A new clinical test to diagnose growth hormone deficiency in saliva of patients with short stature

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Abstract
Growth hormone deficiency (GHD) is one of the most common endocrine disorders with short stature in children. This study aims to suggest a novel test to assess the level of IGFBP-3 in saliva as a new clinical technique to assess patients with short stature. In this study, a total of 60 short stature patients with growth hormone deficiency, ages 4-18 years, were included, along with 60 age-matched healthy volunteers. Serum and salivary IGFBP-3 were determined using ELISA technique. The results obtained indicated the presence of a highly significant decrease (p <0.01) in the levels of IGFBP-3 of serum and saliva for patients (2.74 ± 0.47 ng/mL, 1.57 ± 0.35 ng/mL) as compared to the control levels (8.25 ± 1.32 ng/mL, 3.55 ± 0.56 ng/mL). Moreover, comparison of the levels of IGFBP-3 for GHD patients revealed to the presence of a significant positive linear correlation between salivary IGFBP-3 and serum IGFBP-3, in contrast to no correlation between them in normal subjects. From this, we can conclude that the measurement of IGFBP-3 in saliva can be considered as an additional and useful technique to assess disease activity in GHD patients, as it can adequately reflect the corresponding serum levels. The new saliva-based method of IGFBP-3 measurement is considered a fast diagnostic method that saves time and effort. It is an easy, inexpensive, and economical method, and can be used in place of the old method which depends on patient's serum. The new technique has important clinical applications in the field of medical diagnostics that help to speed up diagnosis of GHD patients. It also has applications in the field of clinical research that help researchers to conduct health related studies. In summary, the main conclusion that can be drawn is that saliva can be relied upon to diagnose HGH deficiency by measuring IGFBP-3.

Key Words: Short stature; Growth hormone; GHD; Saliva; IGFBP-3.

DOI:  http://doi.org/10.36295/ASRO.2021.24413  Page(s):7-13

Volume/Issue: Volume 24/ Issue: 04

Introduction
Growth hormone (GH), also called somatotropin, is a single polypeptide chain of 191 amino acids with 2 di-sulfide bridges and a molecular weight of 22 kDa [1]. The primary function of GH is the promotion of longitudinal growth in childhood. It does however have wide ranging action on muscle, bone and adipose tissue as well as a number of other organs of the body. GH may act both directly on organs and tissues, and / or indirectly via the insulin like growth factors (IGFs) [2].

Insulin-like growth factor-1 (IGF-1), also called somatomedin C, is a peptide hormone that plays an important role in childhood development and has anabolic effects in adults. IGF-1 is produced mainly by the liver under GH stimulation and is characterized by similarities in the molecular structure of insulin [3]. IGF-1 has a very high affinity for a family of binding proteins to form Insulin-like growth factor binding proteins (IGFBPs) and circulates in a ternary complex. It is found that approximately 98% of IGF-1 is always bound to insulin-like growth factor binding protein-3 (IGFBP-3), the most abundant of a group of IGFBP s. IGFBP-3 is a 264 amino acids polypeptide chain with a M. Wt. of 29 kDa. It is important to transport and control bioavailability and half-life of IGFs, in particular IGF-1 [4]. IGFBP-3 is produced mainly in the liver and secreted into the serum. The hepatic expression level of IGFBP-3 is regulated by GH, and this ensures that with increased IGF-1 secreted in response to GH stimulation, there will be an increased amount of IGFBP-3 to be absorbed into the circulation [5].
Short stature is a term that applies to a child whose height is two or more standard deviations (SD) less than the average for children of the same sex and age. Short stature is a common problem in children, and it occurred due to many causes; these causes may be genetic, environmental or chronic diseases. The most common etiologies for short stature are growth hormone deficiency either congenital or acquired. Diagnosis of short stature can be achieved by two combined ways: physical examination and laboratory tests, where the treatment of short stature depends on the right diagnosis and its causative agent. Growth hormone deficiency (GHD) is a medical condition due to a lack of growth hormone if it begins in childhood or infancy. It has been reported that the cause of this condition is unknown in 75 percent of patients, and in most cases brain malformations occur. GHD may be isolated or combined with other anterior and/or posterior hormonal deficiencies. GHD has been found to be rare but important cause of short stature in children. The diagnosis of growth hormone deficiency is currently based on clinical, auxological, biochemical and neuro-radiological investigation. Provocative tests of GH secretion using physiological/pharmacological stimuli are used to confirm GHD. The clonidine test (CT) is widely used to assess GH secretory status. In addition, estimation of serum IGF-I and IGFBP-3 levels are also used to assess GHD.

