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Efficacy of salivary alkaline phosphatase enzyme as a non-invasive biomarker for skeletal maturation indicator in growing subjects - An Invitro study

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Abstract

Background and Objectives: The use of non invasive biomarkers as maturation indicator which potentially eliminates any supplemental radiation exposure is definitely advantageous over the radiographic methods since they are totally subjective and can have magnification errors.This study aims to detect ALP levels in saliva and to correlate it with the skeletal age and to project it as a noninvasive tool for assessment of skeletal growth.

Materials and Method: 100 subjects were selected of age 9-16 years old with lateral cephalogram taken within past 6 months. 1 ml of un-stimulated saliva was collected in a sterile container and refrigerated immediately and stored overnight .Samples were centrifuged and 20uL of saliva was added to 1000uL ERBA Manheim kit ALP reagent. ALP values were analysed in the auto analyser. The ALP values were compared with the CVMI stage 1, 2, 3, 4, 5 and 6 and results were analysed. using Statistical analysis

Results: Salivary alkaline phosphatase activity was in correlation with the cervical vertebral maturation showing peak values at the Stage 3 (Transition) correlating with the peak of puberty.

Conclusion: Salivary ALP values can be used as a non-invasive biochemical diagnostic aid for skeletal maturity assessment.

Keywords: salivary alkaline phosphatase; CVMI; skeletal maturity; lateral cephalogram; saliva; Biomarker.

Introduction

The assessment of growth potential is essential because of individual variation in timing, duration and velocity of growth[1]. The classic method of assessing skeletal maturity is by the use of a hand wrist radiographs[2-7].

Assessing the degree of cervical vertebrae maturation stage[2,3,6,8-11] on lateral cephalometric radiograph and recording MP3 Stages [11-15] on periapical X ray films are also used to identify peak skeletal maturity. Assessing of maturity by clinical and radiographic examination of different stages of tooth development and by evaluation of frontal sinus using lateral cephalogram. Radiographic methods are highly subjective techniques involving radiation exposure[1]. The use of biomarkers has been proposed as a promising aid in assessing individual skeletal maturity, with the advantage of being true indicator pertaining to the physiology of the patient whereas traditional radiographic method are morphological and purely subjective in nature and has magnification errors too. Most commonly used laboratory diagnostic procedures involve the analyses of the cellular and chemical constituents of blood serum. The very scarce data reported to date include molecular constituents from the serum, such as insulin-like growth factor 1 (IGF-1)[16-19] or from GCF, such as alkaline phosphatase (ALP). [20-22] Bone biomarkers used are ALP, Serum Osteocalcin[23], Pro peptide puridinoline cross link of type 1 collagen[24]. ALP is essential for bone mineralisation and proposed as a diagnostic aid as its expression increases during osteo blastic activity[25]. ALP has often been measured as possible indicators of gingival inflammation and bone metabolism[26]. Christesen[27], Takimato[28], Ins oft[29], have shown increase in serum ALP levels during puberty. Baccetti[30] showed increase in GCF ALP levels during pubertal peak. But as it is an invasive procedure, many times its objected by patients[31]. But collection of GCF is a tedious procedure. It can be hypothesized that there will be increases in salivary ALP during puberty. Salivary collection is a non-invasive procedure, easy to perform, does not clot and requires less manipulation than blood[31]. The procedure is economical. Saliva is easily collected, shipped, and stored, resulting in decreased overall costs. Despite these favorable attributes, the use of saliva as a diagnostic fluid has yet to become a mainstream idea[31]

The purpose of this study was to associate salivary ALP levels with stages of CMVI so as to assess if salivary ALP levels can be used as pubertal maturity indicator. Also to find the peak salivary ALP levels and its correspondence with CVMI stages.

Materials and Methods

100 subjects (50 males and 50 females) were selected from the outpatient or patients undergoing orthodontic treatment of age 9-16 years old at Department of Orthodontics and Dentofacial Orthopedics ,Rajarajeswari Dental College and Hospital, Bangalore based on the exclusion and inclusion criteria with lateral cephalogram taken within the past 6 months. The inclusion criteria were 1) Healthy individuals 2) Individuals aged between 9-16 years . The exclusion criteria were 1) Any individual diagnosed with a medical condition 2) Systemic diseases 3) Intra oral infections 4) Bone disorders 5) Craniofacial disorders 6) Taking medications that affects the growth or bone metabolism. Subjects were explained in detail about the study and an informed consent was obtained from the parents/guardian accompanying the subject before including them in the study. 1 ml of un-stimulated saliva was collected in a sterile container. The samples were refrigerated (at 2-8 O C) immediately and stored overnight to get rid of air bubbles. Samples were centrifuged at 3000 rpm for 15 minutes. 20uL of saliva was added to 1000uL ERBA Manheim kit ALP reagent. ALP value was analysed in the auto analyser. The normal values of salivary ALP is found to be 20-140 U/L[32] .The values were again compared with the CVM stage 1,2 ,3, 4, 5 and 6 and results were analysed. Results were

analysed using the software Statistical Package for Social Sciences (SPSS) for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp. The collected data were analyzed statistically by Kruskal Wallis test followed by Mann Whitney's post hoc test was used to compare the mean ALP levels between different CVMI stages. Spearman's correlation test was used to estimate the relationship between age and ALP levels in different CVMI stages. The level of significance was set at P<0.05.

