Assessment of Alkaline Phosphatase activity in Gingival Crevicular Fluid as a Skeletal Maturity Indicator

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ABSTRACT

BACKGROUND AND OBJECTIVES: The aim of the study was to evaluate gingival crevicular fluid (GCF) alkaline phosphatase (ALP) activity in growing subjects in relation to stages of skeletal maturation, i.e., the growth phase, as prepubertal, pubertal, and postpubertal. METHODS: Sixty healthy growing subjects (27 girls and 33 boys; age range, 9 – 18 years) were enrolled in this study that followed a double-blind, prospective, cross-sectional design. Collection of GCF was performed at the mesial and distal sites of both central incisors, for the maxilla and mandible. Growth phase was assessed through the hand wrist radiograph using Bjork and Grave method. GCF parameter was expressed as total ALP activity. RESULTS: The total GCF ALP activity showed a peak for the pubertal growth phase. No differences were seen between the maxillary and mandibular sites, or between the sexes, for any GCF parameter. CONCLUSION: The GCF ALP activity has potential as a diagnostic aid for identification of the pubertal growth phase in individual subjects.

Keywords: Gingival crevicular fluid; Alkaline phosphatase activity; Growth phase; Skeletal maturity; Orthodontics

INTRODUCTION

The association between diabetes mellitus and tuberculosis and their synergistic role in causing human disease has been recognised centuries ago. Ancient literature describes the symptoms of patients with a magnitude of ailments which range from obesity to impotence, thirst, and glycosuria, and ultimately lead to tuberculosis or death1. One of the most important factor in orthodontic treatment planning is the growth potential of the patient. Human growth and development are not uniform, with accelerations and decelerations in the growth velocity of different skeletal components at various developing maturational stages.

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The onset of puberty varies with sex, population, and the environment. Researchers have tested many biological indicators to determine growth potential in adolescents such as body weight2, body height3, menarche4, sexual maturation characteristics5, chronological age6, dental development7 and skeletal development. Skeletal age has been shown to be a more reliable and precise indicator than chronological age in assessing the progress of an individual towards maturity. Skeletal maturation staging from radiographic analysis is a widely used approach to predict the timing of pubertal growth velocity and to estimate the proportion of remaining growth. Although use of insulin like growth factor (IGF)9 and frontal sinus10 has been reported, skeletal maturation is generally determined by using stages in the ossification of bones of the hand and wrist11 or by evaluating the cervical vertebrae12. The assessment of the degree of cervical vertebral maturation (CVM) is used to assess skeletal maturation. But few improvements are still needed to make the method easier and applicable to the vast majority of patients. However, small sample size used to devise the CVM method and lack of validation raise doubts about its effectiveness, especially in the later stages of development when the growth tapers off.
Moreover, some children go through a prolonged period of accelerated growth without a distinct growth peak. It is unclear whether CVMI method can be an effective predictor in these subjects. Bone growth and remodeling is not only under the control of local factors but systemic factors also play a crucial role. Biomarkers represent agents that are involved directly in growth and remodeling. Biomarkers avoid radiographic exposures also. Research is being done to explore the role of biomarkers for determination of skeletal maturation. Gingival crevicular fluid (GCF) is a potential source of biomarkers, with molecular constituents that are derived mainly from the serum but also from the interstitial fluid of periodontal tissues. The enzyme alkaline phosphatase (ALP) plays a role in bone metabolism. It is a membrane bound glycoprotein produced by many cells, such as polymorpho nuclear leukocytes, osteoblasts, macrophages and fibroblasts within the area of periodontium and gingival crevice. ALP is essential for bone mineralization and proposed as a diagnostic aid in periodontology and orthodontics. Insoft et al have shown an increase in serum ALP level during puberty. But as detection of serum ALP involves an invasive procedure, many times it is objected by patients and parents. GCF alkaline phosphatase (ALP) activity has been shown to be related to the pubertal growth spurt and has thus been proposed as a noninvasive diagnostic aid for the determination of optimal treatment timing in functional jaw orthopedics. The aims and objectives of the present study are to compare the mean alkaline phosphatase levels in gingival crevicular fluid at different stages of skeletal maturity using hand wrist radiographs and to assess whether alkaline phosphatase activity in gingival crevicular fluid can be used as a skeletal maturity indicator.

