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ASSESSMENT OF THE LEVELS OF ALKALINE PHOSPHATASE AND CATHEPSIN B IN GINGIVAL CREVICULAR FLUID DURING ORTHODONTIC CANINE RETRACTION

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Abstract

Introduction: The biochemical activities of gingival crevicular fluid (GCF) suggest the various alterations that occur in the gingival tissue during the orthodontic tooth movement. Alkaline phosphatase (ALP) and Cathepsin-B (CAB) act as markers in bone metabolism. This longitudinal study was conducted to examine the activity of ALP and CAB in GCF.

Materials and Method: A split mouth study was conducted using both left and right maxillary quadrants for 20 sites in 10 subjects. After 6 weeks of placement of archwire, the canine retraction was initiated bilaterally on the 0.019x0.025" stainless steel base archwire. The initial force of approximately 200 gm was applied by using NiTi closed coil spring.

GCF was collected using calibrated micropipette from mesial and distal sites of maxillary canines at different time intervals: T_0 (before extraction), T_1 (one hour prior to retraction), T_2 (after 1 day), T_3 (after 7 days), T_4 (after 14 days) and T_5 (after 21 days). After collection, GCF was transferred to eppendorf tube and stored at -20^{0}C.

Results: Both ALP and CAB achieved peak level after applying the force for 24 hours followed by a significant reduction at 1st, 2nd and 3rd week.

Conclusion: The levels of both biomarkers achieved peak level after the first day of force application followed by a significant decrease at 1 week, 2 weeks and 3 weeks. After 3 weeks, it was reduced to the

initial level i.e. before application of retraction force. CAB was shown to have higher levels as compared to ALP at all time intervals.

Keywords: ALP, CAB, GCF, orthodontic canine retraction.

Introduction

Orthodontic tooth movement (OTM) results from the response of periodontium to the orthodontic force through receptor cells signaling cascades causing bone remodeling.[1]

While applying the orthodontic forces, the periodontal tissues respond to the mechanical stimulus by altering the flow of blood and the localization of the electro-chemical environment by secreting various biologically active biomarkers which result in biochemical and cellular reactions through the generation and propagation of signaling cascades. These biomarkers are associated with inflammatory process, bone remodeling, PDL changes, vascular and neural responses.[2]

The rate of flow of GCF is related to the degree of inflammation of gingiva. This inflammation occurs due to prolonged application of mechanical forces that trigger the cascade of biological activities which are related to the remodeling of bone.[3]

Osteoblasts are associated with ALP activity. Thus, variations in the levels of ALP in serum as well as in bone can be utilized as markers for bone metabolism.[4] The lysosomal cysteine protease Cathepsin-B play a vital role in initiation and perpetuation of inflammatory processes and hence is required for the generation of resorption lacunae by degradation of intra-cellular and extra-cellular collagenous and non-collagenous matrix. [5,6]

The following study was conducted to evaluate and compare the levels of ALP and CAB in GCF during orthodontic canine retraction by enzyme-linked immunosorbent assay (ELISA), utilizing the miniaturization in chip format to quantify vast quantities of proteins in a smaller sample volume thereby providing a captivating approach for GCF. [7]

Materials and Method

10 participants were selected who were requiring the fixed orthodontic therapy with first premolar extraction. The inclusion criteria includes the good general as well as the periodontal health i.e. <3 mm of probing depth values in the entire dentition with no evident radiographic signs of bone loss.

Informed consents were taken from the patients and the parents for the patients who were minors. The participants had not taken any anti-inflammatory drugs during the month preceding the study. The participants with mixed dentition, poor periodontal health, craniofacial anomalies, conditions like pregnancy, lactation, with any known systemic disease or medically compromised condition influencing ALP and CAB were excluded.

A split mouth study was conducted using both left and right maxillary quadrant making it to a total of 20 sites for the study. The subjects were bonded with 0.022x0.028" MBT prescription brackets, following maxillary first premolar extraction. Arches were aligned and levelled with a series of NiTi archwires followed by 0.019x0.025" stainless steel archwire which was left in place for 6 weeks following which the canine retraction was initiated using the NiTi closed coil spring delivering a 200 gm of force. A dynamometer was used to measure the force (Figure 1).

