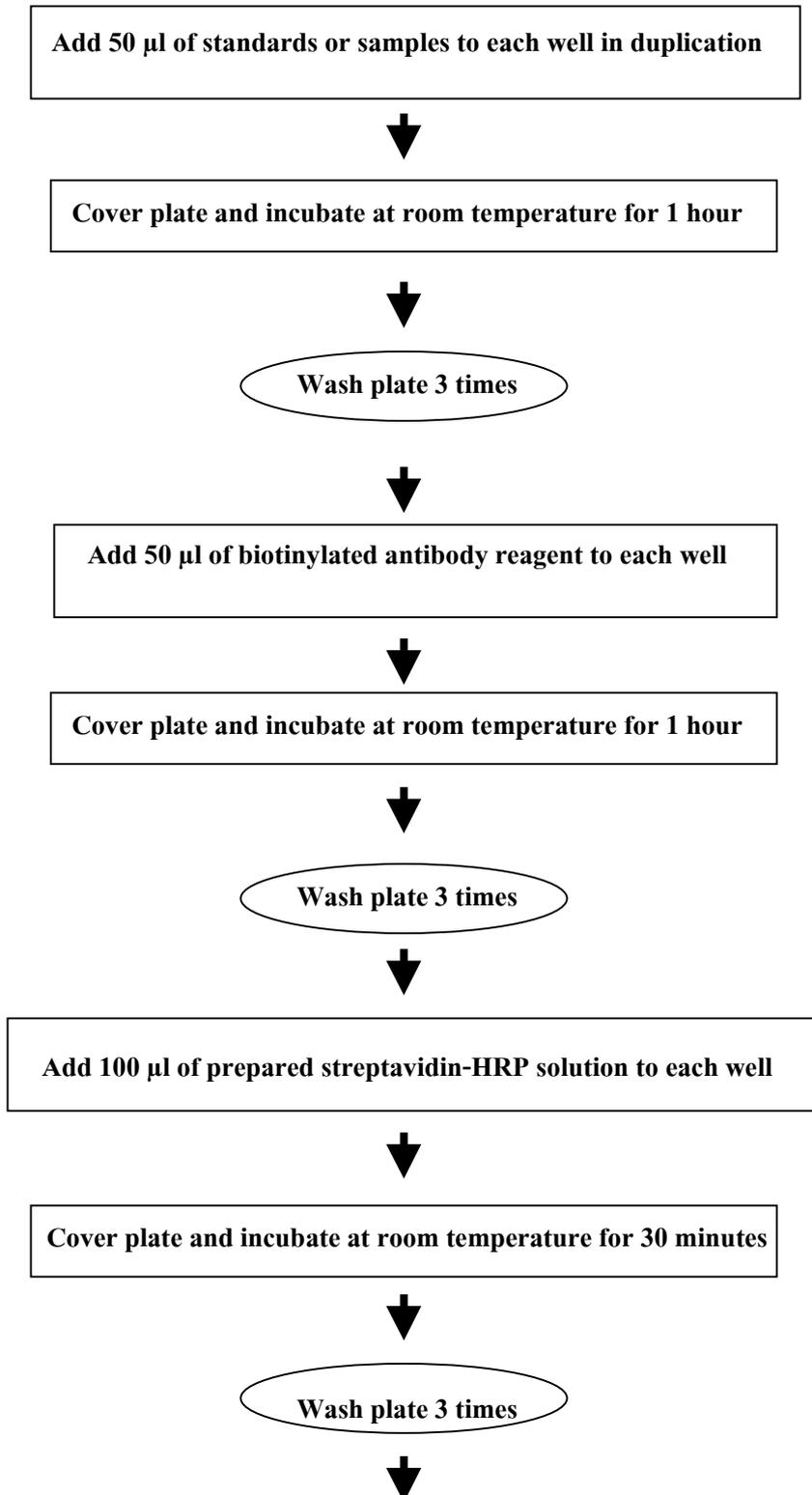


Fig.12: Step of interlukin-1 β analysis 3: the stop solution was added when blue substrate turned yellow. Then the optical density was measured at 450 nm.