MATERIALS AND METHODS

The sample used in this study consisted of 60 subjects (33 boys and 27 girls) in the age range of 9-18 years divided into three groups using Bjork, Grave and Brown method of hand wrist radiographs i.e. pre-pubertal, pubertal and post-pubertal group, with 20 subjects in each group. The subjects were randomly selected from patients visiting the Departments of Orthodontics and Dentofacial Orthopedics and Oral Medicine and Radiology, Mahatma Gandhi Dental College & Hospital, Jaipur, Rajasthan. The ethical clearance for the study was obtained from Institutional Ethical Committee of Mahatma Gandhi Dental College and Hospital, Jaipur.

Inclusion criteria

Age between 9 to 18 years.
Good general health and absence of nutritional problems.
No use of anti-inflammatory drugs or antibiotics in the month preceding & during enrolment for the study.

Exclusion criteria

Subjects who are undergoing orthodontic treatment.
Compromised oral hygiene conditions.

At the first clinical examination, the subjects were explained about the investigation and informed consent was obtained from those who were ready to participate in the study. At the second visit the subjects underwent a session of professional supragingival and subgingival scaling and subjects were recalled after 7-10 days for GCF collection. The patients were asked to rinse their mouth with 0.012% chlorhexidine mouthwash twice daily during this period. The patients were also informed not to take any anti-inflammatory or antibiotic drugs during this period. In the last session the GCF was collected by using microcapillary pipette. Then it was transferred to the eppendorf tubes containing 200 µl of buffer solution and sent to laboratory for determination of level of alkaline phosphatase by spectrophotometry. Along with GCF collection the hand wrist radiograph was also taken at the same visit. The hand wrist radiographs were staged by using the 9 stage SMI technique described by Bjork, Grave and Brown staging, and the 9 stages were regrouped in to 3 phases. The first phase, including SMIs 1 through 3, was considered prepubertal; 20 out of 60 subjects were in this group. The second phase, which included SMIs 4 and 5, was considered peak pubertal phase; 20 subjects were in this group. The third phase, SMIs 6 through 9, was considered post-pubertal; which included 20 subjects.

GCF sample collection procedure

The patients were seated in an upright position on the dental chair with proper light condition. The site for GCF collection was identified to the eburnated line or gingival margin at the mesial and distal embrasures of maxillary and
mandibular central incisors. The selected site was dried and isolated using cotton rolls. GCF samples were obtained by placing calibrated microcapillary pipette extra-crevicularly over the site. Then 5 µl GCF sample was collected from each site. 19

**Biochemical assays**

The biochemical assays were performed by a single operator who was double blinded to the maturational stages of the subjects. The GCF samples from both the maxillary and mandibular sites were resuspended in 200 µl buffer containing 100 mM Tris and 20 mM MgCl2 (pH 9.8 ± 0.1) and 6 mM p-nitrophenol phosphate. The samples were then incubated at 37°C (±0.1°C fluctuations) for 2 hours, whereby the alkaline phosphatase in the samples hydrolyses the p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The reactions were then stopped by adding 5 µl 3 M NaOH, and the rates of increase in absorbance were read with a spectrophotometer at 405 nm wave-length. The relevant control for each analysis consisted of the reagent and the Tris buffer without the sample. Using 18.45 as the p-nitrophenol mM absorptivity, the absorbance was converted into International units per litre enzyme activity units. (1 unit = 1 mmol of p-nitrophenol released per minute at 37°C). 20

