Comparisons of the chondroitin sulphate levels in orthodontically moved canines and the clinical outcomes between two different force magnitudes


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SUMMARY The aims of this study were to compare the chondroitin sulphate (CS) levels in gingival crevicular fluid (GCF) of moved canines using either 70 or 120 g of orthodontic force, and to compare the rate of tooth movement and the amount of pain between these two force magnitudes. Sixteen patients (6 males and 10 females; aged 16.91 ± 2.99 years), with class I malocclusion, who required orthodontic treatment with first premolar extractions, were recruited. The force magnitudes used to move the maxillary canines distally were controlled at 70 and 120 g on the right and the left sides, respectively. GCF samples were collected with Periopaper® strips before and during orthodontic tooth movement. Competitive ELISA with monoclonal antibody was used to measure the CS levels. The distance of tooth movement and the amount of pain assessed by visual analog scale (VAS) scores were evaluated. The medians of CS levels during the loaded period were significantly greater than those during the unloaded period (P < 0.05). The differences between the medians of CS levels of 70 g and 120 g retraction force during each 1 week period were not significant. There was no significant difference in the rates of canine movement between these two force magnitudes. However, using 120 g, the medians of VAS scores were significantly greater than those with 70 g (P < 0.05). Collectively, 70 g retraction force appears to be sufficient and more suitable than 120 g force as it causes no difference in biochemically-assessed bone remodelling activity, the same rate of tooth movement, reduced pain and better comfort.

Introduction

Orthodontic tooth movement has been defined as a result of the coupling of bone resorption at compressive sites and bone deposition at tensile sites adjacent to the periodontal ligament (Melsen, 1999). Extrinsic mechanical stimulation leads to remodelling of supporting periodontal tissues, especially bone. The optimum orthodontic force magnitude should produce a maximal rate of tooth movement with minimal tissue damage and with maximal patient comfort. The recommended force magnitudes are broadly reported to range from 1.2 to 1200.0 g (Ren et al., 2003), but no specific or evidence-based force magnitude has so far been recommended for appropriate efficiency in clinical orthodontics.

The assessments of tooth movement include clinical, histological, biomechanical, and biochemical assessments. Biochemical markers of bone formation and resorption provide a new and potentially important clinical tool for assessing and monitoring bone metabolism (Christenson, 1997). Furthermore, monitoring changes in gingival crevicular fluid (GCF) constituents is a non-invasive method of investigating such clinically unnoticeable changes and is a rapid biochemical assessment of metabolic changes in periodontal tissue (Pender et al., 1994). Up to the present, various biochemical markers have been studied and used to assess bone remodelling and to monitor periodontal health and disease (Lowney et al., 1995; Insoft et al., 1996; Nozoe et al., 2002; Dudic et al., 2006; Yamaguchi et al., 2006).

There are at least three major types of biochemical markers that can be used to monitor alveolar bone remodelling during orthodontic tooth movement, including inflammatory mediators, host-derived enzymes, and tissue breakdown products. Chondroitin sulphate (CS) is categorized as one of the tissue breakdown products, especially from alveolar bone. CS is a main component of glycosaminoglycans (GAGs) found in alveolar bone (Waddington et al., 1989), consisting of a high quantity of chondroitin-4-sulphate but a low amount of chondroitin-6-sulphate (Waddington and Embery, 1991).

By an electrophoretic method, several previous studies have demonstrated an increase in CS levels in GCF of teeth undergoing orthodontic tooth movement, which reflects changes in the deeper periodontal tissues during orthodontic
treatment. The CS levels in GCF from compressive side of orthodontically moved teeth were significantly greater than those detected in the control group (Last et al., 1988). The longitudinal investigation of GCF volume and GAGs components in GCF before and during orthodontic tooth movement of various types over a 9 month period demonstrated an increase in GCF volume during orthodontic movement with fixed appliances (Samuels et al., 1993). This study also showed the significant change in CS levels in GCF from the group treated by fixed appliances producing vertical and horizontal movement. Monitoring of orthodontic treatment over a 2 year period demonstrated raised GCF volume and CS levels during orthodontic tooth movement (Pender et al., 1994). Therefore, the elevated CS levels during the applied force indicate active turnover of deep periodontal tissues (Baldwin et al., 1999). In this study, we applied our patented WF6 monoclonal antibody, raised against the WF6 catabolic epitope of CS (Pothacharoen et al., 2007), to monitor changes in CS levels in GCF of moved canines under different magnitudes of orthodontic force. The aim of this study was, therefore, to compare the CS (WF6 epitope) levels in GCF as well as the rate of tooth movement and the amount of pain between two different magnitudes (either 70 or 120 g of orthodontic force) during the distal movement of canines.