GCF was collected at different time intervals i.e. at T_0 (before extraction), T_1 (1 hour prior to retraction after completion of leveling and alignment), T_2 (after 1 day), T_3 (after 7 days), T_4 (after 14 days), T_5 (21 days after the initiation of canine retraction). T_0 (before extraction) served as a baseline (Control). A sample of 1 µl GCF was collected with the help of pre-calibrated micropipette and then transferred in eppendorf tube containing 100 µl of PBS (Figures 2 & 3). These eppendorf tubes were stored at -20^0 C till the analysis was conducted.

Sample preparation

ALP ELISA TEST: The microtiter plate wells were coated with purified human ALP antibodies and thereafter the solid-phase antibody was created. The samples were added to the well and then combined with Human ALP antibody. The antibody-antigen-enzyme-antibody complex was created with the combination of Human ALP antibody and the Horseradish peroxidase (HRP) enzyme, thereafter it was washed thoroughly. Tetramethylbenzidine (TMB) substrate solution was added to this complex, due to which this substrate displayed the bluish shade at HRP enzyme-catalyzed reaction. This reaction was terminated by adding the sulphuric acid solution due to which the colour of the solution changed to the yellow, which was quantified spectrophotometrically at 450 nm of wavelength. Human ALP concentration was then determined in the samples by correlating the optical density (OD) of the samples to the standard curve.

CAB ELISA TEST: The microtiter plate wells were coated with the Purified Human CAB antibody due to which the solid phase antibody was produced. Thereafter the samples were added to the wells. The antibody-antigen-enzyme-antibody complex was created by the combination of Human CAB antibody and Horseradish peroxidase, and thereafter it was washed thoroughly. Tetramethylbenzidine (TMB) substrate solution was added to this complex giving the solution a bluish shade at HRP enzyme-catalyzed reaction. This reaction was then terminated by adding the sulphuric acid due to which the colour of the solution turned into a yellowish hue that was subsequently measured spectrophotometrically at a 450 nm of wavelength. Human CAB concentration was then determined in the samples by correlating the optical density of the samples to the standard curve.

Results

SPSS version 24.0 was used to analyze the data. The level of significance was set at $p \leq 0.05$. Paired t-test was used for estimating the relative change with respect to time for the study variables and unpaired t-test was utilized for assessing the difference among the groups.

The level of ALP increased gradually from its pretreatment value at T_0 (before extraction) and T_1 (1 hour prior to retraction after completion of leveling and alignment) to T_2 (after 1 day) achieving the peak value and started decreasing at T_3 (after 7 days), T_4 (after 14 days) and T_5 (21 days after the initiation of canine retraction) getting back to initial value.

ALP activity was recorded to have statistically significantly high at time points T_2, T_3 and T_4 when compared to the baseline level but showing non-significant difference at time points T_1 and T_5 from baseline (Tables 1-3). ALP levels at T_0 and T_1 were statistically insignificantly different but were found to have statistically significant progression from T_1 to T_2. Statistically insignificant change was noted from T_2 to T_3. The statistically significant reduction was recorded from T_3 to T_4 & T_4 to T_5.

Significant changes were observed in the levels of ALP from baseline to time point T2, T3 and T4 but showing non-significant difference at time point T1 and T5 from baseline as p-value is greater than 0.05 in this case (Table 1).

The levels of CAB increased gradually from its pretreatment value at T_0 (before extraction) and T_1 (1 hour prior to retraction after completion of leveling and alignment) to T_2 (after 1 day) achieving the peak value and started decreasing at T_3 (after 7 days), T_4 (after 14 days) and T_5 (21 days after the initiation of canine retraction) getting back to initial value (Tables 4-6).

CAB levels were recorded to be statistically significantly high at time points T_2, T_3 and T_4 when compared to the baseline level but showing non-significant difference at time points T_1 and T_5 from baseline. ALP levels at T_0 and T_1 were statistically insignificantly different but were found to have statistically significant progression from T_1 to T_2. Statistically significant reduction from T_2 to T_3 was noted. The recorded statistically insignificant changes were observed from T_3 to T_4 and statistically significant decrease was recorded from T_4 to T_5.

On comparing ALP and CAB, there is significant difference between mean ALP and CAB at all time points (Table 7). Figure 4 represents the Mean ALP and CAB levels.

