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Alkaline phosphatase activity in gingival crevicular fluid during canine retraction

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Structured Abstract

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Objectives – The aim of the study was to investigate alkaline phosphatase activity in the gingival crevicular fluid (GCF) during orthodontic tooth movement in humans.

Setting and Sample Population – Postgraduate orthodontic clinic. Ten female patients requiring all first premolar extractions were selected and treated with standard edgewise mechanotherapy.

Experimental Variable – Canine retraction was done using 100 g sentalloy springs. Maxillary canine on one side acted as experimental site while the contralateral canine acted as control.

Outcome Measure – Gingival crevicular fluid was collected from mesial and distal of canines before initiation of canine retraction (baseline), immediately after initiation of retraction, and on 1st, 7th, 14th and 21st day and the alkaline phosphatase activity was estimated.

Results – The results show significant ($p < 0.05$) changes in alkaline phosphatase activity on the 7th, 14th and 21st day on both mesial and distal aspects of the compared experimental and control sides. The peak in enzyme activity occurred on the 14th day of initiation of retraction followed by a significant fall in activity especially on the mesial aspect.

Conclusions – The study showed that alkaline phosphatase activity could be successfully estimated in the GCF using calorimetric estimation assay kits. The enzyme activity showed variation according to the amount of tooth movement.

Key words: gingival crevicular fluid; alkaline phosphatase activity; canine retraction

Introduction

Gingival crevicular fluid (GCF) is an osmotically mediated inflammatory exudate in the gingival sulcus. It has recently been suggested that enzyme activity of

GCF could be an important predictor in orthodontic tooth movement (1,2).

The biological response incident to orthodontic tooth movement ultimately involves alterations in the surrounding bone architecture (3). Alkaline phosphatase is commonly associated with bone metabolism with osteoblasts showing high alkaline phosphatase (4). Although the enzyme is present in all body tissues, about half the total serum activity comes from the skeleton. It is of particular interest in bone conditions associated with increased osteoblastic activity such as osteitis deformans where it rises 10–25-folds. Moderate increase in activity is observed in osteomalacia, third trimester of pregnancy and hyperparathyroidism. Transient increase in activity is also observed during healing of bone fractures and physiologic bone growth (5).

Acid and alkaline phosphatases are released by injured, damaged, or dead cells into extracellular tissue fluid. As a result of orthodontic force application, these enzymes produced in the periodontium diffuse into the GCF. Thus monitoring of phosphatase activities in the GCF could be suggestive of the tissue changes occurring during orthodontic tooth movement. An experimental study in rats suggested that phosphatase activities reflect bone turnover in orthodontically treated tissues (6). A clinical study in humans (three cases) correlated alveolar bone remodeling with changes in GCF phosphatase activities (7). Early elevation in alkaline phosphatase activity was observed between the first and third week when no or little tooth movement occurred. These observations suggest that changes in phosphatase activities in GCF may reflect local biologic processes associated with orthodontic tooth movement. There exists a temporal and spatial difference in alkaline phosphatase activity in GCF from orthodontically treated and control sites in the same individual. This study was undertaken to investigate and assess alteration in the alkaline phosphatase enzyme activity during orthodontic tooth movement during a 21-day period in a larger sample size.

Material and methods

Ten female patients (age range 12–21 years) requiring orthodontic treatment with all first premolar extractions were taken for this study, after informed consent. The patients were in permanent dentition and good periodontal health (probing depth <3 mm, plaque and gingival index of ≥1 with no radiographic evidence of periodontal bone loss). Patients were ruled out for pregnancy and were free from any medical condition that could influence the study. A detailed menstrual history of the patients was taken so that the role of the estrus cycle on the enzyme activity could be evaluated. The patients were treated with conventional standard edgewise (0.022×0.028) mechanotherapy. Following leveling and alignment the retraction of the canine was initiated on a base wire of $0.019 \times 0.025''$ on one side with the other side acting as control. Canine retraction was performed using recalibrated sentalloy spring capable of delivering 100 g of constant force.