Materials and methods

Subjects

Sixteen patients (6 males and 10 females; aged 16.91 ± 2.99 years; range 12.3–22.5 years) were selected. These patients met the following criteria: (1) good general and oral health; (2) lack of antibiotic therapy during the previous 6 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, distal canine movement, and maximum anchorage control. All patients received repeated oral hygiene instruction, and the gingival health was controlled and maintained throughout the entire study. This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University. Informed consent was obtained from all subjects.

Experimental design

In each patient, two miniscrew implants (2.0 mm in diameter and 10.0 mm in length; Bio-Ray, Syntec Scientific Corporation, Chang Hua, Taiwan) were placed buccally into infrapyogmonic bone between the maxillary first permanent molar and the maxillary second permanent molar (Figure 1). At placement and during the unloaded and the loaded periods, the success rate of the miniscrew implants was 100 per cent, since all miniscrew implants were clinically immobile. Transpalatal arches were also placed in all patients to secure the anchorage in case of miniscrew failure. Orthodontic pre-adjusted brackets (Roth prescription slot 0.018 × 0.025 inches; 3 M Unitek Inc., Monrovia, California, USA) were bonded on all teeth 3 weeks after first premolar extractions. GCF was collected with Periopaper® strips (ProFlow Inc., Amityville, New York, USA) from maxillary right and left canines before and during orthodontic tooth movement. Mandibular canines not needing orthodontic tooth movement were used as controls.

During the unloaded period, the GCF samples around maxillary right and left canines were collected as baseline data. During the loaded period, a Nickel–Titanium closed coil spring (Tomy, Tokyo, Japan) was used to connect the miniscrew implant head to the canine bracket in order to move the maxillary canine distally on a 0.016 × 0.016 inch stainless steel wire (Figure 1). The orthodontic force magnitudes were controlled at 70 and 120 g on the right and the left sides, respectively. The force was activated and calibrated every fourth week by a force strain gauge (Dentaurum, Ispringen, Germany). The GCF samples
were collected from the distal gingival sulcus of maxillary canines (Figure 1) every week for 8 consecutive weeks.

**GCF collection**

The GCF collection was conducted as described previously (Khongkhunthian et al., 2008). Briefly, 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 gently dried with an air syringe. GCF was collected with Periopaper® strips 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 individually transferred to microcentrifuge tubes. All strips were stored at −80°C 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 (over 90 per cent) from each strip was determined by a dye-binding assay, using known concentrations of sulphated GAGs as standards (Ratcliffe et al., 1988).

**Determination of the distance of tooth movement**

The study casts were made before and after orthodontic canine movement every 4 weeks until the 12th week. The distance of maxillary canine movement was measured by using an ABSOLUTE digimatic caliper (Mitutoyo Corporation, Kawasaki, Japan). The measurement, which measures the maximum distance from the cusp tip of an orthodontically moved canine to the buccal groove of the first permanent molar, was performed (Dixon et al., 2002). The rate of canine movement in millimeters (mm) per month was then calculated. To assess the intra-examiner reliability and error of the method, the study models were re-measured by the same investigator 1 week later. The measurements were compared to the initial measurements using a paired t-test. There was no statistically significant difference between these two measurements.

**Evaluation of the amount of pain**

The visual analog scale (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 (first to eighth week) for 8 consecutive weeks. The linear scale properties ranged from 0 (absence of pain) to 10 (worst possible or unbearable pain).

