Time-related salivary cathepsin B levels and periodontal status in different orthodontic force magnitudes

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ABSTRACT

Background: Biologically active substances, such as Cathepsin B (CAB) which is a lysosomalcystein protease may be involved in periodontal metabolism in the degradation of organic bone matrix containing collagen fibers in response to mechanical stress from orthodontic appliance. The aims of study were to determine and compare salivary levels of CAB, pH as well as clinical periodontal parameters (Plaque index PLI and gingival index GI) with different orthodontic force magnitudes at different time intervals.

Materials and methods: A twenty-four patients (both gender) with age range (17-23) years had Angle's Class II division 1 malocclusion with GI <0.5 enrolled in this study. The level of salivary CAB and pH, in addition to the clinical periodontal parameters (PLI and GI) were measured before (baseline), 1 hour (1H), 1 day (1D), 1week (1W), 2 weeks (2W) and 3 weeks (3W) after fixed orthodontic appliance placement with different forces applied to the teeth (F1 (40 gm), F2 (60 gm) and F3 (80 gm)).

Results: The highest mean concentration of salivary CAB was (12.057) at F1 in 1D visit with highly significant differences among the visits as well as among the forces ($p \leq 0.05$). Weak correlations were revealed between all pairs of forces as well as between each visit with the baseline visit about salivary CAB except the strong negative correlations between F1 with F3 at 3W and baseline with 3W visits at F3. Maintenance of good oral hygiene during the study period demonstrated that the highest mean values of PLI and GI were (0.2) and (0.25) respectively, in addition to the non-significant differences regarding pH among the visits.

Conclusion: The concentration of salivary CAB was increased following fixed orthodontic appliance insertion. The very light continuous orthodontic force could produce significant increase of this enzyme activity and give enough effectiveness to produce tooth movement as compared to the higher forces.

Key words: Cathepsin B, Saliva, Orthodontic Tooth Movement, Periodontal ligament. (J Bagh Coll Dentistry 2015; 27(2):115-122).

INTRODUCTION

Host response to orthodontic force alters the vascularity and blood flow of periodontal ligament (PDL), resulting in local synthesis and release of different mediators involved in alveolar bone remodelling[(1)]. These molecules can evoke many cellular responses by various cell types in and around teeth, providing a favorable microenvironment for bone resorption or apposition. Although these effects are both physical and biochemical in nature and are frequently intertwined and interdependent [(2)], the evidence has shown large inter-individual differences in both human researches [(3)] and animal experiments [(4)]. In other words, with standardized, constant, and equal forces, the rate of orthodontic tooth movement (OTM) may vary substantially, while with considerably different forces, the rates of OTM may be almost the same among and even within individuals [(5)].

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The sequence of events following OTM can be characterized using suitable biomarkers. A biomarker is a "substance that is measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [(6)].

Applying orthodontic forces to teeth will ultimately result in movement. The main phenomena, both before and after tooth movement, are alveolar bone remodelling, tissue inflammation, and root resorption. Each of these events can potentially be detected using suitable markers [(7)]. Many of the human studies regarding the biology of OTM have focused on the assessment of these biomarkers in gingival crevicular fluid (GCF) [(8-10)]; however, it is difficult to draw firm conclusions because the number of studies concerning variations in the levels of bone remodelling biomarkers through the different phases of OTM is sparse and has yielded contradictory results [(9,11)].

Cathepsin B (CAB), an intracellular lysosomal cysteine proteinase, isolated from many mammalian species [(12)]. It can decompose the extracellular components including collagen and lead to the protein turnover in the lysosomal system *in vitro* [(13)].A significant level of CAB

existed in GCF from gingivitis patients in a study conducted by Eisenhauer et al. [14]; they concluded that it might play an important role in intercellular protein catabolism in periodontal tissue.

Although the clinical follow-up examination remains the basis for patient's evaluation in addition analysis of saliva, which is a complex fluid that contains the systemically and locally derived markers, compelling reasons exist to use saliva as a diagnostic fluid. It meets the demands for in expensive, non invasive and easy to use and collect diagnostic method [15].