The GCF was collected by micropipettes from the mesial and distal side of maxillary canines before canine retraction was initiated (a), immediately after initiating canine retraction (0 day) (b), and on 1st (c), 7th (d), 14th (e) and 21st day (f). The tooth movement was measured intraorally on the 7th, 14th and 21st day of initiation of canine retraction. A digital caliper (Mitutoyo UK Ltd, Hampshire, UK; accuracy up to 0.01 mm) was used to measure tooth movement between mesial of the canine bracket to V- bend, and distal end of the canine bracket to the mesial end of the premolar bracket. A mean value was calculated.

Patient preparation

Leveling and alignment was completed by 3.5 months (average) of the start of treatment. After anchorage preparation and bite opening, canine retraction 0.019×0.025 stainless steel wires were ligated. The first premolar extractions were done. The canine retraction was initiated 3 months later so that the bone remodeling occurring due to the healing socket should not influence our study. No force was applied on the left maxillary canine (control site) during the experimental period. Canine retraction on the experimental side canine was initiated using sentalloy closed coil spring (9 mm) of 100 g force manufactured by GAC (Central Islip, NY, USA).

Experiment design

Thorough oral prophylaxis was done 1 week prior to collection of samples. All patients complied with strict

oral hygiene instructions to rinse twice daily with 0.5 ounces of 0.2% chlorhexidine gluconate twice a day throughout the study period. Patients were not allowed to take any medications or drugs including NSAIDS during the study period.

Gingival crevicular fluid collection

Gingival crevicular fluid was collected using volumetric micropipettes of 1 μl capacity (Borosil Company Limited, New York, NY, USA). Before the sample collection gingival massage was given to stimulate the GCF. The patient was asked to gargle vigorously with a glass of sterile water to cleanse the oral cavity. The isolation of the teeth was obtained using a self-retaining retractor, suction and cotton rolls. The micropipette was placed extracrevicularly and 1 μl of GCF was collected from mesial and distal sides of the active and control sites. In case of inadequate sample, collection was done twice or thrice.

Sample preparation

One microliter of GCF was diluted to 100 μl with Sorensens media containing 0.05% bovine serum albumin in phosphate-buffered saline (pH 7.0). The samples were centrifuged at 2000 rpm in a refrigerated microcentrifuge for 1 min to remove the bacterial and cellular debris. The samples were analyzed immediately or stored at −70°C after adding a drop of acetic acid stabilizer.

Assay of alkaline phosphatase activity

The enzyme activity was assayed using quantitative kits (Boerhinger-Mannheim, Mannheim, Germany). The buffer used was a combination of diethanolamine [1 mol/l (pH 9.8)] and magnesium chloride (0.5 mmol/l). The substrate comprised of *N*-nitrophenylphosphate (10 mmol/l).

The reagent solution was prepared by dissolving the buffer and the substrate. One microliter of the reagent solution was added to the GCF sample and the absorbance measured in a spectrophotometer at 405 nm. Readings were noted immediately after initiation of the reaction (A1), 1 min later (A2), 2 min later (A3) and 3 min later (A4). The change in absorbance was noted by summation of the changes over the 3-min period starting from A1 to A4 [(A2 − A1) + (A3 − A2) + (A4 − A3)] and was designated as delta A. Mean change in absorbance per minute was calculated (delta A/min).

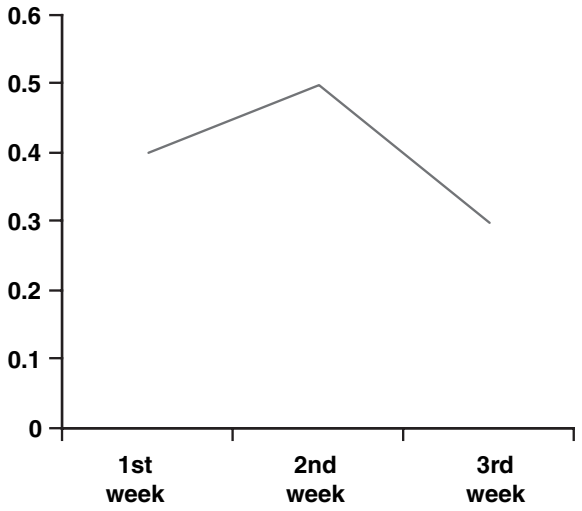

Fig. 1. Amount of tooth movement measured in mm.