Discussion

The following prospective study was planned to examine the relationship between the orthodontic tooth movement and the applied force at a weekly basis by determining the ALP and CAB activities in GCF by performing ELISA on the biomarkers activity. ALP and CAB enzyme activity were assessed in 20 subjects during the canine retraction done by NiTi closed coil spring. The study was conducted for 21 days and GCF was collected at different time intervals T_0 (before extraction), T_1 (one hour prior to retraction), T_2 (after 1 day), T_3 (after 7 days), T_4 (after 14 days) and T_5 (after 21 days). The underlying purpose was to recognize the changes in the enzymatic activities occurring throughout the initial stages of OTM and to investigate them with initial and lag phases of OTM. Pre-extraction enzymatic activity was taken as a control (T_0), and as a guide to the actual phase of tooth movement when the base archwire was kept for 3 weeks passively in the oral cavity followed by the leveling of the arches.

Davidovitch et al stated that when orthodontic appliances are inserted in the oral cavity, various mechanical stresses are created that leads to the secretion of biologically active substances, expressed by the cells of the periodontium.[8] Osteoblasts show the ALP activity and enzymatic changes in the serum and bone which serve as markers for the metabolism of bone in the numerous disorders. Variations in alveolar bone in the localized areas can be revealed by the rise in ALP in the GCF.[4]

Osteoclasts resorbs the bone by reducing the pH in the resorption lacuna following which the proteolytic enzymes are secreted. The extracellular components like collagen and protein turnover in the lysosomal system are degraded by the CAB enzyme.[9,10] According to the various investigations, majority of times the long-lasting proteins are only degraded by cathepsin and the lysosomal enzymes.[11]

Goseki et al conducted an in-vitro study and stated that the action of CAB is enhanced by the progress of cellular ageing in the cells of periodontium, indicating that the periodontal cells play a vital role in the generation of this CAB enzyme.[12] Another study by Ishibashi et al quoted that the resorption of bone occurring during the OTM is also influenced by the activity of CAB enzyme.[13] In addition to this, various previous studies stated that the CAB plays a crucial role in initiating and continuing the bone resorption process.[14,15,16]

Sugiyama et al investigated the levels of cathepsin B in GCF for 10 patients throughout the OTM and concluded that cathepsin B levels were highest at 24 hours of OTM.[15] The finding of this study was in agreement of our study, which reported peak value of CAB at 1 day.

In our study, the levels of both ALP and CAB remained increased following 1 day, 1 week and 2 weeks after application of retraction force of 200 gms bilaterally as compared to the initial level i.e. before application of retraction force but later reduced to the initial level at 3 weeks. Both ALP and CAB attained peak level after 1 day of force application following which both the biomarkers recorded significant decrease at 1 week, 2 weeks and 3 weeks. After 3 weeks, it was reduced to the initial level i.e. before application of retraction force.

This pattern of biomarkers is in agreement with previous research done by Wahab et al, Sugiyama et al who further noticed the reduced levels of their respective biomarker on 21st day on experimental site which is associated with the force applied during OTM in response to the periodontal tissues.[15,17]

It was found that the CAB enzyme level was always higher than ALP levels which can be best explained with the help of "*Biphasic Theory of Tooth Movement"* that states the catabolic phase precedes the anabolic phase, where catabolic phase determines the osteoclast-driven bone resorption and anabolic phase determines the osteoblast-driven bone formation.[18]

Insoft et al performed a study on 3 female patients, where they illustrated the action of phosphatase enzyme after applying 100 gm of force. They revealed that the highest level of phosphatase activity is observed between 1st and 3rd week, followed by a gradual decrease.[19] Our study showed increase in ALP at 1 day, 1 week and 2 weeks after application of retraction force but later reduced to the initial level at 3 weeks. However, the peak of ALP activity was recorded after 24 hours.

Perinetti et al assessed the ALP levels on 28th day of canine retraction which was performed by using NiTi open coil spring that exerted the 150 gm of force and showed the elevated levels of ALP in samples of GCF.[20]

A study was conducted by Batra et al to determine the levels of ALP in GCF during maxillary canine retraction using 100 gm force by using sentalloy springs. ALP activity was determined by estimating the GCF levels from mesial and distal aspects of canine before starting the canine retraction (baseline), immediately after initiating the retraction and on 1st, 7th, 14th and 21st day. The results showed the highest level of enzymatic activity on the 14th day of retraction and thereafter ALP activity was reduced especially on the mesial aspect of the tooth.[21]

Wahab et al observed the peak activity of ALP at 2 weeks in comparison with the control by applying the force of 100 gm whereas with 150 gm of force, the peak activity was reached within the one week of force application.[22] These studies are in contrast to our study as we found that ALP achieved peak level after 1 day of force application following which the significant decrease was recorded at 1 week, 2 weeks and 3 weeks. After 3 weeks, it was reduced to the initial level i.e. before application of retraction force.

