Original Article

Evaluation of 1, 5-anhydroglucitol as a salivary biomarker in Type 2 diabetes mellitus patients

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Introduction

Diabetes mellitus (DM) being one of the oldest diseases known to man was reported in Egyptian manuscript during 3000 years ago. It is caused due to low levels of insulin hormone secreted by beta-cells of islet of Langerhans in the pancreas. Insulin resistance at the level of insulin receptors, effector enzymes, or genes signal transduction system is responsible for these metabolic abnormalities occurring mainly in skeletal muscles, adipose tissue, and liver. Insulin-dependent diabetes (Type 1) is mainly due to autoimmune destruction of pancreatic β-cells through T-cell-mediated inflammatory response (insulites) and humoral (B-cell) response. Non-insulin-dependent DM (Type 2) consists of insulin resistance and defects in the function of pancreatic β-cells resulting in increased demand for insulin, accounting for 85–90% of all cases.

DM accounts for 4.6 million deaths annually worldwide and seen more in low- and middle-income countries with 50% of cases remain undiagnosed. India has the highest number of diabetic patients in the world. According to the International Diabetes Federation, incidence rate of diabetes in India is reported to increase 10-fold from 1.2% in 1971 to 12.1% in 2006 and further to 7.7% by 2030 posing enormous health problems and making India the diabetic capital of the world.

The screening tests for diabetes using serum collection following invasive techniques include hemoglobin A1C (HbA1C), fasting blood plasma, impaired glucose tolerance, and impaired glucose tolerance test. Demand for rapid and non-invasive diagnostic tests has developed exponentially in the past decade, leading to widespread research on saliva as biological fluid for clinical diagnosis. Saliva has several advantages compared to blood and urine such as easy collection of sample, economical for storing and transportation, and non-invasive method of collection decreases anxiety and discomfort in apprehensive patients. Consequently, saliva can be used as a non-invasive diagnostic tool for diabetic screening.

1, 5-anhydroglucitol (1, 5-AG) is a small monosaccharide supplied from diet. It is sourced in high quantities from rice, soybeans bread, beef, and other plant sources and remains stable in the body due to balance between intestinal absorption, urinary...
reabsorption, and secretion through intestinal tract.\textsuperscript{10} 1, 5-AG levels in saliva are correlated with 1, 5-AG in blood which is inversely proportional to blood glucose levels. Hence 1, 5-AG has a strong relationship with Type 2 DM.\textsuperscript{11}

Clinical studies conducted by Selvin \textit{et al.}, 2011,\textsuperscript{12} in serum samples determined 1, 5-AG by Glycomark and Kyowa Medex assays demonstrated 1, 5-AG in plasma samples by liquid chromatography (LC)–electrospray tandem spectrometry.\textsuperscript{12} All of the above studies required an invasive method for diabetic screening and established that 1, 5-AG in blood as a reliable marker of short-term glycemic control.

As there is a need for a non-invasive screening technique for diabetes, our studies focus on developing a non-invasive marker for diabetic screening by the evaluation of 1, 5-AG level in saliva and validate it with plasma levels by LC and mass spectrometry (LC–MS/MS) method.

\section*{Materials and Methods}

The study was carried out after obtaining permission from the institutional ethical committee, informed written consent from participating subjects, and recording a detailed case history. Subjects were selected from the outpatients of our institution.

\subsection*{Inclusion criteria: For G I (study group)}

The following criteria were included in the study:

\begin{itemize}
  \item Patients diagnosed with Type II DM (HbA1C confirmed)
  \item Patient between the age group of 30 and 65 years.
\end{itemize}

\subsection*{Inclusion criteria: For G II (control group)}

\begin{itemize}
  \item Healthy individuals who are non-diabetic (HbA1C confirmed) were included in the study.
\end{itemize}

\subsection*{Exclusion criteria}

\begin{itemize}
  \item Patients with other underlying systemic disorders were excluded from the study.
\end{itemize}

\subsection*{Procedure of saliva collection}

Non-fasting, unstimulated whole saliva was collected by spitting method after advising the subjects to refrain from eating ½ h before collection. Rinse mouth with water at least 10 min before collection of saliva. Subjects allow the saliva to accumulate in mouth and then expectorate into graduated cylindrical test tube, for every 60 s for 2–5 min till sufficient sample (2–3 ml) is collected. Saliva samples were stored between 2 and 8°C and transported to laboratory within 6 h of sample collection. Samples were centrifuged at 2500 rpm for 10 min, aliquoted, and subjected to MS on the same day.

\subsection*{Procedure for blood collection}

Under aseptic conditions, 2–3 ml of blood was collected from antecubital fossa. Blood samples were stored between 2 and 8°C and transported to laboratory within 6 h of sample collection. Samples were centrifuged at 5000 rpm for 5 min, aliquoted, and subjected to MS on the same day.