Total alkaline phosphatase activity was calculated using the formula:

$$U/L = 3300 \times \text{delta/min}$$

The rate of tooth movement on the experimental site was 0.4 mm in the first week, 0.5 mm in the second and 0.3 mm in the third week after initiation of canine retraction with an average of 0.4 mm/week (Fig. 1).

A master chart was prepared for the enzyme activity, according to the readings obtained in the spectrophotometer. The mean level of alkaline phosphatase activity was calculated and the standard deviation of the mean values of the enzyme at the two sites was determined. ANOVA and LSD multiple comparison tests were done for comparison of enzyme activity among the predetermined time intervals. The SPSS computer program was used to carry out the statistical evaluation.

Results

Alkaline phosphatase activity at mesial and distal surfaces of experimental and control sites

The pretreatment basal alkaline phosphatase activity on the mesial surface was higher on the control site when compared with the experimental site (Table 1). The correlation between the enzyme activity on day 0 and day 1 was not statistically significant ($p = 0.9071$).

Table 1. **Mean alkaline phosphatase activity**

	Alkaline phosphatase activity (IU/l) ± SD			
	Experimental site		Control site	
	Mesial surface	Distal surface	Mesial surface	Distal surface
Pre-treatment	12.430 ± 5.658	12.760 ± 7.428	14.520 ± 6.555	12.980 ± 7.564
Day 0	12.210 ± 5.325	12.210 ± 5.738	10.120 ± 6.953	13.970 ± 9.520
Day 1	16.390 ± 6.568	13.750 ± 8.727	11.770 ± 7.479	11.550 ± 7.447
Day 7	27.170 ± 12.446	17.600 ± 9.086	12.210 ± 5.738	11.770 ± 7.727
Day 14	52.360 ± 16.964	21.560 ± 10.793	11.990 ± 6.444	12.320 ± 6.576
Day 21	21.230 ± 7.916	17.160 ± 7.919	13.310 ± 6.297	12.760 ± 6.942

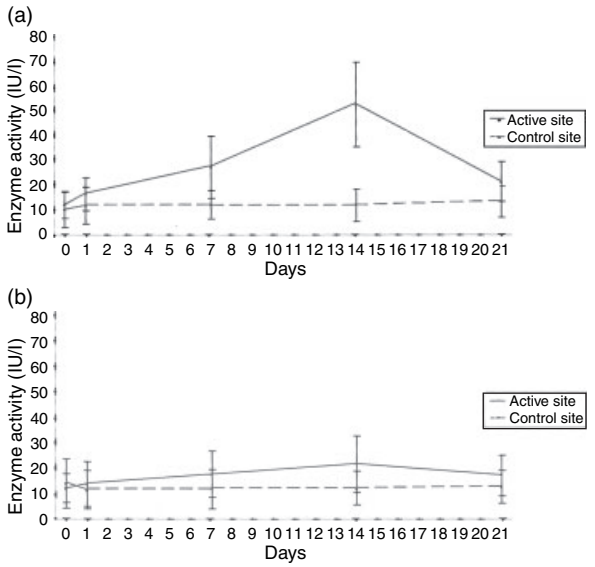

Fig. 2. (a) Mean alkaline phosphatase activity – mesial surface; (b) mean alkaline phosphatase activity – distal surface.

However on 7th day enzyme activity on the experimental site was 2.2-fold higher than that on the control site ($p < 0.05$) and this rose to 4.4-folds ($p < 0.05$) on the 14th day (Table 1, Fig. 2a). On the 21st day, the enzyme activity showed a steep fall (60%) at the experimental site even though it was still 1.6-fold higher when compared with the control site ($p < 0.05$) (Table 1, Fig. 2a).