Results

The distribution of the study population among the six CVMI Stages is presented in Graph 1. The comparison of Mean ALP levels [IU/L] between different CVMI Stages (Table 1) demonstrate that mean ALP levels increases gradually from stage 1 to stage 2 . At stage 3 there was a shootout of mean ALP followed by a gradual decline in mean from stage 4 to 5 and 6 .This mean difference in the ALP levels between 6 CVMI stages was statistically significant at P<0.001. Multiple comparison of Mean ALP levels [IU/L] levels b/w diff. groups (Table 2) showed that stage 3 showed significantly highest ALP levels as compared to other CVMI stages at P < 0.001 & with stage 4 at P=0.01. This was then followed next with stage 4 showing significantly higher mean ALP levels as compare to stages 5 & 6 at P<0.001. This was followed later with stages 1 & 2 showing significantly higher mean ALP levels as compared to stage 5 & 6 at P<0.001 respectively. Spearman's correlation coefficients for age of study subjects and ALP levels [IU/L]. shows a statistical significance at stage 3 and stage 4 (Table 3)

DISCUSSION An understanding of growth is of primary importance in the practice of clinical orthodontics[33]. Maturational status can have considerable influence on diagnosis, treatment goals, treatment planning, and the eventual outcome of orthodontic treatment. In the recent past tremendous concomitant research activities are taking place in the field of bio markers. The present study is based on one such important biomarker ALP. Alkaline phosphatases are true isoenzymes because they catalyze the same reaction throughout the body[34].In diseased state, elevated levels of ALP in the blood are most commonly caused by liver or bone disorders. In healthy individuals elevated levels of ALP can be found in bone tissues during active bone growth and remodeling. The sources of Alkaline Phosphatase are almost all biological fluids also which include Serum, GCF, and Saliva etc. Tobiume et al (1997)[35] stated increased serum ALP activity during puberty. Perinetti et al (2011)[30] reported that ALP activity peak in GCF was during the pubertal growth spurt and Tarvade et al (2015)[25] also found higher levels of salivary ALP during the growth spurt. As far as collection of the samples are considered, obtaining sample from serum is invasive and collecting sample from GCF involves a difficult and tedious. Since the Mantra in any treatment concept is “Do No Harm”, it is prudent to develop and utilize other alternative systems which is devoid of any potential complication. In our study too, we assessed the possibility of ruling out the additional radiation exposure by avoiding any other supplemental x rays and introducing a patient friendly new assessment method such as estimation of salivary ALP as a non- invasive biomarker to determine skeletal age as well as comparative evaluations of their reliability Results of this study showed in Stage 1 (Initiation) , mean Salivary ALP levels were , 1353.00 ± 22.69 IU/ L ,where there is an increase in ALP values from normal range which associates with the initiation of the pubertal growth spurt of an individual. Stage 2 (Acceleration), mean Salivary ALP levels were 1409.70 ± 38.84 IU/L, a higher mean ALP values were seen as compared to stage 1(Initiation) which associates with the acceleration in the pubertal growth of the individual. In Stage 3 (Transition)