Since, currently, there are no reports on estimating the level of salivary CAB during treatment with fixed orthodontic appliances, this study aimed to determine and compare the levels of salivary CAB , pH and clinical periodontal parameters (Plaque index PLI and Gingival index GI),before orthodontic appliance placement at baseline and then at different five-time intervals after application of different orthodontic force magnitudes (40gm, 60gm, 80gm) according to the study design, in order to know the ongoing process occur during orthodontic treatment which can result in an appropriate choice of the mechanical loading as well as shortening the treatment period and preventing the adverse effects associated with orthodontic treatment, such as resorption of root or loss of bone.

MATERIALS AND METHODS

Human Sample

A total of twenty-four orthodontic patients (age ranged 17-23 years) ; who were attending the postgraduate clinic of Orthodontic Department at College of Dentistry/Baghdad University, were selected to participate in this study according to the following criteria:

- All patients had Angle's Class II division 1 malocclusion with no crowding or with mild crowding about 2-3mm [16]. They all required extraction of bilateral maxillary first premolar teeth as part of their orthodontic treatment.
- Smoker, drinker, pregnancy and women taking contraceptive pills were excluded from the study
- No history of any systemic diseases.
- No periodontal treatment and not use of any anti-inflammatory or antimicrobial medications 3 months prior the study period.
- Clinically healthy periodontium, mean without pocket nor attachment loss and no radiographic evidence of periodontal bone loss.
- No missing teeth.

We certify that this study involving human subjects is in accordance with the Helsinki declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

Study Design

Two weeks before orthodontic appliance placement, all patients underwent a session of motivation, oral hygiene instructions and accurate supra and sub gingival ultrasonic scaling. Oral hygiene status of the patients was determined before initiating the experimental procedures. Thus only patients with GI<0.5 [17] were included in this study.

The fixed orthodontic appliance design used in this study was according to a previous study conducted by Abdul Ameer [18] which consisted of sectional stainless steel straight wire (Ortho Technology, USA), a bracket (Roth 0.022 slot, Orthoclassic) on the maxillary first premolar which was indicated for extraction, and a molar tube (Ortho Technology, USA) on the maxillary first molar for each side. The arch wire was bent just mesial to the molar tube in a labial direction to apply a labial force on the maxillary first premolar. Those patients were divided into three groups (8 patients for each group) according to different continuous orthodontic force magnitudes applied, group F1: subjected to 40 gm force, group F2: subjected to 60 gm force and group F3: subjected to 80 gm force. This force was measured by a strain gauge (0-500 grams, Ortho Technology, USA); and the arch wire was ligated to the maxillary first premolar bracket by a stainless steel ligature wire.

Saliva Collection

The patients should not drink or eat within at least one hour before collection of the saliva. The patient asked to sit in a comfortable position and spit 5 ml of unstimulated whole saliva into sterile plane plastic test tube within 10 minutes and put in cooling box to stop the growth of bacteria. The samples were taken from each patient and salivary pH was measured by using an electronic pH meter prior to fitting the orthodontic appliance at baseline and then 1 hour (1H), 1 day (1D), 1week (1W), 2 weeks (2W) and 3 weeks (3W) after force application to the teeth.

After collection, the whole saliva was centrifuged at 3000 rpm for 20 min to remove insoluble material by using centrifuge machine. The supernatants saliva were collected by pipette into eppendrof tubes and frozen at -20^{o} C until biochemical analysis.

Clinical Periodontal Parameters Examination

Clinical periodontal parameters examination was performed according to GI [17] and PLI [19] systems after salivary sample collection at six time intervals by using Michigan O periodontal probe on four surfaces (buccal/ labial, lingual/ palatal, mesial and distal) of all teeth except 3rd molar.

Assay of Salivary CAB Level

Frozen saliva supernatant should be thawed at room temperature and salivary CAB level (mg/ml) was detected by mean of Enzyme Linked Immune Sorbent Assay (ELISA) kit (Shanghai Crystal Day Biotech Co., LTD, China) according to the manufacturer instructions.

Statistical Analysis

Statistical analyses were made with the Windows statistical software SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). Descriptive statistics including means and standard deviations (SD), and inferential statistics for inter-group comparisons including one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) and Correlation Coefficient were used. The Level of significance was set at $p \leq 0.05$.

