Salivary glucose and antioxidant defense markers in type II diabetes mellitus

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Background/aim: To evaluate salivary antioxidant defense markers, their correlation with salivary glucose, and glycemic status in type II diabetes mellitus (DM).

Materials and methods: The study included 53 diabetic patients and 40 healthy subjects. Salivary glucose, blood glucose, and uric acid (UA) were determined by specific enzymatic methods. Total antioxidant activity (AOA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and total protein were determined spectrophotometrically.

Results: Salivary UA (3.12 vs. 1.89 mg/dL), GSH (47 vs. 11.92 µg/mL), and total protein (375.12 vs. 202.23 mg/dL) were significantly higher (P < 0.001; r = 0.455, 0.735, 0.498 respectively) and AOA (653.1 vs. 897.3 µmol/L) was significantly lower in the DM group (P < 0.001, r = –0.431) compared to healthy controls. Among the antioxidant enzymes, CAT was significantly lower (1214 vs. 9468.9 kat) in the DM group (P < 0.001, r = –0.886). Spearman correlation analyses within the diabetic group showed a strong positive association between salivary glucose and blood glucose (P < 0.001, r = 0.9), salivary glucose and GSH, and salivary glucose and UA. Salivary glucose showed a negative correlation with AOA and CAT (P = 0.008, r = –0.447) in the diabetic group.

Conclusion: Findings of this study, showing a strong correlation between salivary glucose and blood glucose as well as changes in antioxidant components in the DM group, suggest that saliva can be used for the diagnosis and management of DM.

Key words: Antioxidant activity, catalase, diabetes mellitus, glutathione, oxidative stress, saliva, superoxide dismutase, uric acid

1. Introduction
Diabetes mellitus (DM), a chronic endocrine metabolic disorder, has become a worldwide epidemic affecting both developed and developing countries. The global incidence of DM was 366 million cases in 2011. In India alone there were around 61.3 million cases and it is predicted that this may increase to 101.2 million by 2030 (1). This suggests that its diagnosis and management will become one of the major health challenges of the 21st century. An alternate simple approach for detection and routine monitoring of glucose and other related parameters of DM are the need of the hour. Recently, the use of saliva as an investigative tool for disease processes and disorders has begun attracting wide attention (2). Studies have explored the diagnostic potential of saliva in cardiovascular diseases, autoimmune disorders, cancer, oral conditions, etc. (3). Collection of saliva is noninvasive and requires minimal expertise.

DM is characterized by chronic hyperglycemia, a result of defects in insulin secretion and/or insulin action that cause disturbances in carbohydrate, fat, and protein metabolism (4). There are many reports on the increased production of free radicals leading to oxidative stress in DM, which plays an important role in intensifying DM-associated complications such as retinopathy, nephropathy, etc. (4,5). Antioxidant defense mechanisms of the body work towards minimizing this damage. The human antioxidant system comprises 2 major groups, enzymatic and nonenzymatic. Key enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase. SOD is an oxidoreductase that catalyzes dismutation of highly reactive superoxide radicals (O₂⁻) to O₂ and H₂O₂, the latter being subsequently decomposed to harmless H₂O and O₂ by CAT (6,7). Nonenzymatic antioxidants are many and include macromolecules such as albumin, ceruloplasmin, and ferritin as well as low-molecular-weight molecules like ascorbic acid, reduced glutathione (GSH), uric acid (UA), and bilirubin. All of these act synergistically to maintain

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or reestablish redox homeostasis. Total antioxidant activity (AOA) is a measure of nonenzymatic antioxidants. Several methods have been developed to assess the AOA of human serum or plasma because of the difficulty in measuring each antioxidant component separately (8). The measured AOA of a sample depends on the methodology and the free radical generator or oxidant used in the measurement (8–10). An assessment of a few individual antioxidant parameters and AOA in healthy subjects and in controlled and uncontrolled DM cases may help in its better management.

Accumulating data on sialochemistry show that saliva reflects human plasma/serum biomolecular composition associated with DM and hence the systemic condition (2). However, contradicting results from earlier studies of DM present a pressing need for the reevaluation of such changes in salivary components. The purpose of this study was to investigate the feasibility of a noninvasive approach by using saliva for routine monitoring of glucose in DM as well as antioxidant status in controlled and uncontrolled DM cases as compared to healthy subjects.