mean Salivary ALP levels were 1981.91 ± 528.47 IU/L, showing peak mean ALP values which correlates with the peak of the pubertal growth spurt. In Stage 4 (Deceleration), mean Salivary ALP levels were 1608.00 ± 425.78 IU/L, the mean ALP values were lower than stage 3 correlating with deceleration in the pubertal growth. In Stage 5 and Stage 6 mean Salivary ALP levels were 793.13 ± 217.21 IU/L and 767.40 ± 236.89 IU/L respectively. The ALP values were significantly reduced marking the end of pubertal growth of an individual. Hegde et al[36] assessed B-ALP levels in saliva and correlated it with different skeletal maturity stages of hand-wrist radiographs comprising 90 individuals. The salivary B-ALP values were significantly higher in subgroup 3 (MP3) as compared to subgroup 1 (So) and subgroup 5 (R-J). The salivary B-ALP levels showed a steady increase in mean value from subgroup So to subgroup MP3, followed by a steady decline thereafter as revealed by our study. Alhazmi et al[37] investigated the relationship between salivary ALP, protein concentration, and chronological age with CVMS comprising 79 subjects. Salivary ALP reached peak at early pubertal stage and then declined with a significant difference. A significant positive correlation between age and CVMS was found concluding that the combination of salivary ALP activity and chronological age may provide the best CVMS prediction similarly as obtained by our study. Overall the results of our study, Peak stages had highest mean ALP values which was similar to findings obtained by Gupta [38], Ishaq[39],Gupta[40],Tarvade[25], Baccetti [21], Tripathi [23] The results of our study shows a new horizon of assessment of growth maturation by using noninvasive, simple, easy procedure demanding minimal patient discomfort without compromising the validity of the desired results. It also also proved that it could be possible to take the diversion from the most commonly used supplemental hand wrist radiographs for growth assessment to the non-invasive method of assessing the skeletal maturation with the salivary ALP. Although numerous authors have reported several issues regarding using salivary ALP .One of the limitations of this study was the interexaminer reliability for using the CVM method. As this method is subjective and poorly reproducible and the interexaminer reliability is low. Due to the presence of existing lateral cephalometric radiographs and ethical concerns with repeating them, saliva collection was not achieved exactly at the same time as lateral cephalometric radiographs but within 6 months. Although this study included a large number of subjects, all were Bangalore, India. Thus, the data sets a landmark for future studies with larger and more ethnically diverse sample populations and a long -term longitudinal study to investigate the potential use of salivary ALP and other salivary proteins as biomarkers for skeletal maturity[37].

Conclusion

From this study report, it is concluded that, Salivary alkaline phosphatase activity was in correlation with the CVMI showing peak values at the pubertal stages.Hence salivary alkaline phosphatase can be used as a biochemical marker for identification of skeletal maturation.

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Legend Graphs ans Tables

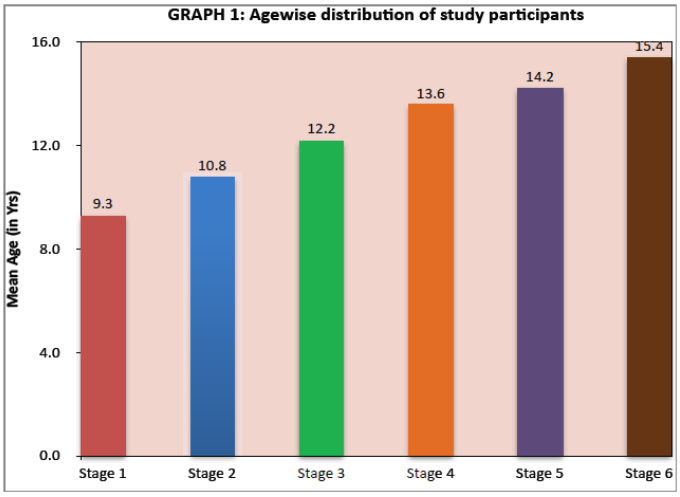

TABLE 1 : Comparison of Mean ALP levels [IU/L] between different Cervical Vertebral Maturation stages using Kruskal Wallis test

CVMI	N	Mean	SD	Min	Max	P-Value
Stage 1	10	1353.00	22.69	1324	1388	<0.001*
Stage 2	23	1409.70	38.84	1358	1509	
Stage 3	22	1981.91	528.47	1456	2647	
Stage 4	14	1608.00	425.78	1019	1963	
Stage 5	16	793.13	217.21	510	977	
Stage 6	15	767.40	236.89	541	1023	

TABLE 2 : Multiple comparison of Mean ALP levels [IU/L] levels b/w diff. groups using Mann Whitney Post hoc Analysis Test

Group [I]	Group [J]	Mean Diff.	95% CI for the Diff		P-Value
			Lower	Upper	
Stage 1	Stage 2	-56.70	-411.29	297.90	1.00
	Stage 3	-628.91	-985.94	-271.88	<0.001*
	Stage 4	-255.00	-642.60	132.60	0.40
	Stage 5	559.88	182.11	937.64	<0.001*
	Stage 6	585.60	203.02	968.18	0.001*
Stage 2	Stage 3	-572.21	-851.39	-293.04	<0.001*
	Stage 4	-198.30	-515.64	119.03	0.46
	Stage 5	616.57	311.50	921.65	<0.001*
	Stage 6	642.30	331.28	953.31	<0.001*
Stage 3	Stage 4	373.91	53.86	693.96	0.01*
	Stage 5	1188.78	880.88	1496.69	<0.001*
	Stage 6	1214.51	900.72	1528.30	<0.001*
Stage 4	Stage 5	814.88	471.92	1157.83	<0.001*
	Stage 6	840.60	492.35	1188.85	<0.001*
Stage 5	Stage 6	25.725	-311.075	362.525	0.86

TABLE 3: Spearman's correlation coefficients for ageof study subejcts and ALP levels [IU/L]

Age	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
r	0.42	0.33	0.73	0.61	0.15	0.33
P-Value	0.23	0.12	<0.001*	0.02*	0.59	0.23