RESULTS

Table (1) revealed that the highest mean value of PLI was (0.2) present in patients subjected to F3 at 1W visit with highly significant differences among the visits at each force. While (0.25) was the highest mean value of GI presented by subjects with F1 and F2 at 1H and 1D visits respectively with highly significant differences among the visits at F2and F3, whereas anon-significant difference at F1. The highest mean values of pH were (7.499) at F1, (7.223) at F2 and (7.299) at F3 demonstrated at 1H visits in all force groups with non-significant differences among the visits at each force.

The highest mean concentrations of salivary CAB were (12.057) and (10.445) at F1 and F2 demonstrated in 1D visit, while at F3 it was (11.788) in 2W visit, with highly significant differences among the visits at each force, as well as among the forces at each visit except the significant difference at 1W visit, as shown in (Table 2, Figure 1).

From (Table 3) highly significant differences of salivary CAB levels were revealed between all pairs of forces at each visit except the significant differences between F2 with F3 and F1 at 1D and 1W visits respectively and non-significant differences between F1 with F2 at 1H and 3W visits, as well as, between F1 with F3 at 1W visit.

Furthermore,(Table 4)illustrates the comparisons between all pairs of visits at each force regarding the levels of salivary CAB, the results were highly significant differences except the non-significant differences between 1H with 1W at F1 and F2,baselinewith 2W at F2, as well as 1H with 3W and 1D with 1W at F3.Hence a significant difference presented between baseline with 2W at F1.

Weak correlations were demonstrated between all pairs of forces at each visit about the mean concentrations of salivary CAB except the strong negative correlation between F1 with F3at 3W visit, hence, moderate negative correlation between F1 with F2 at 2W visit was revealed, (Table 5). However, the correlations between each visit with baseline visits at each force were weak except the strong negative correlation of 3W visit at F3, and the moderate correlation of 1D and 1H at F1and F2 respectively that were negative, while it was positive at 3W in F1, (Table6).

Generally, weak correlations were revealed between all pairs of visits (excluded the baseline visits) at each force, but the results showed strong and moderate negative correlations between 1H with 3W and 2W visits respectively at F1, while at F2,strong positive correlation presented between 2W with 3Wvisits, on the other hand, regarding F3, strong positive correlations demonstrated between 1D with 1H as well as, 2W with both 1D and 1W visits, while they were strong negative between 3W with 1W and 2W visits.

Table 1: Statistical analysis of PLI, GI and pH for all visits at each force

Forces	Periodontal parameters	Statistical analysis	Visits						ANOVA	
			Baseline	1 H	1 D	1 W	2 W	3 W	F-test	P-value
F1	PLI	Mean	0.000	0.125	0.175	0.138	0.113	0.088	6.575	0.000 ***
		SD	0.000	0.100	0.089	0.052	0.035	0.064		
	GI	Mean	0.000	0.250	0.150	0.112	0.237	0.137	2.372	0.055
		SD	0.000	0.200	0.169	0.124	0.243	0.159		
	pH	Mean	7.184	7.499	7.386	7.124	6.959	7.129	0.565	0.727
		SD	0.603	0.637	0.802	0.712	0.975	0.630		
F2	PLI	Mean	0.000	0.069	0.125	0.188	0.194	0.188	9.812	0.000 ***
		SD	0.000	0.088	0.071	0.083	0.062	0.083		
	GI	Mean	0.000	0.150	0.250	0.138	0.100	0.088	5.966	0.000 ***
		SD	0.000	0.120	0.139	0.092	0.093	0.064		
	pH	Mean	6.929	7.223	7.083	6.688	6.966	6.835	0.480	0.789
		SD	0.460	0.654	0.609	1.068	0.802	0.835		
F3	PLI	Mean	0.000	0.125	0.175	0.200	0.188	0.163	8.872	0.000 ***
		SD	0.000	0.089	0.046	0.076	0.095	0.069		
	GI	Mean	0.000	0.175	0.125	0.100	0.110	0.075	4.631	0.002 ***
		SD	0.000	0.139	0.089	0.076	0.011	0.046		
	pH	Mean	6.916	7.299	7.078	6.986	6.730	6.970	1.661	0.165
		SD	0.373	0.406	0.373	0.428	0.495	0.383		