**Competitive inhibition ELISA with WF6 monoclonal antibody (mAb)**

The quantitative ELISA to determine the WF6 epitope of CS was performed using a protocol described previously (Pothacharoen et al., 2006). In brief, microtiter plates (Maxisorp®, Nunc, Roskilde, Denmark) were coated overnight at room temperature with 10 µg/mL shark PG-A₁ fraction (100 µL/well) in coating buffer (20 mM sodium carbonate buffer, pH 9.6). On 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 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. After washing, 100 µL/well of the mixture, samples or standard competitors (Shark PG-A,D, fraction: range 39.06–10 000 ng/mL) in mAb against the WF6 epitope (1:100) were added for 60 minutes at 37°C. Subsequently, the plates were washed, and the IgM-specific peroxidase-conjugated anti-mouse immunoglobulin (100 µL/well; 1:2000) was added and incubated at 37°C for 60 minutes. Then, the plates were washed, and 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 4 M H₂SO₄. The absorbance ratio at 492:690 nm was measured using a Tietertek Multiskan® MCC/340 multiplate reader (ICN/Flow Laboratories, Costa Mesa, California, USA). The minimal detection level of CS (WF6 epitope) was 19 ng/mL.

**Protein assay**

Total protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) based on the Bradford dye-binding procedure, a simple colorimetric assay for measuring total protein concentration. The known concentrations (0–1000 µg/mL/well) of BSA standards and the GCF samples were added to the microtiter plates (10 µL/well) in triplicate. A mixture between dye reagent and de-ionized distilled water at 1:4 was added to each well (200 µL/well). The plates were incubated at room temperature for 5 minutes, and the absorbance was measured at 620 nm. Protein concentrations were determined from a standard curve of BSA standards.

**Statistical analysis**

The data were analysed using the Statistical Package for Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The Kolmogorov-Smirnov test was used to determine the distribution of CS (WF6 epitope) levels, the rates of canine movement and VAS scores. The differences between the CS levels, during the unloaded and the loaded periods were determined by the Wilcoxon signed-rank test; the differences between the two levels of force magnitude (70 and 120 g) during each experimental period were determined by the Mann-Whitney U-test. The difference between the mean rate of canine movement with...
70 and that with 120 g of orthodontic force was determined by the Independent-T test. The differences in VAS scores between the two levels of force magnitude (70 and 120 g) during each experimental period were determined by the Mann-Whitney U-test. The results were considered statistically significant at $P < 0.05$.

Results

Elevated levels of CS (WF6 Epitope) in GCF of orthodontically moved canines

During the unloaded period, the medians of CS levels around the right and the left control mandibular canines were 0.767 (0.001–3.446) and 0.597 (0.007–3.652) ng/µg of total protein, respectively, and the medians of CS levels around the right and the left experimental maxillary canines were 0.794 (0.003–3.269) and 0.669 (0.116–1.762) ng/µg of total protein, respectively. There were no significant differences in the median CS levels between control and experimental canines and between right and left canines.

During the loaded period, the median of CS levels around the right experimental maxillary canines (70 g retraction force) was raised to 1.421 (0.042–28.615) ng/µg of total protein and was significantly greater than that during the unloaded period ($P = 0.002$; Figure 2), while the median of CS levels around the left experimental maxillary canines (120 g retraction force) was elevated to 1.861 (0.002–31.106) ng/µg of total protein and was significantly greater than that during the unloaded period ($P = 0.001$; Figure 2). Comparisons of CS levels around the right and the left control mandibular canines during the experimental period for 8 weeks are shown in Figure 3, and it was demonstrated that the medians of CS levels during these periods were not significantly different from each other (Figure 3).

No clinical features of gingival inflammation, such as swelling of gingival margin, color changes, etc., were observed around the maxillary and mandibular canines during the three-month interval of this study in each individual patient (data not shown), implying that patients’ oral hygiene was well controlled throughout the study.

No differences in the medians of elevated CS levels and in the mean rates of tooth movement between two different force magnitudes

The medians of CS levels around the right and the left experimental maxillary canines (70 and 120 g of retraction force, respectively) during each 1 week period (the unloaded and the first to the eighth loaded weeks) are shown in Figure 4. The differences between the medians of CS levels around the right and those around the left experimental maxillary canines (70 and 120 g of retraction force) during each 1 week period were not significantly different (Figure 4).