The basal enzyme activity at the distal surface was slightly higher at the control site when compared with the experimental site (Table 1, Fig. 2b). Day 0 and day 1 enzyme activity did not show any significant differences. On 7th day enzyme activity at the experimental site was 1.5-fold higher than that at the control site ($p < 0.05$) (Table 1, Fig. 2b). The 14th day enzyme activity at experimental site was 1.75-fold the activity when compared with the control site ($p < 0.05$). On the 21st day, however, the enzyme activity on the experimental site dropped to 1.3 times the activity at the control site ($p < 0.05$) (Table 1, Fig. 2b).

The patients in the study sample ranged between 12 and 21 years of age (Table 2). Most patients belonged to a middle socioeconomic group, which ruled out the effect if any of variation in nutritional status of these patients on the enzyme levels. The weight to height ratio was also considered and related to the level of activity. It was found that in all patients the weight to height ratio inversely correlated to the difference in the maximum and minimum level of enzyme activity. The difference in enzyme activity was related to the duration of leveling.

Discussion

Biochemical analysis of the GCF can provide valuable information on the underlying changes in periodontium. Out of more than 50 components of the GCF studied to date, alkaline phosphatase, beta-glucuronidase, aspartame transaminase, prostaglandins and immunoglobulin G4 have been shown to be related to periodontal disease progression (8,9).

Orthodontists employ different mechanics to move teeth but some individuals exhibit rapid tooth movement while in others tooth movement is slower. Analysis of the enzymes associated with bone metabolism such as acid and alkaline phosphatase could be indicators of the histological and biochemical changes in bone turnover and therefore the rate/amount of tooth movement. Alkaline phosphatase activity in GCF can be influenced

Table 2. **Enzyme activity and related parameters**

Patient no.	Age (years)	CVMI score (% of growth left)	Socioeconomic status	Duration of pre-canine retraction phase (days)	Weight/height ratio (kg/cm)	Alkaline phosphatase activity (max – min) (IU/l)
1	15	5 (5–10)	Upper middle class	174	64/171 (0.374)	52.8 – 2.2 = 50.6
2	21	6 (minimal)	Upper middle class	71	56/148 (0.378)	45.1 – 2.2 = 42.9
3	16	6 (minimal)	Upper middle class	85	60/164 (0.366)	63.8 – 5.5 = 58.3
4	17	6 (minimal)	Middle class	135	69/163 (0.423)	38.5 – 7.7 = 30.8
5	19	6 (minimal)	Lower middle class	140	52/154 (0.337)	75.9 – 6.6 = 69.3
6	19	6 (minimal)	Lower middle class	54	65/164 (0.396)	61.6 – 22.0 = 39.6
7	16	5 (5–10)	Middle class	142	70/169 (0.414)	41.8 – 3.3 = 38.5
8	13	3 (25–65)	Middle class	81	67/154 (0.435)	33.0 – 4.4 = 28.6
9	13	3 (25–65)	Lower middle class	106	42/110 (0.381)	45.1 – 5.5 = 39.6
10	12	2 (65–85)	Poor	67	56/162 (0.345)	75.9 – 11.0 = 64.9

by alveolar bone turnover events and therefore reflects metabolic changes in the periodontium (10–13).

The duration in this study was 21 days and the time interval of collection for GCF was programmed so as to identify and understand the enzymatic changes occurring during the early stages of orthodontic tooth movement and to coincide with initial and lag phases of tooth movement. The pre-canine retraction enzyme activity served as a control, as well as a guide to the actual phase of movement as the base edgewise wire was passively left in the mouth for at least 3 months following leveling prior to initiation of canine retraction. The immediate changes occurring in the periodontium and the rapid tooth movement within the periodontal ligament space was best estimated by finding out the enzyme activity immediately after canine retraction and the first day of force application. The 7th day enzyme activity is expected to coincide definitively with the lag phase of tooth movement as the hyalinization sets in, and the 14th and 21st day enzyme activity are pointers to the continuity of this phase or the beginning of the post-lag phase. Considering the above facts, the time interval of this study was selected as pretreatment, day 0, day 1, day 7, day 14 and day 21.