Thus, the present study suggests that the levels of ALP and CAB could be used as the biological indicators of orthodontic tooth movement.

Conclusion

1) No notable variations in the levels of ALP and CAB are observed when measured at post-alignment stage as compared to that before extraction.
2) Both ALP and CAB achieved peak level after 1 day of force application following which both the biomarkers recorded significant decrease at 1 week, 2 weeks and 3 weeks. After 3 weeks, it was reduced to the initial level i.e. before application of retraction force.
3) CAB was shown to have higher levels as compared to ALP at all time intervals.

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Note: There is no conflict of interest.

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Table 1: Descriptive Statistic analysis of ALP levels (ng/ml) at different time from T0 – T5 (n=20)

ALP	Mean	Std. Deviation	SE	95% Confidence Interval		Range	
				Lower Bound	Upper Bound	Minimum	Maximum
T0	7.04	0.34	0.075	6.877	7.193	6.4	7.5
T1	6.87	0.45	0.101	6.658	7.082	6.2	7.7
T2	7.88	0.48	0.107	7.650	8.100	7.3	8.8
T3	7.62	0.39	0.086	7.435	7.795	7.1	8.6

T4	7.33	0.34	0.076	7.171	7.489	6.9	8.1
T5	6.99	0.48	0.108	6.758	7.212	6.2	8.2

Table 2: P value of Change in Alkaline phosphatase(ng/ml) at different time from T0(n=20)

ALP	T0	T1	T2	T3	T4	T5
P value		0.24	<0.001***	<0.001***	0.007**	0.711

P<0.05 Significant*, P<0.01, Highly Significant**, P<0.001 Very highly Significant***

Table 3: comparison of Alkaline Phosphatase (ng/ml) at different time intervals (n=20)

ALP	Paired Differences					Sig.(2tailed)	Cohen's d
	Mean Difference	Pooled Std. Deviation	Std. Error	95% Confidence Interval of the Difference			
				Lower	Upper		
T0 v/s T1	-0.1650	0.6020	0.1346	-0.4467	0.1167	0.999	0.2740
T1 v/s T2	1.0050	0.4861	0.1087	0.7775	1.2325	**<0.0001****	2.0674
T2 vsT3	-0.2600	0.3560	0.0796	-0.4266	-0.0934	0.061	0.7303
T3 v/s T4	-0.2850	0.2323	0.0519	-0.3937	-0.1763	**<0.0001****	1.2268
T4 v/s T5	-0.3450	0.2964	0.0663	-0.4837	-0.2063	**0.001*****	1.1639

* P=.05; ** P=.01; *** P=.001; ****P=.0001

Table 4: Descriptive Statistic analysis of Cathepsin-B levels (ng/ml) at different time from T0 – T5 (n=20)

Cathepsin	Mean	Std. Deviation	SE	95% Confidence Interval		Range	
				Lower Bound	Upper Bound	minimum	Maximum
T0	13.26	1.19	0.26	12.70	13.82	10.7	14.6
T1	13.25	0.99	0.22	12.78	13.71	11.4	14.9
T2	15.14	1.05	0.24	14.65	15.63	13.7	16.8
T3	14.46	1.25	0.28	13.87	15.04	10.9	16.7
T4	13.94	0.69	0.15	13.62	14.26	12.9	15.7
T5	13.30	0.83	0.19	12.92	13.69	11.9	15

Table5: P value of Change in CAB (ng/ml) at different time from T0(n=20)

CAB	T0	T1	T2	T3	T4	T5
P value		0.94	<0.001***	<0.001***	0.005**	0.852

* P=.05; ** P=.01; *** P=.001; ****P=.0001

Table 6: comparison of CAB (ng/ml) at different time intervals (n=20)

Cathepsin							
	Paired Differences					Sig.(2tailed)	Cohen's d
	Mean Difference	Pooled Std. Deviation	Std. Error	95% Confidence Interval of the Difference			
				Lower	Upper		
T0 v/s T1	-0.02	0.82	0.18	-0.40	0.37	0.999	0.0182
T1 v/s T2	1.89	0.76	0.17	1.54	2.24	**<0.0001*** **	2.5030
T2 v/s T3	-0.69	0.69	0.16	-1.01	-0.36	**0.005****	0.9789
T3 v/s T4	-0.51	1.09	0.24	-1.03	-0.01	0.726	0.4717
T4 v/s T5	-0.64	0.67	0.15	-0.95	-0.32	**0.006****	0.9541