Nonetheless, a cyclical pattern of rises in CS levels 1 week after force application (the first and the fifth weeks) was noticeable with a gradual decline after that (Figure 4). Similarly, the mean rates of canine movement (mm/month) induced with 70 and with 120 g of orthodontic force were not significantly different between these two force magnitudes (Table 1).

However, the VAS scores, representing the amount of pain that the patients felt with two different magnitudes of orthodontic force, showed that the medians of the VAS scores were significantly greater with 120 g of orthodontic force than with 70 g of orthodontic force at the first, second, third, fifth, sixth, and seventh weeks, but no significant differences between these two force magnitudes were found at the fourth and eighth weeks (Figure 5). Moreover, under both force magnitudes, the VAS scores showed a similar cyclical pattern to the rises in CS levels, which showed a peak 1 week after the first force application (first week) and then gradually decreased, and again showed a peak 1 week after the reactivation force application (fifth week), then gradually declined again (Figure 5).

Discussion

Mechanical force applied during orthodontic tooth movement causes dental and periodontal remodelling. Various
biochemical markers have been used to assess bone remodelling and to monitor periodontal health. CS comprises approximately 94 per cent of the total GAGs in alveolar bone (Waddington et al., 1989), 60 per cent of the total GAGs in cementum (Bartold et al., 1988), 17 per cent of the total GAGs in gingival tissue (Bartold, 1987), and a minor component in periodontal ligament (Gibson and Pearson, 1982). The fact that the high concentration of CS is found in human alveolar bone suggests that alveolar bone is a major source of CS in GCF. Therefore, altered levels of CS in GCF are likely to reflect early changes in alveolar bone, which cannot be clinically observed (Last et al., 1988; Samuels et al., 1993; Pender et al., 1994; Waddington et al., 1994; Smith et al., 1995; Kagayama et al., 1996).

The effects of force magnitude on tissue reaction during orthodontic tooth movement have been studied in various experiments (Kohno et al., 2002; Nozoe et al., 2002; Ren et al., 2004; Batra et al., 2006; Yamaguchi et al., 2006; Gonzales et al., 2008; Thiruvencatchari et al., 2008; Gonzales et al., 2009; Yee et al., 2009; Luppanapornlarp et al., 2010), in which the species of subjects, the ranges of force magnitude, the specific teeth chosen for the experiment, directions of tooth movement, durations of experiment, and force reactivation intervals varied. Nevertheless, a range of force magnitude of 70 to 120 g has been suggested for bodily tooth movement (translation), as it causes a maximal rate of tooth movement, most comfort for patients, and minimal tissue damage (Proffit et al., 2007). Thus, the suggested lower limit of force magnitude (70 g) and the upper limit (120 g) were selected for comparison in this study by monitoring both biochemical changes, i.e. the CS levels in GCF around moved canines using our patented WF6 monoclonal antibody, and clinical assessments.

The results of this study have shown that the medians of CS levels around the experimental maxillary canines, which were loaded with 70 and with 120 g retraction force during the loaded period, were significantly greater than those during the unloaded period, whereas the medians of CS levels around both the right and the left control mandibular canines during the experimental period for 8 weeks were not significantly different from each other. These findings are consistent with the studies by (Last et al., 1988), and (Kagayama et al., 1996), which reported increases in CS levels in GCF in association with the compression condition of teeth during orthodontic movement. However, analyses of the CS levels in their studies were done by the electrophoretic and the immunohistochemical methods, whereas a better quantitative ELISA with our WF6 monoclonal antibody was performed in this study. The WF6 monoclonal antibody has been successfully used to detect the elevated CS levels in sera of patients with joint diseases (Pothacharoen et al., 2006). In dentistry, the WF6 monoclonal antibody has also been used to quantify the CS levels in GCF of patients with periodontal diseases.
In our study, the median of CS levels was highest 1 week after force application with 70 or 120 g of retraction force, and then it gradually decreased. After 4 weeks, the force was reactivated, and the CS levels increased and reached peak levels again 1 week after force reactivation (the fifth week). These results showed a cyclical pattern of alveolar bone remodelling during the 4 week loaded period in experimental maxillary canines in contrast to control mandibular canines, which showed a non-cyclical pattern. Bone remodelling is a process, involving cellular functions directed toward co-ordinated resorption of old bone and formation of new bone, which can be regulated by systemic hormones and local factors. The sequences of events in the remodelling cycle are osteoclastic bone resorption, a reversal phase, followed by osteoblastic bone formation. The bone resorption cascade involves a series of steps removing both the mineral and organic constituents of bone matrix by osteoclasts (Hill, 1998). Osteoclasts act as major resorbing cells in the bone resorption process and have a limited life span of 12.5 days (Roodman, 1996; Hill, 1998). Therefore, the peak levels of CS 1 week after force activation or reactivation found in this study are consistent with the average life span of human osteoclasts, as well as with the fact that our WF6 monoclonal antibody literally detects the catabolic epitope of CS (Pothacharoen et al., 2007).