Saliva is an aqueous substance that produced in the salivary glands, 98% of water, and found in the mouth. Saliva has multiple functions, including moisturizing the mucous membrane of the upper digestive system, which makes saliva indispensable for ingestion of food. Saliva usually contains many important substances, including electrolytes, mucus and several enzymes. It is found that the speed at which hormones are transferred from the blood to the saliva is controlled by passing through the lipophilic layers in the capillaries and glandular epithelial cells. Therefore, lipophilic molecules, such as steroids, are transported through these barriers more quickly than hydrophilic molecules, such as peptides. It has been found that salivary diagnosis is an increasingly interesting field in dentistry, endocrinology, pediatrics, immunology, clinical pathology, psychology, and sports medicine. Moreover, an increasing number of drugs, hormones and antibodies can be monitored in saliva. Therefore, salivary diagnosis is suggested as particularly beneficial in cases where repeated samples are required and when blood drawing is impractical or unethical or both.

Serum levels of IGFBP-3 are constant throughout the day and closely dependent on the growth hormone. This feature led to the use of IGFBP-3 assessment as a reliable and simple screening test in the follow-up of children with short stature. In this study, the aim is to develop a novel and simple technique to diagnose HGH deficiency in short stature patients by measuring the level of IGFBP-3 in saliva as a substitute of blood.

**Materials and methods**

**Subjects and study design**

The present study is conducted in Chemistry department/ College of Science/ Mustansiriyyah University in cooperation with Pediatric department/ National Diabetic Center (NDC)/ Mustansiriyyah University, Baghdad, Iraq, between March, 2019 and January, 2020. The study included one hundred twenty children and adolescents aged 4 to 18 years. Sixty healthy individuals as control group, and sixty short stature patients with growth hormone deficiency were selected from outpatients who attended to the Pediatric department/ NDC and were diagnosed by an endocrinologist.

**Sample collection**

From each individual, (2-5 ml) of blood was drawn, after an overnight fasting, through a vein puncture using disposable syringes and collected in a gel tube. The blood sample was centrifuged at 2000 xg for 10 minutes, and the resulting serum was stored at -20 °C until the time of analysis. In addition, saliva samples were collected from each individual. Within 5-10 minutes of collection, each saliva sample was centrifuged (a typical volume about 1 mL) at 2000 xg for 10 minutes, and the resulting supernatant was collected carefully and stored at -20 °C until the time of analysis.

**Sample analysis**

Insulin like growth factor binding protein-3 in serum and saliva samples was estimated by Enzyme-Linked Immune sorbent Assay (ELISA) technique based on the sandwich principle method, and following the protocol of the available IGFBP-3 kit supplied by (Melsin /China).
Statistical analysis:
Data was analyzed using SPSS statistical software, version 26. Independent-Samples Student t test were performed between patients and control groups, and the resulting values were expressed as standard deviation, standard error, range, Inter quartile and probability (p) values. The Pearson correlation coefficient was also carried out to determine the relationships between all study variables. The statistical tests were consider significant at p<0.05 and highly significant at p<0.01 with a confidence interval of 95%.