\section*{Quantification of 1, 5-AG in blood and saliva samples by LC–MS/MS}

\subsection*{Standard preparation}

1, 5-AG was purchased from Toronto Research Chemicals. Standard stock of 100 ppm solution is prepared in methanol:water (50:50) further diluted to 10–500 ppb in same diluents.

\subsection*{Saliva sample preparation}

About 0.5 ml of saliva sample was taken in 2 ml microcentrifuge tube and 1.5 ml methanol was added, shaken vigorously for 1 min, and centrifuged for 5 min at 10,000 rpm. 1 ml of supernatant was taken, 1 ml of water was mixed thoroughly and filtered through 0.42 µ syringe filter, and injected to LC–MS/MS.

\subsection*{Blood sample preparation}

After centrifugation of blood samples, serum extract was subjected to MS. MS instrument condition was evaluated and method validation was done based on specificity, precision, lineants, recovery, limit of detection, and limit of quantification. Saliva and blood samples were subjected to LC–MS for a period of 9 min; data obtained were subjected to statistical analysis.

\section*{Results}

Values obtained were entered into Microsoft Excel sheet and statistical analysis done using the SPSS v.22 (IBM, Corp.) for Windows software.

The sociodemographic characteristics analysis with age as parameter among G1 and G2 subjects included a mean age of 54.1 years among G1 and mean age of 51.6 years among G2 with no significant difference seen among mean age range between G1 and G2 subjects.

The gender parameter analysis among G1 shows a higher female predominance of 53.3% and G2 shows a higher male predominance of 56.7% with no significant difference seen between the two groups with \( P = 0.4 \).

In Table 1 – the correlation coefficient is denoted by “rho.”

\begin{table}[h]
\centering
\begin{tabular}{ |c|c|c| }
\hline
\textbf{Group} & \textbf{Variables} & \textbf{Values} & \textbf{1, 5-AG saliva} & \textbf{1, 5-AG serum} \\
\hline
\textbf{Group 1} & 5-AG-saliva & \textbf{Rho} & 1 & 0.41 \\
 &  & \textbf{P-value} & - & 0.027* \\
 &  & \textbf{N} & 30 & 30 \\
\hline
\textbf{Group 2} & 5-AG-saliva & \textbf{Rho} & 1 & 0.47 \\
 &  & \textbf{P-value} & - & 0.009* \\
 &  & \textbf{N} & 30 & 30 \\
\hline
\end{tabular}
\caption{Relationship between salivary and serum 1, 5-AG levels in G1 and G2 using Spearman’s correlation test}
\end{table}

1, 5-AG: 1, 5-anhydroglucitol, *Statistically significant
1, 5-AG salivary biomarker in DM patients
Asha, et al.

Discussion

Type 2 DM is highly common, primarily due to increase in the prevalence of sedentary lifestyle and obesity. Although there are various diabetes screening tests such as fasting plasma glucose, glucose tolerance test, and the gold standard HbA1C, at least quarter of DM cases in India remain undiagnosed. In recent times, screening for diabetes using saliva has been suggested as an effective approach to identify affected individuals. Diabetes screening using saliva rather than blood and urine could facilitate a non-invasive technique for diabetes screening. Studies done by Yoshioka et al. have established 1, 5-AG as a glycemic marker in the serum. The present study evaluated 1, 5-AG levels in saliva and validated it with serum among type 2 DM patients using LC–MS method.

In the present study, G1 DM subjects consisted of 14 males and 16 females with higher female predominance of 53.3%. G2 consisted of 17 males and 13 females with a higher male predominance of 56.7%. Therefore, a female predominance was noted which could be attributed to lower attendance of male patients during the specific study period or due to difference in the prevalence of type II DM in the population studied. This is in agreement with findings reported by Cheekurthy et al. There is wide variation in gender distribution of Type 2 DM. Type 2 DM mainly affects older age group; many people in younger age group are also being affected with this disease. In our study, G1 was 38–66 years and G2 was 36–66 years. This could be attributed to higher attendance of subjects belonging to the age group between 6 and 66 years during specific study period. It is in agreement with studies done by Gyawali et al. The variation in age distribution could be due to difference in the prevalence of type 2 DM in population studied.

The mean HbA1C level among G1 subjects is significantly higher than G2 with \( P < 0.01 \) [Table 2]. It is in agreement with studies done by Monnier et al. The reason for this variation could be attributed to hemoglobin glycosylation rate which depends on the glucose concentration and, therefore, reflects long-term glycemic control associated with erythrocyte turnover.

The mean salivary and serum 1, 5-AG levels in G1 is less than G2 and is statistically significant with \( P < 0.001 \) [Table 3]. It is in agreement with studies done by Barnes et al. The reason attributed to decrease in serum and salivary 1, 5-AG levels among G1 could be due to inhibition of 1, 5-AG reabsorption in renal tubules by glucosuria which leads to reduction in 1, 5-AG levels in serum and saliva.