***** Highly significant, **Significant, significance was set at p≤0.05**

Table 2: Statistical analysis of salivary CAB concentration (mg/ml) for all visits at each force

Visits	Salivary CAB							
	F1		F2		F3		ANOVA d.f. = 23	
	Mean	SD	Mean	SD	Mean	SD	F-test	P-value
Baseline	6.015	0.722	4.886	0.727	8.238	0.539	52.128	0.000***
1 H	8.735	0.319	8.421	0.680	10.778	0.398	54.348	0.000***
1 D	12.057	1.256	10.445	0.299	9.410	0.376	23.600	0.000***
1 W	8.814	0.603	8.053	0.739	9.224	0.800	5.467	0.012**
2 W	6.758	0.418	5.112	0.996	11.788	0.821	157.643	0.000***
3 W	4.156	0.489	3.854	0.679	10.769	0.704	306.513	0.000***
F-test	121.047		101.808		33.783		ANOVA d.f. = 47	
P-valeu	0.000***		0.000***		0.000***			

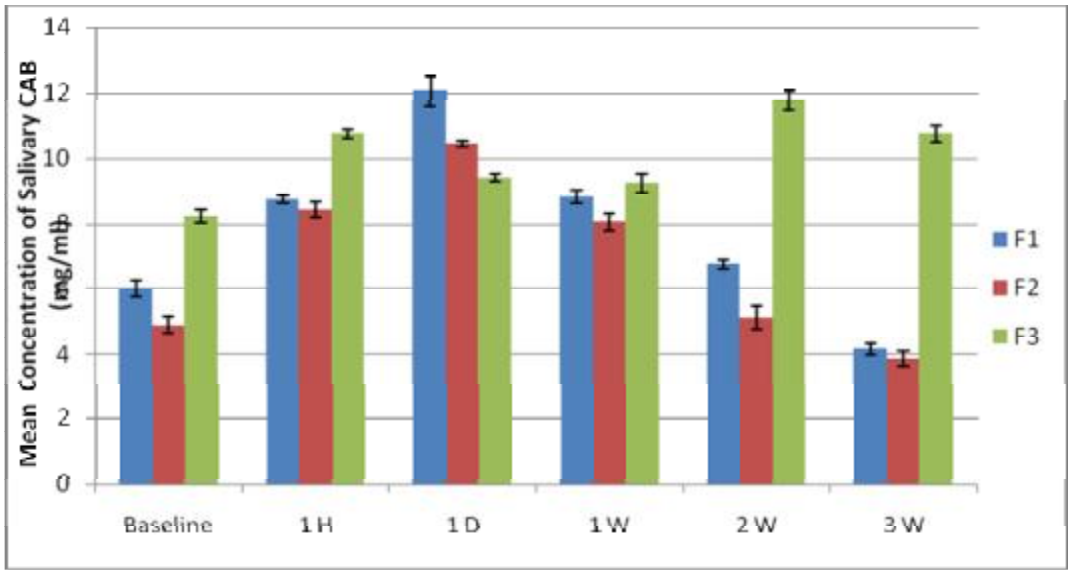

Figure 1: Mean concentration of salivary CAB (mg/ml) for the three forces at each visit. Bars represent mean ± SE: (set at 95% CI for mean).

Table 3: Inter-forces comparisons of the mean concentrations of salivary CAB at each visit

Visits	Forces		P-value
1 H	F1	F2	0.215
	F1	F3	0.000***
	F2	F3	0.000***
1 D	F1	F2	0.000***
	F1	F3	0.000***
	F2	F3	0.015**
1 W	F1	F2	0.046**
	F1	F3	0.267
	F2	F3	0.004***
2 W	F1	F2	0.000***
	F1	F3	0.000***
	F2	F3	0.000***
3 W	F1	F2	0.349
	F1	F3	0.000***
	F2	F3	0.000***

Table 4: Inter-visits comparisons of the mean concentrations of salivary CAB at each force