2. Materials and methods

This study was conducted at the Center for Post Graduate Studies, Jain University, Bangalore, India, for a period of 1 year (August 2011 to August 2012). The Institutional Ethics Committee of Bhagwan Mahaveer Jain Hospital (Bangalore, India) approved the study protocol.

2.1. Study group

The study group included in-house diabetic patients diagnosed by the Medical Faculty of the Endocrinology Department of Bhagwan Mahaveer Jain Hospital, Bangalore. Prior to subject recruitment, patients with oral infections, inflammatory conditions, and smoking habits were identified (with assistance from consultants/questionnaires) and excluded from the study. Informed consent was obtained from all participants. A thorough clinical history was collected by means of a questionnaire. Patients were either on insulin or oral hypoglycemic drugs. Fasting blood glucose values of chosen DM patients (analyzed on the same day as saliva) were collected from the hospital registry. Based on the glucose values, these cases (n = 53) were divided into patients with controlled DM, n = 27 (fasting blood glucose of <140 mg/dL) and patients with uncontrolled DM, n = 26 (fasting blood glucose of ≥140 mg/dL), to form 2 groups (11). The average duration of disease was 8.73 ± 7.29 years, excluding 2 patients that were >30 years. The third group was a control group (n = 40) and comprised healthy age- and sex-matched nondiabetic subjects with blood glucose within normal limits (Table 1). Considering the number of parameters assayed for each sample, methods used for data collection, and the duration of the study, a sample size large enough to be an accurate representative of the population to achieve significant results and control errors was calculated.

2.2. Sample collection and preparation

Unstimulated fasting saliva from patient and control groups was collected between 0700 and 0800 hours by expectoration method (12,13). Subjects were asked to rinse their mouth thoroughly with water, bend their heads forward, and allow saliva to flow into an ice-chilled sterile polypropylene tube. The tubes were brought immediately to the laboratory from the collection site in chilled conditions. Saliva samples were checked for blood and phlegm and rejected if found to be contaminated. This was followed by centrifugation at 4000 rpm for 15 min to remove any particulate matter. The supernatant was freeze-preserved or immediately used for analysis. Fasting blood samples from patients were collected on the same day as saliva collection by standard venipuncture by the hospital attendant and blood glucose was estimated in the hospital laboratory. These results were collected from the hospital documents. Some of the healthy volunteers were also asked to have their fasting blood glucose analyzed on the same day as saliva collection.

2.3. Chemicals

All chemicals were procured from HiMedia (Mumbai, India) and Merck (Bangalore, India). Kits for glucose, protein and UA assays were procured from Accurex Biomedical Pvt. Ltd. (Thane, India). All chemicals were of analytical grade.

2.4. Analysis of physical characteristics

The pH of saliva samples was measured by dipping pH strips into them and comparing the color change with the standard color bar provided. Volume and froth were measured by comparing the collection tube with a precalibrated tube and reported in milliliters. Salivary flow rate (volume of saliva secreted per minute) was obtained by dividing the total volume of saliva in the container with duration of collection and reported as milliliters per minute.

2.5. Salivary enzymatic antioxidants

CAT activity was measured by Sinha’s method (14). Estimation of SOD was carried out based on Kakkar et al’s method (15).

2.6. Salivary nonenzymatic parameters

Total AOA assay was carried out according to Koracevic et al. (9). GSH was measured according to Ellman’s method with slight modification (16), wherein to eliminate saliva turbidity contribution to the absorbance values, an internal control was prepared for every sample assayed. UA was assayed using the Infinite Uric Acid Liquid Kit (Accurex Biomedical). Glucose was assayed using the Eco-Pak Glucose Kit (Accurex Biomedical). Total protein was determined by the biuret method using the Autozyme.
Total Protein Biuret Kit (Accurex Biomedical). When using the commercial kits to quantify salivary UA, glucose, and total protein, the volume of sample used was appropriately increased so that the concentration was closer to the standard provided by these kits.