Various authors have given different magnitude of optimum force for canine retraction (14–18). Most agree to 100 g of constant force as the optimum. Therefore, we used a 100-g sentalloy spring (GAC). The Iso ALP kit manufactured by Boerhinger-Mannheim for small sample volumes was used keeping in mind the sensitivity of the kit (19).

It has been reported that bone remodeling may continue for long period after the appliance decay (20). This has a significant influence on our study as the canine retraction on a base arch wire which was left in mouth unactivated for 1 month only. The residual bone activity could be responsible for the large variation in pretreatment enzyme activities at experimental and control sites. Even though the control tooth was in the same patient and in a similar intraoral environment yet the intrabone environment was variable. Thus the pretreatment enzyme activity level is not the true basal activity of the enzymes in the sulcus as the dynamics of bone remodeling continued even 1 month after ligation of canine retraction archwire. There could be several reasons for the variable pretreatment enzyme activity. It could be due to the difference in the total time required to achieve leveling. Leveling and precanine retraction mechanics (bite opening and anchorage preparation) were complete by 3.5 months (mean) of beginning of treatment. However, the duration of pre-canine retraction phase varied from 54 to 174 days (in patient 6 and 1, respectively) (Table 2). It appears that if the leveling phase took longer, the enzyme activity was less due to normalizing of the enzyme activity, which had risen during leveling. In other words, in-patients where leveling was achieved faster, the pre-canine retraction enzyme activity was high. Thus in patients where pretreatment and day 0 activity was high, faster tooth movement could be expected as the bone was in a favorable dynamic state. High enzyme activity is an expression of greater cellular activity. It

was observed that the alkaline phosphatase activity peaked on the 14th day in most patients, followed by a sharp fall by the 21st day. The fall in activity is related to removal of the hylanized zone. Yokoya et al. (21) reported that osteoclasts on the pressure side increased in number up to the 7th day but fell rapidly by the 14th day. When the enzyme activity was high the tooth movement rate was greater. This implies that the alkaline phosphatase activity followed the rate of tooth movement during the initial phases.

In the hard bony tissues alkaline phosphatase has been implicated in the process of mineralization. However, few (22–24) believe that the enzyme is involved in the synthesis and laying down of the organic matrix only. Active osteoblasts and osteocytes give an intense staining reaction for alkaline phosphatase. No enzyme activity is found in bone matrix *per se* except in close association with matrix synthesizing cells. Alkaline phosphatase activity is found at much higher levels in the periodontal ligament than in other connective tissues (25). The alkaline phosphatase activity decreases with rise in compressive force. Thus the fall in activity from day 0 to day 1 on distal surface of the experimental site in-patients 1, 2, 4 and 9 was probably due to orthodontic forces being heavier than the physiological range. This was the initial response of the periodontal ligament fibroblasts.

A study on rats concluded that alkaline phosphatase activity in the tension side of the periodontal ligament showed a significantly higher increase than that in the alveolar bone from these zones (20). The osteogenic cells in the periodontal ligament respond to the tensional forces with an increase in the maturation rate. In the periodontal ligament the fibroblast proliferation and collagen has been shown to increase in the tension sites. In addition, the osteoprogenator cell pool responds by increased proliferation and differentiation. The second messengers thus transmit the responses from the periodontal ligament fibroblasts to the osteogenic cells. In contrast to the tension sites on the pressure side a decrease in alkaline phosphatase activity is observed. The alkaline phosphatase activity is low in the compressed hyalinized zones of the periodontal ligament. The osteoblast is now perceived as the cell that regulates both the formative and resorptive phases of the bone remodeling cycle in response to hormonal and mechanical stimuli (26). The bone remodeling process is more complex with resorptive activity initially (3–5 days) and is followed by its reversal (5–7 days). Subsequently, a late phase of bone deposition (7–14 days) occurs in both tension and pressure sites of the alveolar wall. In the early phases bone resorption is more than bone deposition, but in the later phase, resorption and deposition become synchronous (13). This might be due to increased acid phosphatase activity that has been observed in the early phases of orthodontic tooth movement. High levels of alkaline phosphatase have been described after 7 days, when bone deposition begins. In this study too alkaline phosphatase activity at the experimental site increased significantly before peaking on day 14 (6).