* P=.05; ** P=.01; *** P=.001; ****P=.0001

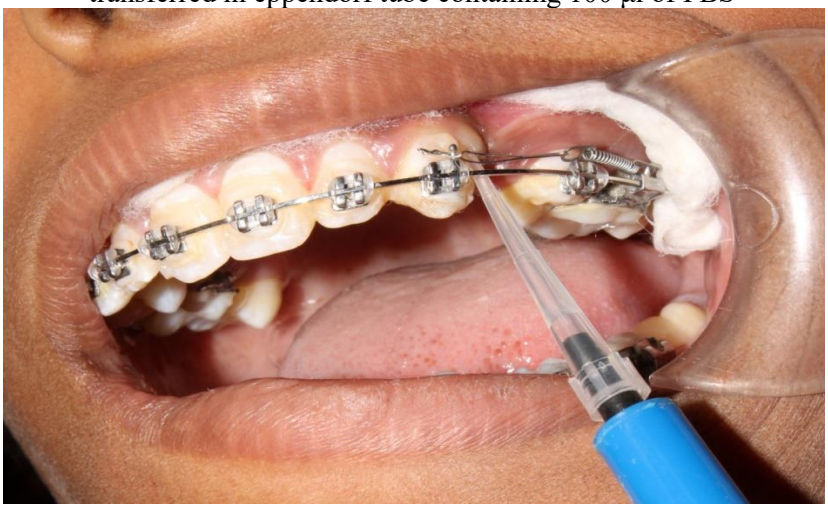
Table 7: Represents the comparison of mean values of ALP and CAB((ng/ml) at different time intervals (n=20).

Group Statistics					
	Group	N	Mean	Std. Deviation	p-value
T0	ALP	20	7.04	0.34	<0.001
	CAB	20	13.26	1.19	
T1	ALP	20	6.87	0.45	<0.001
	CAB	20	13.25	0.99	
T2	ALP	20	7.88	0.48	<0.001
	CAB	20	15.14	1.05	
T3	ALP	20	7.62	0.39	<0.001
	CAB	20	14.46	1.25	
T4	ALP	20	7.33	0.34	<0.001
	CAB	20	13.94	0.69	
T5	ALP	20	6.99	0.48	<0.001
	CAB	20	13.30	0.83	

* P=.05; ** P=.01; *** P=.001; ****P=.0001

Figure 1: Dynamometer used to measure the force

Figures 2 & 3: A sample of 1 µl GCF was collected with the help of pre-calibrated micropipette and transferred in eppendorf tube containing 100 µl of PBS



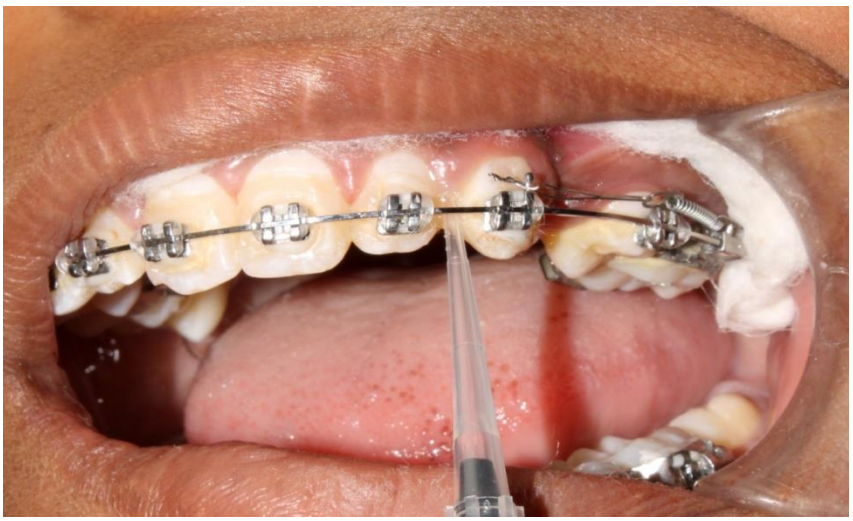


Figure 4: Mean ALP and CAB levels