Visits		F1	F2	F3
		P	P	P
Baseline	1 H	0.000***	0.000***	0.000***
Baseline	1 D	0.000***	0.000***	0.001***
Baseline	1 W	0.000***	0.000***	0.003***
Baseline	2 W	0.041**	0.530	0.000***
Baseline	3 W	0.000***	0.006***	0.000***
1 H	1 D	0.000***	0.000***	0.000***
1 H	1 W	0.825	0.310	0.000***
1 H	2 W	0.000***	0.000***	0.003***
1 H	3 W	0.000***	0.000***	0.979
1 D	1 W	0.000***	0.000***	0.558
1 D	2 W	0.000***	0.000***	0.000***
1 D	3 W	0.000***	0.000***	0.000***
1 W	2 W	0.000***	0.000***	0.000***
1 W	3 W	0.000***	0.000***	0.000***
2 W	3 W	0.000***	0.001***	0.002***

Table 5: Correlations of the mean concentrations of salivary CAB between all pairs of forces at each visit

Visits	F1-F2		F1-F3		F2-F3	
	r	p	r	p	r	p
1 H	-0.311	0.454	-0.227	0.589	0.183	0.665
1 D	-0.256	0.540	0.403	0.323	-0.464	0.247
1 W	0.208	0.621	0.132	0.756	0.124	0.769
2 W	-0.587	0.126	0.226	0.591	0.166	0.694
3 W	0.006	0.989	-0.822	0.012**	-0.361	0.379

Table 6: Correlations of the mean concentrations of salivary CAB between different visits with baseline visits at each force

Visits	F1		F2		F3	
	Baseline		Baseline		Baseline	
	r	p	r	p	r	p
1 H	-0.191	0.650	-0.508	0.199	-0.150	0.723
1 D	-0.562	0.147	0.294	0.480	0.124	0.770
1 W	0.045	0.915	0.303	0.466	0.190	0.652
2 W	0.124	0.770	0.407	0.317	0.197	0.640
3 W	0.643	0.085	0.226	0.591	-0.765	0.027**

DISCUSSION

In the present study, very minimal plaque accumulation with excellent gingival health were detected during the study period, this is due to the oral hygiene instructions and motivation given to each participant before and throughout the study. It has been reported in previous studies that oral hygiene and gingival health maintenance were possible during orthodontic treatment when good knowledge, attitude, practice of gingival health were applied [20,21].

As well as, there were very low non-significant increase in salivary pH in all groups of forces throughout the study and reach its maximum level after 1H of appliance placement. This is may be attributed to the maintenance of good oral hygiene through the study period and due to the fact that there was an increase in salivary flow usually occur after placement of orthodontic appliance [22]. People experience changes in salivary function over time, and these changes have a long-term of clinical significance [23-25]. Some studies have detected associations between fixed orthodontic appliances, microbial outcomes, and measures of salivary function; hence the results were not consistent. Lara-Carrillo et al. [23] concluded in their study that orthodontic treatment changes the oral environmental factors including promotes a major salivary stimulated flow rate and increases its buffer capacity and salivary pH, whereas Alessandri Bonetti et al. [26] found in their study that after 1 year of the placement of fixed orthodontic appliances the salivary pH, buffer capacity and flow rate were not change when compared with the baseline assessment.

Previous studies showed an increase in CAB level in GCF of orthodontic patients with a higher relation to the periodontal diseases [27- 29]. Eley and Cox [27] found a positive correlation between CAB activities in GCF with clinical periodontal parameters of untreated chronic periodontitis. Then those authors found a reduction in all these parameters after periodontal treatment [28]. Therefore, prevention of plaque-induced inflammation with excellent gingival health and normal salivary pH allowed this study to focus mainly on the process of mechanical stimulation of salivary CAB in during orthodontic treatment at different force magnitudes. Various investigations considered different orthodontic duration to examine the effect of several enzymes and biological mediators in their studies, whereas in this study the appropriate duration of an orthodontic cycle was 21 days had been considered, in order to identify and understand the enzymatic changes occur during early stages of orthodontic force application in coincidence with initial and lag phases of tooth movement, therefore, in our study salivary sample collection continued until the end of 3weeks after appliance placement.