2.7. Statistical analysis
Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) (17). Analysis of variance (ANOVA) and the Spearman correlation test were used. P < 0.05 was considered significant.

3. Results
Samples from 93 subjects were included in the study, out of which 53 were diabetic patients and 40 were healthy controls. The diabetic patients were divided into 2 groups: controlled DM (fasting blood glucose of <140 mg/dL) and uncontrolled DM (fasting blood glucose of ≥140 mg/dL) (Table 1).

A significant increase in the mean salivary glucose concentration (P < 0.001) was observed in the diabetic group when compared to healthy controls. SOD and CAT, major antioxidant enzymes, showed different responses. SOD showed a marginally higher activity in diabetic cases, with no difference in the subgroups. However, a drastic reduction in CAT activity was seen in diabetic samples, with a 7.11 and 8.66 times reduction in the controlled and uncontrolled groups, respectively (Table 2).

The AOA was found to be lower in diabetic patients, with maximum reduction observed in the uncontrolled group. GSH level was higher in diabetic (both controlled and uncontrolled) patients. Mean UA was approximately 1.7 times higher in diabetic patients than in healthy controls. Salivary total protein was found to be almost 2 times higher in the uncontrolled group and about 1.5 times higher in the controlled DM group (mean: approximately 1.8 times). Table 2 compares the mean values of the salivary parameters analyzed.

3.1. Spearman correlation analysis
In order to determine whether the differences observed could be linked to glycemia, the parameters recorded in the diabetic group were compared to salivary glucose levels. Fasting salivary glucose showed strong positive correlation with fasting blood glucose (all diabetic patients: r = 0.9, P < 0.001; controlled cases: r = 0.9, P < 0.001; uncontrolled cases: r = 0.922, P < 0.001). Association between UA and salivary glucose in the diabetic group was found to be positive. AOA showed a significant negative correlation with salivary glucose mainly because of the uncontrolled diabetic category. GSH showed positive correlation with salivary glucose. Among the endogenous enzymes, CAT showed a significant negative correlation with salivary glucose in the diabetic group, with the uncontrolled category exhibiting a higher contribution. SOD showed a negative correlation with salivary glucose. Tables 3 and 4 show results of Spearman correlation analyses.

Flow rate and pH decreased with increase in salivary glucose content. Salivary total protein was also significantly higher in diabetic patients who suffered from other pathological conditions (hypertension, cardiac-related ailments, etc.) in addition to DM. An increase in froth and a marginally low salivary flow rate along with pH were other significant observations in the diabetic group. Other associated observations included male diabetic patients showing a significantly high GSH level compared to females. With age, CAT activity and AOA showed a negative association; however, there was a positive association with GSH.

Table 1. Study group details.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Age, years</th>
<th>Fasting blood glucose, mean ± SD, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>40</td>
<td>53.50 ± 10.67 (34–71)</td>
<td>86.30 ± 4.8 (80–102)</td>
</tr>
<tr>
<td>Males (18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>53</td>
<td>61.96 ± 13.5 (33–84)</td>
<td>160.64 ± 73 (80–340)</td>
</tr>
<tr>
<td>Males (32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females (21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic patients (controlled cases)</td>
<td>27</td>
<td>63.29 ± 14.3 (33–84)</td>
<td>109 ± 15.9 (80–140)</td>
</tr>
<tr>
<td>Diabetic patients (uncontrolled cases)</td>
<td>26</td>
<td>60.63 ± 13 (43–84)</td>
<td>211.85 ± 70.62 (140–340)</td>
</tr>
</tbody>
</table>