**RESULT AND DISCUSSION**

The data so obtained were subjected to statistical analysis for obtaining correlation between hand wrist radiograph and alkaline phosphatase levels in GCF. Kruskal-Wallis test was used for inter-group comparison of GCF alkaline phosphatase activity in maxilla and mandible among three groups. Mann-Whitney U test was used for multiple comparisons of GCF alkaline phosphatase activity among three groups, as well as for site wise comparison for GCF alkaline phosphatase activity in maxilla and mandible. Chi-square test was used to assess the gender wise difference in GCF alkaline phosphatase activity. Table 1 shows descriptive statistical analysis, which indicates that GCF alkaline phosphatase levels were significantly higher in pubertal stage, than pre and post-pubertal stages. Table 2 shows inter-group comparison of GCF alkaline phosphatase levels among maxilla and mandible using **Kruskal-Wallis test**. It depicts the statistically significant difference in GCF alkaline phosphatase levels among three groups in maxilla as well as mandible. Table 3 shows multiple comparisons of GCF alkaline phosphatase levels among the groups in maxilla and mandible using Mann-Whitney U-test. There was statistically significant difference in GCF alkaline phosphatase levels, in both maxilla and mandible, between pre-pubertal and pubertal groups (p<0.05). However difference between pre-pubertal and post-pubertal group among maxilla and mandible was statistically non-significant with P value of 1.00. Table 4 shows site wise comparison of GCF alkaline phosphatase levels in pre-pubertal, pubertal and post-pubertal groups. There was statistically non-significant difference among pre-pubertal, pubertal and post-pubertal group in maxillary and mandibular sites. Table 5 shows chi-square test for gender-wise comparison of GCF alkaline phosphatase levels among maxilla and mandible in pre-pubertal, pubertal and post-pubertal group which was statistically non-significant in males and females. Successful treatment of many orthodontic problems depends to a great extent on the amount of mandibular growth that patients experience during treatment. The use of skeletal age has been shown to be more reliable and precise than chronological age in assessing the progress of an individual towards maturity. 8 Traditionally, evaluation of hand wrist radiographs and cervical vertebrae have been used to predict the timing of the mandibular growth spurt. According to Ball et al 21, cervical vertebral maturation stages cannot predict the onset of the peak in mandibular growth and should be used with other methods of biologic maturity assessment. Moreover, the difference between horizontally rectangular, square and vertically rectangular shape of cervical vertebrae depend on researcher’s arbitrary decisions. 22 Previous studies 17 have correlated CVMI stages of skeletal maturation with GCF alkaline phosphatase levels. In the present study we have correlated the hand wrist skeletal maturation stages with GCF alkaline phosphatase levels. The radiograph of the hand wrist has been the most frequently used area of the skeleton for predicting skeletal
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Table 1: Descriptive data of the study groups

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-pubertal</td>
<td>20</td>
<td>10.00</td>
<td>21.00</td>
<td>15.275</td>
<td>3.30</td>
</tr>
<tr>
<td>Pubertal</td>
<td>20</td>
<td>20.00</td>
<td>29.00</td>
<td>24.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Post-pubertal</td>
<td>20</td>
<td>10.00</td>
<td>20.00</td>
<td>15.25</td>
<td>3.09</td>
</tr>
</tbody>
</table>

Table 2: Kruskal-Wallis test Multiple comparison (inter-group)

<table>
<thead>
<tr>
<th></th>
<th>Among maxilla</th>
<th>Among mandible</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Mean</td>
<td>Std. Deviation</td>
</tr>
<tr>
<td>Pre-pubertal</td>
<td>20</td>
<td>15.95</td>
</tr>
<tr>
<td>Pubertal</td>
<td>20</td>
<td>25.15</td>
</tr>
<tr>
<td>Post-pubertal</td>
<td>20</td>
<td>15.25</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>18.78</td>
</tr>
</tbody>
</table>

Table 3: Mann-Whitney U-test for Multiple comparison (inter-group)

<table>
<thead>
<tr>
<th></th>
<th>Among maxilla</th>
<th>Among mandible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Difference</td>
<td>Sig.</td>
</tr>
<tr>
<td>Pre-pubertal</td>
<td>Pubertal</td>
<td>-9.02</td>
</tr>
<tr>
<td>Pubertal</td>
<td>Post-pubertal</td>
<td>0.7</td>
</tr>
<tr>
<td>Post-pubertal</td>
<td>9.9</td>
<td>0.04*</td>
</tr>
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</table>