The mean salivary 1, 5-AG levels are slightly lower in males compared to that of females in G1 (2163.69%, 342.7%) and vice versa in G2 and are in agreement with studies done by Moork–Kanamori et al. This is attributed to the duration of DM and when duration was adjusted that the difference between male and female was not significant. On the contrary, studies done by Yousri et al. reported that female has lower mean salivary 1, 5-AG levels compared to males. This difference could be due to the physical differences in the renal excretion between genders.

The mean serum 1, 5-AG levels in female is slightly lower than that of male in G1 (131.205, 4230.9%) and vice versa in G2 subjects. It is in agreement with studies done by Wang et al. This is attributed to physical differences in renal excretion between genders. On the contrary, studies done by Kim et al. revealed that 1, 5-AG level in male is slightly lower than that of female among type 2 DM subjects. This difference could be attributed to differences in the duration of DM and when duration was adjusted that the difference between the men and women was not significant.

In the present study, a moderate positive correlation between 1, 5-AG level in serum and saliva among G1 and G2 subjects is seen and is significant with \( P \) value of 0.03 and 0.009 [Table 1, Graph 1]. It is also revealed the prediction of serum 1, 5-AG levels using salivary 1, 5-AG levels, based on simple linear regression analysis which reveals, for every 1 unit increase in salivary 1, 5-AG levels the serum levels increase by 7.7 units in diabetic group; that is, serum 1, 5-AG levels = 7.71 \times \text{salivary 1, 5-AG levels}.

![Graph 1: Scatter plot depicting relationship between salivary and serum 1, 5-anhydroglucitol levels in the study group. (Green line of fit depicts the healthy control subjects and orange line of fit depicts the type 2 diabetes mellitus subjects)](image)

### Table 2: Comparison of mean HbA1c levels between G1 and G2 using independent Student’s t-test

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>N</th>
<th>Mean±SD</th>
<th>Mean Diff.</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>Group 1</td>
<td>30</td>
<td>8.49±0.76</td>
<td>2.57</td>
<td>13.924</td>
<td>&lt;0.000*</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>30</td>
<td>5.92±0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HbA1c: Hemoglobin A1C, SD: Standard deviation, *Statistically significant

### Table 3: Comparison of mean salivary and serum 1, 5-AG levels between G1 and G2 using Mann–Whitney U-test

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>N</th>
<th>Mean±SD</th>
<th>Mean Diff.</th>
<th>Z</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 5-AG</td>
<td>Group 1</td>
<td>30</td>
<td>305.7±198.56</td>
<td>−269.26</td>
<td>−3.844</td>
<td>&lt;0.000*</td>
</tr>
<tr>
<td>in saliva</td>
<td>Group 2</td>
<td>30</td>
<td>574.9±277.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 5-AG</td>
<td>Group 1</td>
<td>30</td>
<td>4177.7±3775.75</td>
<td>−5588.26</td>
<td>−5.101</td>
<td>&lt;0.000*</td>
</tr>
<tr>
<td>in serum</td>
<td>Group 2</td>
<td>30</td>
<td>9766.0±2986.26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1, 5-AG: 1, 5-anhydroglucitol, SD: Standard deviation, *Statistically significant.
levels + (1821.30). For every 1 unit increase in salivary 1, 5-AG levels the serum 1, 5-AG levels significantly increase by 5.13 units in non-diabetic group; that is, serum 1, 5-AG levels = 5.3 × salivary 1, 5-AG levels + 6815.14 [Table 4]. The present study is in unanimous agreement with studies done by Barnes et al.,[19] Mook-Kanamori et al.[11] and Youssi et al.[30]

A study of this magnitude provides a comprehensive association of metabolic pathway with DM in two biofluids from the same patient. Taken together, our data suggest that 1, 5-AG in saliva constitutes a non-invasive marker for deregulated short-term glycemic control, reflecting glycemic status over the previous 48 h–2 weeks. Sequential 1, 5-AG assessments may be useful in monitoring patient’s compliance and pharmaceutical response. Hence can be used as a screening tool for the diagnosis of undetected diabetes.

Conclusion

This is a baseline study that was carried out during the period of 2017–2018 for evaluation of 1, 5-AG as a salivary biomarker in Type II DM patients. The results of the study infer the detection of 1, 5-AG in saliva and serum of healthy controls and detection of serum and salivary 1, 5-AG levels of HbA1C confirmed Type II DM patients and assest the correlation between 1, 5-AG in saliva and plasma of Type II DM subjects.

Future studies involving large sample size and categorization of study population based on fasting and non-defined fasting state should be conducted to further elucidate the present study. Research for further optimization of the assay for 1, 5-AG measurements in saliva will likely result in a versatile tool for diabetic screening and monitoring that will improve type II DM diagnosis, especially in pediatric patients and could be considered as one of the future study goals.

References

21. Wang Y, Yuan Y, Zhang Y. Serum 1, 5 anhydroglucitol level as