The differences between the medians of CS levels around experimental maxillary canines affected with 70 and with 120 g of force magnitude during each 1 week period were not significant. The explanation may be that both force

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<th>Force magnitude</th>
<th>Rate of canine movement (mm/month)</th>
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<tr>
<td></td>
<td>Minimum</td>
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<tr>
<td>70 g</td>
<td>0.67</td>
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<td>120 g</td>
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Figure 4 A boxplot graph of the chondroitin sulphate (CS; WF6 epitope) levels around the right and the left experimental maxillary canines [70 and 120 g of retraction force, respectively], during each 1 week period from Week 0 (unloaded) to Week 8. Note the cyclical pattern of a dotted line connecting the median CS levels in gingival crevicular fluid (GCF) samples of right maxillary canines, and of a solid line connecting the median CS levels in GCF samples of left maxillary canines. The small asterisks represent the extreme values.
magnitudes (70 and 120 g) are within the optimal range, so the alveolar bone remodelling activities are not different. Correspondingly, the rates of tooth movement under these two selected force magnitudes are also not significantly different. However, it is interesting to note that the left experimental maxillary canines, which were moved by 120 g retraction force, showed increased tipping movement in comparison to the right experimental maxillary canines, which were moved by 70 g retraction force (data not shown).

Moreover, the VAS scores for patient discomfort under 120 g retraction force were significantly greater than those under 70 g retraction force.

In the present study, we have shown that the CS types in GCF around moved canines are heterogeneous in nature, comprising mainly CS-6 with the minority of CS-4 by our WF6 monoclonal antibody that can recognize the certain configurations and sequences of heterogeneous CS (ΔDCCC and ΔCCAD; where C and D represent CS-6, while A represents CS-4; Pothacharoen et al., 2007). In addition, ELISA is a more sensitive technique than immunohistochemical staining and provides quantitative data of CS levels along the entire course of experiments. Therefore, our results not only agree with and support the findings from the study by Kagayama et al. (1996) but also explore and provide additional knowledge for biochemical analysis of CS quantity and for timing of increased CS levels in GCF during orthodontic movement.

Our initial research protocol was designed to monitor all parameters in an experimental period of 8 weeks according to our previous study (Intachai et al., 2010). However, measuring the rate of canine movement was extended further for 4 additional weeks (up to 12 weeks) in order to show a clearer picture of canine movement due to the nature of slow orthodontic canine movement. Nevertheless, we speculate that the cyclical pattern of CS levels in GCF as well as that of VAS scores seen in the first and second 4 week periods would be found in the third 4 week period also.

Although both 70 g and 120 g of orthodontic force are within the optimal range for canine retraction, we propose that 70 g retraction force should be more suitable than 120 g retraction force because there is no difference in biochemically assessed bone remodelling activity, the same rate of tooth movement, reduced pain, better comfort, and less tooth tipping. Moreover, our results imply an essential role of CS (WF6 epitope) levels in GCF as a biomarker for alveolar bone remodelling during orthodontic canine movement. In addition, higher force magnitudes used during orthodontic tooth movement may not necessarily produce more efficient tooth movement. On the other hand, they may cause undesirable effects, such as tooth tipping, pain or patient discomfort.

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