IL-8 assay: 50 μ l of each sample and standards were added to each well precoated with anti-human IL-8 antibodies in duplicate. The plate was covered and incubated at room temperature (20 $^{\circ}$ C-25 $^{\circ}$ C) for 1 hour. Then washed plate 3 times with wash buffer and added 50 μ l of biotinylated antibody reagent containing 0.01% thimerosal. The plate was covered and incubated at room temperature for 1 hour. After washing 3 times, streptavidin-horseradish peroxidase (S-HRP) solution, diluted 1:400 with S-HRP dilution buffer, was applied and the plate was incubated at room temperature for 30 minutes. After washing 3 times, 100 μ l of premixed tetramethylbenzidine substrate was added and allowed to react at room temperature for 30 minutes in the dark. 100 μ l of stop solution (0.18 mol/l sulfuric acid) was added to terminate the reaction and the optical density was measured at 450 nm. The total protein level (pg/ml) of each sample was calculated from the constructed linear standard curve (Fig.13-16).

IL-8 assay

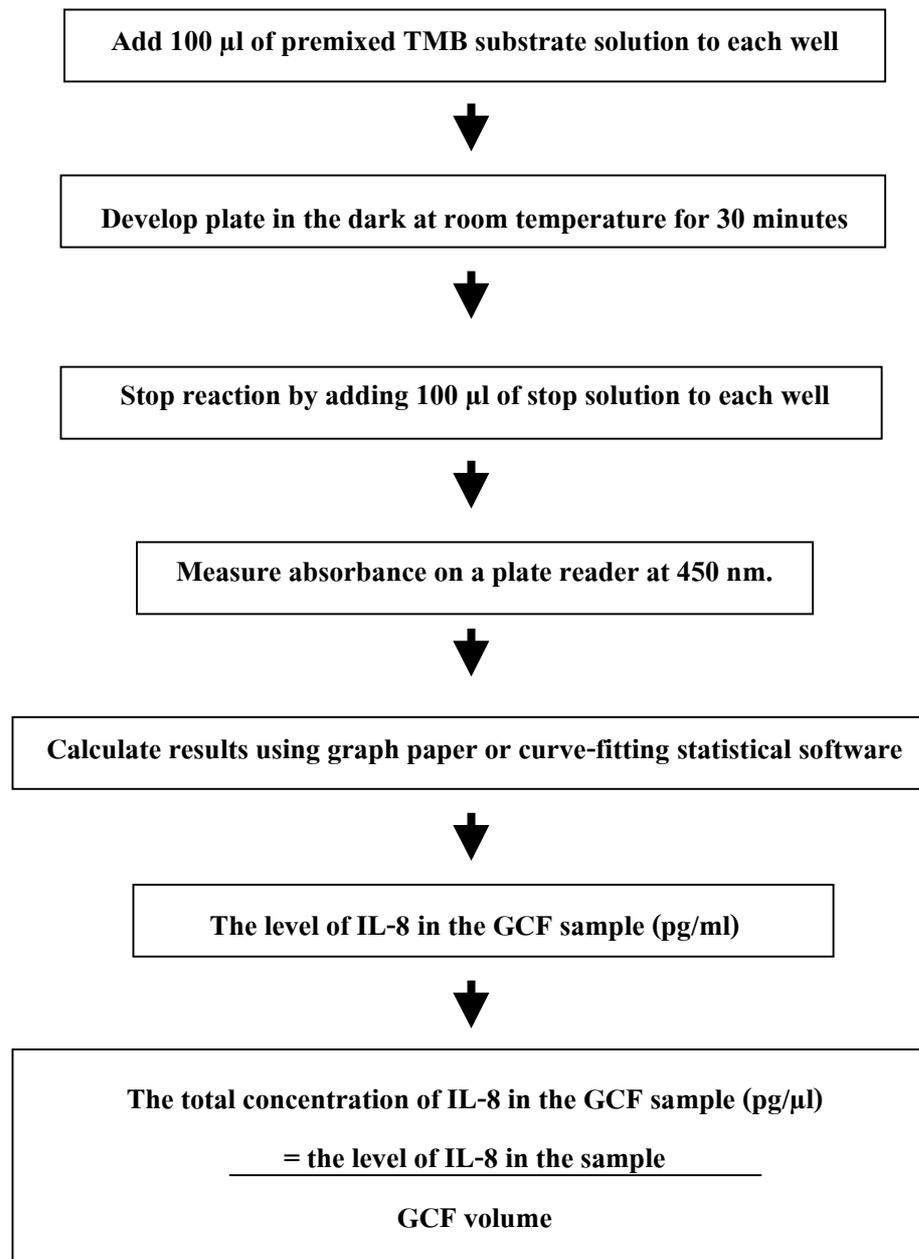


Fig.13. Diagram shows steps of interleukin-8 analysis.