Controlled DM: blood glucose of <140 mg/dL; uncontrolled DM: blood glucose of ≥140 mg/dL.
**Table 2.** Mean values of salivary parameters analyzed.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Parameter</th>
<th>Diabetic subjects Mean (SE)</th>
<th>Healthy controls Mean (SE)</th>
<th>P-value (r-value) between healthy subjects and all diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diabetic subjects</td>
<td>Uncontrolled cases</td>
<td>Controlled cases</td>
</tr>
<tr>
<td>1</td>
<td>Fasting glucose (mg/dL)</td>
<td>161.07 (10)</td>
<td>214.69 (14.1)</td>
<td>109.44 (3.1)</td>
</tr>
<tr>
<td>2</td>
<td>UA (mg/dL)</td>
<td>3.12 (0.21)</td>
<td>3.26 (0.3)</td>
<td>3.03 (0.2)</td>
</tr>
<tr>
<td>3</td>
<td>GSH (µg/mL)</td>
<td>47 (2.8)</td>
<td>49.9 (2.3)</td>
<td>44.8 (4.2)</td>
</tr>
<tr>
<td>4</td>
<td>AOA (µmol uric acid eq/L)</td>
<td>653.10 (45)</td>
<td>542.86 (51)</td>
<td>759.30 (68)</td>
</tr>
<tr>
<td>5</td>
<td>SOD (U/mL)</td>
<td>0.26 (0.01)</td>
<td>0.26 (0.01)</td>
<td>0.26 (0.01)</td>
</tr>
<tr>
<td>6</td>
<td>CAT (kat)</td>
<td>1214 (68)</td>
<td>1092.22 (61.1)</td>
<td>1330.37 (116)</td>
</tr>
<tr>
<td>7</td>
<td>Salivary glucose (mg/dL)</td>
<td>5.83 (0.5)</td>
<td>8.34 (0.8)</td>
<td>3.41 (0.1)</td>
</tr>
<tr>
<td>8</td>
<td>Total protein (mg/dL)</td>
<td>375.12 (26)</td>
<td>407.80 (40.6)</td>
<td>346 (32.3)</td>
</tr>
</tbody>
</table>

Controlled DM: blood glucose of <140 mg/dL; uncontrolled DM: blood glucose of ≥140 mg/dL; P < 0.05 is considered significant. SE: standard error of mean.

**Table 3.** Correlative analysis of salivary glucose with various parameters in type II diabetic patients.

<table>
<thead>
<tr>
<th>Correlations</th>
<th>All diabetic patients</th>
<th>Uncontrolled cases</th>
<th>Controlled cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. glucose–S. glucose</td>
<td>r-value 0.9*</td>
<td>0.922*</td>
<td>0.9*</td>
</tr>
<tr>
<td></td>
<td>P-value &lt;0.001</td>
<td>&lt;0.001</td>
<td>&gt;0.001</td>
</tr>
<tr>
<td>UA–S. glucose</td>
<td>r-value 0.258</td>
<td>0.409</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>P-value 0.079</td>
<td>0.072</td>
<td>0.847</td>
</tr>
<tr>
<td>GSH–S. glucose</td>
<td>r-value 0.003</td>
<td>–0.217</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>P-value 0.97</td>
<td>0.318</td>
<td>0.533</td>
</tr>
<tr>
<td>CAT–S. glucose</td>
<td>r-value –0.447*</td>
<td>–0.934*</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>P-value 0.008</td>
<td>&lt;0.001</td>
<td>0.544</td>
</tr>
<tr>
<td>SOD–S. glucose</td>
<td>r-value –0.428*</td>
<td>–0.458</td>
<td>0.181</td>
</tr>
<tr>
<td></td>
<td>P-value 0.004</td>
<td>0.018</td>
<td>0.364</td>
</tr>
</tbody>
</table>

*: Significant, S: salivary, B: blood. Controlled DM: blood glucose of <140 mg/dL; uncontrolled DM: blood glucose of ≥140 mg/dL.
4. Discussion

The present study demonstrates that glucose and antioxidant levels in saliva from diabetic patients exhibit significant differences compared to control samples.

There was a remarkable increase in fasting salivary glucose levels in the diabetic group. Another significant observation was a parallel increase in fasting salivary glucose with fasting blood glucose levels, which coincides with previous observations made by Hegde et al. (18). To analyze the potential of saliva in reflecting the glycemic picture, correlation analysis between salivary glucose and blood glucose levels was carried out. A value of P < 0.001 supports the suitability of saliva as a substitute for blood for monitoring the glycemic status (19). There are also a few reports contradicting the above observations (11,20,21).