In this study, the alkaline phosphatase activity on the experimental and control sites on the mesial surface showed a consistent trend in almost all the patients. This finding is substantiated in literature (7,13,20) where late changes in activity of alkaline phosphatase have been reported. It is obvious that as a forerunner to bone formation the number of fibroblasts and osteoblasts increase in areas of tension. This increase occurs as a result of increase in cell number by mitotic cell division. In histologic studies it has been observed that in marginal tensional areas cell proliferation occurs between 36 and 50 h and lasts for 10 days or 3 weeks. The tension causes shape changes and osteoblasts move slightly apart. Shortly after cell proliferation osteoid is deposited on the tension side which fills the lacunae in 80–120 days. The rapid formation of osteoid is marked during the secondary period after the undermining bone resorption on the pressure site is completed. But the fall in activity on the 21st day does not conform to other studies in literature (7,13,21). It is also difficult to explain increased levels of alkaline phosphatase activity at 7 days; this is early for osteoblast activation. However, it could be influenced by the magnitude of force exerted by the sentalloy coil spring.

Although the immediate day 1 response may be varied depending on the cytoskeletal features of the osteoblasts and fibroblasts, day 7, 14 and 21 response is likely to fall into a fixed pattern. The pattern is an initial steep rise from day 7 to day 14 and then a fall (in most cases the enzyme activity on the 21st day is still higher than the day 1 activity). The result offers a clinical asset in cases where an extremely long lag phase is observed or patient complains of pain and mobility after application of orthodontic force.

The alkaline phosphatase activity on distal surface of the experimental as well as control site exhibited unexpected levels. On the compression side bone resorption would occur and osteoclastic activity would be high with little or no osteoblastic activity. However, alkaline phosphatase activity followed a similar trend as on the mesial side (although activity was much less and the rise and fall in activity was subtle). The predominant bone remodeling activity at the early times in a bone remodeling cycle is resorptive. However, alkaline phosphatase is not a resorptive enzyme. These findings may indicate significant role played by osteoblasts in bone formation and resorption. Alkaline phosphatase activity has been reported to be high in osteoblasts and osteocytes (osteoprogenator cells) (27, 28). These stem cells (osteoprogenator cells) could perhaps be responsible for the increased activity on the resorption site. It is also being presumed that the differentiating stem cells into the osteoclastic cell types might retain the alkaline phosphatase producing potential. What needs discussion is that the increases in alkaline phosphatase activity could be due to increased enzyme activity per cell or greater numbers of bone forming cells. However, this can be substantiated only histologically.

When an attempt was made to relate the enzyme activities with other influencing biological factors (Table 2) no significant correlation was found with the socioeconomic status as well as dietary habits of the patient. No correlation could be derived from the age and state of skeletal maturity of the patient, except in-patients with higher weight to height ratio, the difference (max − min) in the enzyme scores was inversely related. But the sample size is too small to derive any significant biological and clinical inference from such a finding.

Conclusions

Under healthy gingival conditions, alkaline phosphatase activity could possibly be a biological indicator of the activity in the periodontium and therefore orthodontic tooth movement. It can be concluded that:

(1) The calorimetric technique can be reliably employed for estimating the alkaline phosphatase activity in the GCF during orthodontic tooth movement in patients free from periodontal disease.

(2) There was a significant variation in the alkaline phosphatase activity in GCF on the side undergoing canine retraction (100 g force) when compared with the control site (0 force).

(3) In this study, the maximum change in alkaline phosphatase activity occurred on the 14th day of force application followed by a fall in activity.

The study has limitations in terms of sample size, sex and duration. The study is limited to female patients, in a sample of 10 patients and the duration is of 21 days. The findings of this study need further corroboration on a larger sample size including both sexes and for a longer duration.

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