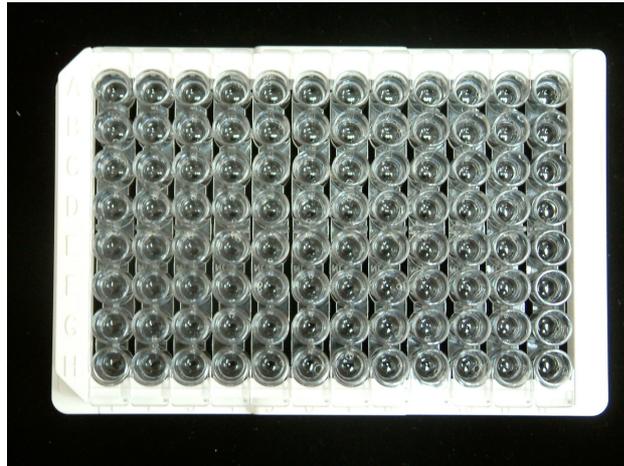


Fig.14: Step of interleukin-8 analysis 1: each sample and standard were added to each well precoated with anti-human IL-8 antibodies in duplicate.

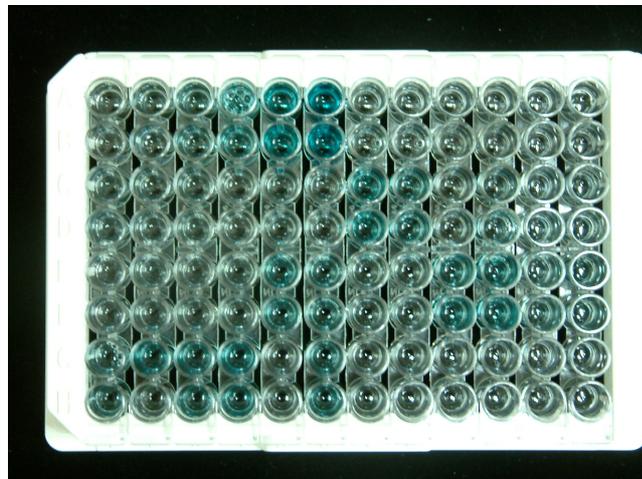


Fig.15: Step of interleukin-8 analysis 2: after tetramethylbenzidine substrate was added and allowed to react at room temperature for 30 minutes in the dark.

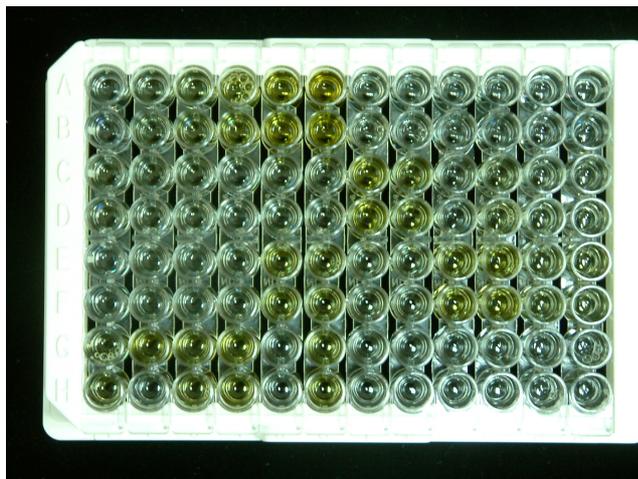


Fig.16: Step of interleukin-8 analysis 3: the stop solution was added when the blue substrate turned yellow. Then the optical density was measured at 450 nm.

Measurement of Canine Movement

The upper arch was taken impression with alginate after the 0.018 inch stainless steel arch wire was placed for 4 weeks (T_0) and after canine retraction 2 months (T_1) for reference models and measured the distance of canine movement. Landmarks, the median palatal raphe, the right and left medial end of the third palatal rugae, were marked on the upper casts using a 0.5 mm graphite pencil (Fig.17). Then the study casts were scanned into a computer with the use of scanner (HP scanjet 4570c). Both sets of upper casts were positioned side by side when scanning. Millimeter ruler was placed next to the dental casts to assess magnification. The images were transferred to Microsoft PowerPoint and printed with laser printer (HP deskjet 1180c) (Fig.18). The mean distance of canine movement measured relatively to the medial point of the third palatal rugae on the right and left quadrants of the casts. The medial end of the third palatal rugae was a suitable landmark for model analysis.⁽⁶⁷⁻⁶⁹⁾ From study model, the midpalatal raphe was used to construct a median reference line. The distance between the distal contact point of the canine and the line which was constructed perpendicular to the median reference line through the medial end of the third palatal rugae in each quadrant was measured. The difference of this distance at T_0 and

T_1 was the canine movement. In patient who used Nance holding arch for anchorage, the distance of canine movement was directly measured with a digital caliper from the T_0 and T_1 study models.