Results and discussion
The data in Table 1 illustrates IGFBP-3 levels in the serum and saliva of both studied groups. The results showed a highly significant decrease (p <0.01) in the mean values of serum and salivary IGFBP-3 of patients (2.74 ± 0.47 ng/mL, 1.57 ± 0.35 ng/mL) compared to the control values (8.25 ± 1.32 ng/mL, 3.55 ± 0.56 ng/mL), the data are represented in Figure 1.

Table 1: Levels of IGFBP-3 in serum and saliva of patients and control groups.

<table>
<thead>
<tr>
<th>Variable (ng/mL)</th>
<th>Sample</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>Range</th>
<th>Inter quartile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IGFBP-3</td>
<td>patient</td>
<td>2.74</td>
<td>0.47</td>
<td>0.06</td>
<td>1.51-3.86</td>
<td>0.69</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>8.25</td>
<td>1.32</td>
<td>0.17</td>
<td>5.23-10.34</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>Salivary IGFBP-3</td>
<td>patient</td>
<td>1.57</td>
<td>0.35</td>
<td>0.04</td>
<td>0.94-2.47</td>
<td>0.55</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>3.55</td>
<td>0.56</td>
<td>0.07</td>
<td>2.51-5.02</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Histogram shows levels of IGFBP-3 in serum and saliva of patients and control groups.

Insulin-like growth factor binding protein-3 is the most abundant IGF binding protein, and because its synthesis is stimulated by GH it uses as a biomarker of integrated GH secretion \[15\]. IGFBP-3 level has been proposed as an excellent test for HGH status \[16\]. Although a single GH test remains the most powerful biochemical tool in assessing a child with development failure, the combination of this test with IGF-I and IGFBP-3 can be used very effectively in GHD confirmation. IGF-1 and IGFBP-3 levels are likely to reflect the integrated concentration of secreted GH and are stable during the day when compared to the pulsatile GH secretion. Despite the relatively good correlations between GH secretion and IGF-1 levels in GHD patients, substantial problems remain in assessing the state of GH by measuring IGF-1 in individual patients. IGF-1 values are very low in early life, and the normal range overlaps with the range for those with GHD, and therefore IGFBP-3 levels may be more appropriate in children \[17\].

The results obtained here for serum IGFBP-3 are consistent with previous studies which reported a significant decrease in the mean values for IGFBP-3 of patients compared to the control values \[18-20\].IGFBP-3 level is
usually low in patients with GHD, which reflects reduced GH stimulation to the liver [21]. On the other hand, another study has shown that IGFBP-3 testing is not useful for diagnosing HGH deficiency [22]. This finding was contrary to our results which showed that IGFBP-3 are ideal screening tools for GH and IGF axis disorders. We can suggest that the reason for this difference with our results may be due to sociodemographic factors affecting the GH-IGF-1 axis.

Regarding saliva results, which is the main objective of this study, Table 1 shows a significant decrease (p <0.01) in IGFBP-3 level in saliva of the patient group compared to the control group. To our knowledge, this is the first study in Iraq (and to some extent in the world, where we have not found any previous study identifying IGFBP-3 in GHD patients saliva) to examine the IGFBP-3 level of saliva for children and adolescents with short stature. In fact, compared to IGFBP-3 serum level, this new method and test will strongly facilitate the diagnosis of HGH deficiency in children and adolescents.

Furthermore and in order to study the effect of puberty on the IGFBP-3 levels, the data in Table 2 shows in details the levels of IGFBP-3 in serum and saliva of the two studied groups, for both children and adolescents. The results showed a highly significant decrease (p <0.01) in the mean values of serum and salivary IGFBP-3 for children of healthy control (7.69 ± 1.44 ng/mL, 3.21 ± 0.32 ng/mL) and GHD patients (2.56 ± 0.42 ng/mL, 1.46 ± 0.34 ng/mL) compared to the values for adolescents of healthy control (8.82 ± 0.92 ng/mL, 3.89 ± 0.55 ng/mL) and GHD patients (2.91 ± 0.45 ng/mL, 1.67 ± 0.32 ng/mL), as seen in Figure 2.