Table 4: Mann-whitney U test for Site-wise comparison

Site-wise comparison of pubertal growth and post-pubertal growth

<table>
<thead>
<tr>
<th></th>
<th>Pre-pubertal growth</th>
<th>Pubertal growth</th>
<th>Post-pubertal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Mean</td>
<td>SD</td>
<td>MD</td>
</tr>
<tr>
<td>Maxilla</td>
<td>20</td>
<td>15.95</td>
<td>3.83</td>
</tr>
<tr>
<td>Mandible</td>
<td>20</td>
<td>14.6</td>
<td>2.702</td>
</tr>
</tbody>
</table>

Table 5: Chi-square test for gender-wise comparison

<table>
<thead>
<tr>
<th></th>
<th>Maxillary</th>
<th>Mandible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Pubertal</td>
</tr>
<tr>
<td>Male</td>
<td>12 (60)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Female</td>
<td>8 (40)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>P value</td>
<td>0.18</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The reason for its use is that many centers are available in this area of skeleton that undergo changes at different times and rates. The information from hand wrist radiograph has been used in a number of ways to evaluate the skeletal age of the child. The present study investigated the possible relationships between GCF alkaline phosphatase activity and skeletal maturation. The purpose of this investigation was to provide an additional tool to help determine the growth potential in orthodontic patients. This study is basically a cross-sectional investigation to correlate GCF alkaline phosphatase levels to hand wrist maturation stages and to compare mean GCF alkaline phosphatase levels at each of these stages. The present study is of great importance because it allows skeletal age to be estimated in a non-invasive manner. As GCF alkaline phosphatase activity increases during periodontal inflammation, it is important to exclude any possible unwanted sources of this enzyme. Therefore all subjects received a...
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session of supra-gingival and sub-gingival scaling and showed optimal periodontal conditions. Subjects were also instructed to use 0.012% chlorhexidine mouthwash. Once the local tissue inflammation is excluded, there are two potential sources that might be responsible for variations in GCF alkaline phosphatase activity: 1. serum alkaline phosphatase (as a systemic factor) and 2. maxillary and mandibular growth (as a local skeletal factor). The serum ALP activity, which is the most used biochemical marker for bone turnover, has been reported to increase at puberty and to decrease in adulthood. In this study, subjects were divided into three groups by using Bjork, Grave and Brown method of hand wrist radiograph assessment i.e. pre-pubertal, pubertal and post-pubertal group. When the GCF alkaline phosphatase activities were assessed in relation to the different hand wrist stages, increased activities were seen for both the maxilla and mandible at pubertal stage, compared to pre-pubertal and post-pubertal stages. These differences were statistically significant within the maxillary and mandibular sites. However, no significant differences were seen between the maxillary and mandibular sites within each group. The results of this study revealed that the GCF alkaline phosphatase levels were low in the pre-pubertal phase (mean of 15.275 ± 3.30 IU/L), rise sharply to their peak in puberty (mean of 24.7±2.1 IU/L) and again decline in the post-pubertal phase (mean of 15.25±3.09 IU/L) to approach the pre-pubertal levels. There was a statistically significant difference in GCF alkaline phosphatase levels between puberty and pubertal stage (p<0.05), and also between pubertal and post-pubertal stage (p<0.05). This is in agreement with the study conducted by Perinetti et al. The results of this study are in accordance with the previous research and confirm the usefulness of this technique. Gingival crevicular fluid analysis offers several advantages from a clinical point of view. Its sampling involves a very simple, rapid and non-invasive procedure that can be performed in a clinical setting, even in the case of multiple GCF collections. Moreover, the ALP activity can be determined through routine and cheap laboratory analyses that are easily available.

CONCLUSION
The following conclusions can be drawn from the present study: mean GCF alkaline phosphatase levels were significantly higher in pubertal phase as compared to pre-pubertal and post-pubertal stage. No statistically significant differences in GCF alkaline phosphatase levels were seen between the maxillary and mandibular sites. GCF alkaline phosphatase appears to be a promising diagnostic tool as a non-invasive biomarker of the pubertal growth spurt.

REFERENCES
1. Malgorzata K, Baccetti T. Duration of the pubertal peak in skeletal class I and class III subjects. Angle Orthod 2010;80:54-57