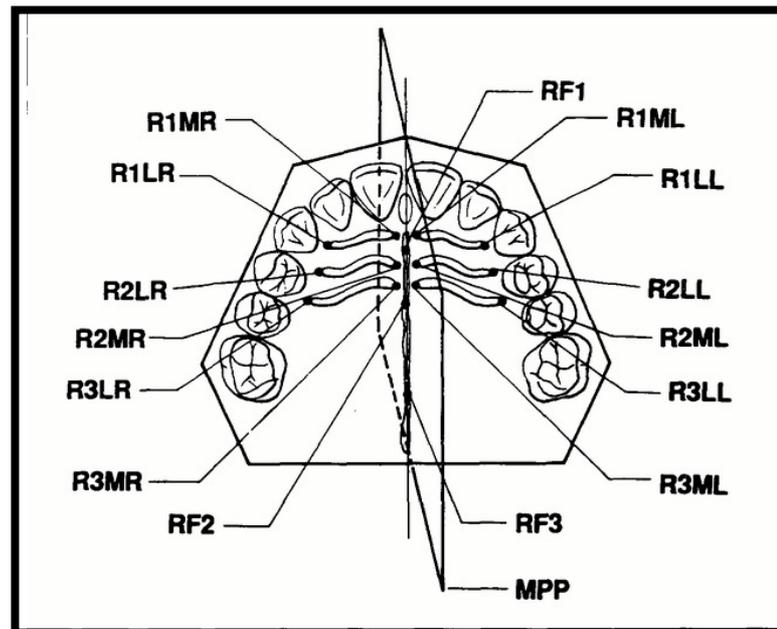


Fig.17: Landmarks marked on the cast: points are located on the median palatal raphe (MPP), the right medial end of the third palatal rugae (R3MR) and the left medial end of the third palatal rugae (R3ML).⁽⁶⁸⁾



Fig.18: The image was scanned study casts with scanner. Both T₀ (upper) and T₁ (lower) study models were positioned side by side when scanning and millimeter ruler was placed next to the dental casts to assess magnification.

Measurement error

Measurement error in assessing space closure

To reduce method error associated with the measurement of the study models, the examiner was blind to the method of space closure used in each quadrant. The study models were measured randomly. Additionally, measurements were taken two times to reduce random error.

Intra-examiner reliability

The analytical balance (Sartorius Model MC 210S) and strain gauge were calibrated before use. The study models were remeasured 1 week later and the mean of these measurements was compared to the mean of the initial measurements using a paired t-test. There was no statistically significant difference between these two results.

Statistical Analysis

The levels of IL-1 β and IL-8 from pretreatment sample group and from after canine retraction group were calculated as subject means and standard deviations. The IL-1 β level and IL-8 level at each time were compared to the baseline level by repeated measurement ANOVA at $p < 0.05$. Using paired t-test at $p < 0.05$ to determine whether there were any significant differences of IL-1 β level and IL-8 level between NT & CH groups at each time. The rate of canine movement was compared between NT group and CH group with paired t-test at $p < 0.05$.

Materials and Equipments

1. Nickel titanium closed coil spring (Fig.19)
2. Elastic c-chain (Fig.19)
3. Paper point No.30 (Fig.20)
4. Sterile phosphate buffer saline
 - NaCl
 - KCl
 - Na₂HPO₄
 - KH₂PO₄
 - Sterile distilled water
5. Human IL-1 β and IL-8 ELISA kit (Fig.21 & 22)
6. Ultrapure water
7. Precision pipettors 20, 100, 200 and 1000 μ l (Fig.23) and disposable plastic tips
8. Plastic pipettes 5-15 ml
9. Glass two-liter container to prepare Wash Buffer
10. A squirt wash bottle
11. 1.5 ml polypropylene or polyethylene tubes
12. Disposable reagent reservoirs
13. 15 ml plastic tube to prepare streptavidin-HRP solution
14. Digital scale analytical balance (Fig.24)
15. A standard ELISA reader (Fig.25)
16. Centrifugal machine
17. Freezer -80 °C



Fig.19: NiTi closed coil spring and elastic c-chain



Fig.20: Paper point No.30 and sterile tube



Fig.21: Human IL-1 β ELISA kit



Fig.22: Human IL-8 ELISA kit



Fig.23: Precision pipettors 20, 100, 200 and 1,000 μl



Fig.24: Digital scale analytical balance (Sortarius model MC 210S)



Fig.25: A standard ELISA reader (Biotrak II)