**Table 2:** Effect of puberty on the serum and salivary IGFBP-3 levels of patients and control groups.

<table>
<thead>
<tr>
<th>Parameters (ng/mL)</th>
<th>Mean ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IGFBP-3 Control</td>
<td>Children: 7.69 ± 1.44, Adolescents: 8.82 ± 0.92</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Patients</td>
<td>Children: 2.56 ± 0.42, Adolescents: 2.91 ± 0.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Salivary IGFBP-3 Control</td>
<td>Children: 3.21 ± 0.32, Adolescents: 3.89 ± 0.55</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Patients</td>
<td>Children: 1.46 ± 0.34, Adolescents: 1.67 ± 0.32</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

![Figure 2](image_url): Histogram shows effect of puberty on the serum and salivary IGFBP-3 levels of patients and control groups.

These results are consistent with previous studies that indicated increased levels of IGFBP-3 in adulthood, as IGFBP-3 levels in the current study increased with age in children, with maximum levels in adulthood [23, 24]. In fact,
IGFBP-3 results in saliva were similar to those in serum as IGFBP-3 increased in adolescents compared to children. Therefore, it can be suggested that saliva can be used as a serum alternative in monitoring IGFBP-3 levels in patients with short stature.

Finally, and to demonstrate saliva dependency in following IGFBP-3 levels as a new diagnostic method for HGH patients, the correlation coefficient between IGFBP-3 values in saliva and serum was studied for both children and adolescents with HGH deficiency and control groups. In the group of patients with GHD, a positive correlation was found between salivary IGFBP-3 and serum IGFBP-3 in both children \((r=0.509^{**}, p=0.004)\) and adolescents \((r=0.463^{*}, p=0.010)\), Figure 3 (a and b). Whereas, in the group of healthy individuals no correlation was observed between serum and salivary IGFBP-3 for both children \((r=0.070, p=0.713)\) and adolescents \((r=0.265, p=0.158)\), Figure 3 (c and d). The new results showed GHD patients to have significantly lower mean values of serum IGFBP-3 and salivary IGFBP-3 levels than the normal subjects. Furthermore, comparison of the levels of IGFBP-3 in serum and saliva of GHD patients pointed to a significant positive linear correlation, in contrast to no correlation in normal subjects.

Conclusions

IGFBP-3 is consider a vital biomarker suitable for GHD prediction in children and adolescents, thus for early diagnosis of short stature. Estimation of serum IGFBP-3 is valuable and useful diagnostic performance in GHD evaluation. In this study, the salivary IGFBP-3 test performed relatively optimally and gave comparable diagnostic performance in the evaluation of GHD when compared with the serum IGFBP-3 test. On this basis, we conclude that the measurement of IGFBP-3 levels in the saliva of GHD patients can be considered as an additional and useful technique to assess disease activity in GHD patients, because it can adequately reflect the corresponding serum levels.

The new saliva-based method for measuring IGFBP-3 is a fast diagnostic method that saves time and effort by getting results quickly, and is an easy, inexpensive and economical method. It is a valuable method for patient
comfort and ease of performance and can be used for the initial follow-up to GHD in children of short stature. Where in the new method, it is possible to take an IGFBP-3 measurement in a patient's saliva at any time to diagnose a child's condition with growth hormone deficiency rather than exposing children or adolescents to tests that stimulate blood growth hormone, which is usually in the form of three to four blood withdrawals consecutive. The main conclusion that can be drawn is that saliva can be relied upon to diagnose HGH deficiency by measuring IGFBP-3.

Acknowledgments:
The authors would like to thank Mustansiriyah University (www.uomustansiriyah.edu.iq), Baghdad, Iraq, for its support in the present work.

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