CAT exhibited a marked reduction in its activity, contributing less to the removal of H$_2$O$_2$. Assessment of salivary CAT activity in DM is sparse. However, increased CAT has been reported in experimental rats under induced diabetic conditions (22–24). Negative correlation of CAT with salivary glucose and contribution by uncontrolled DM in this correlation may indicate its prognostic potential in this disorder. Only a marginal increase in SOD activity was observed in the diabetic group, as also observed by Al-Rawi (17). This marginal increase could be to combat the excess O$_2^-$ possibly produced in this disorder.

UA is a strong antioxidant in a hydrophilic environment (25). An increase in UA concentration, which corroborates with earlier findings (17), was observed in the saliva of diabetic patients in the present study. It showed a positive correlation with salivary glucose. Uncontrolled diabetic patients had higher levels, suggesting its association with severity of this disease. This supports the compensatory antioxidant defense by UA in saliva. However, the defense role of UA is controversial. Recently it was shown that under selected circumstances, the original antioxidant properties of UA paradoxically become prooxidant (9). It is worth noting that hyperuricemia has been found to be associated with obesity, metabolic syndrome, and insulin resistance and consequently with type 2 DM (26).

GSH was found to be present in enhanced quantities in diabetic individuals in this study, whereas work by Savu et al. and Memisogullari et al. reported a decrease in GSH (27,28). Enhanced levels of GSH and UA may possibly be a compensatory response to tackle O$_2^-$ load through free radical scavenging. Reduction in CAT activity could also be a predisposing factor for the observed enhanced GSH level. This can be further substantiated by studying the contribution of GSH under such circumstances.

Total AOA decreased with increase in salivary glucose in the current study. However, a few other reports suggested an increase in AOA in diabetic saliva (18,29). The perplexing observation in this study was a decrease in AOA in spite of elevated levels of UA and GSH in DM. The measured AOA of a sample depends on which method is used in the measurement (8,14). The current study used a method that recommends UA as a standard (9). GSH response to this method is poor. Mean salivary UA concentration in healthy volunteers was 1.89 mg/dL (112 µmol/L), which corresponds to 12.48% of total AOA (897.31 µmol/L). The rest of the measured AOA appeared to be the result of other antioxidant salivary components. In the diabetic group, mean salivary UA concentration was 3.12 mg/dL (180 µmol/L), which represented 27.56% of the total AOA (653.13 µmol/L). Thus, even with an increase in UA, changes in other salivary antioxidant components might have led to the decrease in total AOA.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Parameters</th>
<th>r-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive correlation</td>
<td>CAT–AOA</td>
<td>0.437</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>TP–UA</td>
<td>0.393</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>TP–GSH</td>
<td>0.356</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>GSH–UA</td>
<td>0.244</td>
<td>0.043</td>
</tr>
<tr>
<td>Negative correlation</td>
<td>CAT–GSH</td>
<td>–0.654</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CAT–TP</td>
<td>–0.489</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CAT–UA</td>
<td>–0.471</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>AOA–GSH</td>
<td>–0.440</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CAT–SOD</td>
<td>–0.241</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>AOA–TP</td>
<td>–0.241</td>
<td>0.024</td>
</tr>
</tbody>
</table>
Salivary total protein level was found to be increased in the diabetic group in comparison with that of the controls, as repeatedly reported in various earlier studies (30). Protein antioxidants such as albumin have been recently reported to be important contributors to antioxidant plasmatic barriers (31). However, its role at the salivary level still needs to be probed. The reflection of glycemic status in saliva remains one of the significant observations of the study. Taken together with the observed antioxidant biochemical variations in saliva, this study brings substantial insight into the pathogenesis and evolution of type II diabetes. Whether such alterations predispose one to the development of associated systemic complications remains to be addressed by a follow-up study.

Acknowledgments
The authors thank Dr BM Suraj, Dr TS Ravindra, and Dr SK Supreeth (Department of Internal Medicine, Bhagwan Mahaveer Jain Hospital) for assisting in sample collection. The authors also thank Jain University for the research support, Professor Leela Iyengar for her comments on the project, and the volunteer sample donors.

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