Periodontal tissue vascularity and blood flow usually affected by mechanical loading, thus result in the formation and release of different mediators locally, such as cytokines e.g. Interlukine-6, enzymes, growth factors and the neurotransmitters [7]. The biological mechanisms controlling the change from the stimulus, consist of continuous force application, to the reaction, represented by the displacement of the tooth in the periodontal space, and could be estimated by measuring the lower or higher rate of such biomarkers in GCF or saliva. A study conducted by Frodge et al. [30] showed that different mediators involved in alveolar bone remodelling are continuously washed into saliva from GCF , whole-saliva samples may constitute an easy alternative to individual gingival sulcular samples for determining analytes of bone turnover that are present within the periodontal environment, providing a sensitive and inexpensive detection technique.

Cathepsin B, play a role in the resolve of the organic matrix, the last step in resorption of bone. CAB was elevated around the osteoclasts; it involved in the degradation of exposed collagen fibers and collagen degradation by products [31]. The results from the present study regarding the overall quantitative changes of salivary CAB concentration showed significant increase in its level at all the three force groups immediately after 1H of appliance placement, this is due to the fact that OTM is purely described as an aseptical and transitory inflammatory process [1] and CAB is recognized to play an important role in the starting and continuation of such processes as in previous studies [32-36] that showed higher accumulation of CAB in GCF with OTM and which could be directly reached into saliva. Eventhough, in this study its highly significant increase in quantitative level reaches after 1D of appliance placement in F1 and F2 groups, these findings are similar to those by Sugiyama et al. [35] where high significant increase in CAB level occurs in GCF of the teeth at the treated sites as compared to those at the control sites after 1D of force application. However; comparison with this study is difficult due to many technical variations like study design, GCF collection and the initial force used was 250gm. These results indicate that this enzyme is a product of cells in alveolar bone and PDL like osteoblast, osteoclast, fibroblast and macrophages when they were exerted to any

factor exceeds the normal physiological limits such as pathological condition or mechanical deformation [32-36]. Sasaki and Ueno- Matsuda [32] reported that CAB is involved in the formation of resorption lacunae by means of intra and extracellular degradation of collagen and other non- collagenous matrix proteins of primary teeth. Moreover, Goseki et al. [37] found an in vitro relation between high CAB activities with progression of PDL cellular aging. It has also been reported that interlukine-6 increases CAB activity in human PDL cells [34]. Therefore, CAB may be involved in periodontal metabolism through the degradation of organic bone matrix containing collagen fibers [33]. Immunocytochemical study by Nogimura [36] demonstrated increased detection of CAB in PDL fibroblasts and osteoclasts of rat molar following experimental tooth movement; this proves that cathepsins play an important role in starting OTM by initiating bone resorption. However, there were few studies on the biological role of CAB during OTM hence all these investigations assessed its relation to OTM as a mediator of inflammation and bone remodelling process which can be detected in GCF or in saliva.

In the present study salivary CAB level showed gradual and steady decrease in F1 and F2 groups (however in F1 more than in F2 group) till the end of the study. It has been thoroughly acknowledged that in the earlier phases of tooth movement, bone resorption is greater than bone apposition, but in a later phase, resorption and apposition can become synchronous [38]. Whereas, its level in F3 group showed fluctuant significant differences between every sampling visit, except for the pairs baseline visit with 1D and 1week visits. It is quite probable that the increasing and decreasing trend of salivary CAB values may be due to collagen breakdown resulting from higher magnitude of mechanical loading (80gm), ischemia, and hypoxia, which appear immediately after force application and last throughout most of the initial and lag phase of OTM [11].Although the elevation observed in F1 and F2 groups at 1D visit in comparison to baseline examination, it might be associated with the initial shift of the teeth within the PDL space and early bone resorption observed during the initial phase of OTM [38]. Moreover, in the lag phase (2W visit), salivary CAB level at F3 showed a significant increase regarding baseline examination. These findings could be attributed to the effect of higher force magnitude on periodontium and bone remodelling process. Altogether, these salivary findings might be a reflection of the actual enzymatic profile of GCF and consequently of the biologic activity within the periodontal environment during OTM [39].

In conclusion, CAB can be used as a marker in OTM. The light continuous orthodontic force could produce the significant and reliable increase of this enzyme activity and give enough effectiveness to produce steady tooth movement with less adverse effects on the surrounding structures as could be present in higher forces.

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