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News & Views

Relationship of Flow Rate, Uric Acid, Peroxidase, and Superoxide Dismutase Activity Levels with Complications in Diabetic Patients: Can Saliva Be Used to Diagnose Diabetes?

MOSHE ZLOCZOWER,[1,4] ABRAHAM Z. REZNICK,[2,4] RULA OBEID ZOUBY,[1–4] and RAFAEL M. NAGLER[3,4]

ABSTRACT

Can salivary free radicals and antioxidant parameters be useful in general diagnosis and evaluation of diabetes mellitus Type II (DM)? Serum and salivary redox state of 40 diabetes mellitus patients were examined and compared with 20 controls. The involvement of salivary gland in diabetes mellitus has been suggested based on salivary flow rate and compositional alterations. In addition, the redox state of saliva of diabetes mellitus patients is different than that of normoglycemic control human subjects. This observation unveils the opportunity to use noninvasive saliva-based diagnostics for diabetes mellitus patients. *Antioxid. Redox Signal.* 9, 765–773.

DIAGNOSIS OF DIABETES MELLITUS AS RELATED TO OXIDATIVE STRESS

DIAGNOSIS AND MONITORING OF DIABETIC PATIENTS are based on numerous glucose and hemoglobin A1c (HbA1c) measurements routinely performed on blood samples taken from patients. These measurements are neither sensitive nor accurate enough to properly evaluate the severity and prognosis of the disease. Routine taking of blood is not only an invasive procedure that may spread infection, but it also requires a medical professional and can become expensive over time. Accordingly, having a better means for diagnosing the severity of the disease and predicting its prognosis in light of the pathogenesis of diabetes mellitus would be beneficial.

Recently accumulated data indicate a possible pivotal role for oxidative stress in complication pathogenesis (1, 5, 6, 21). Multiple pathways, such as nonenzymatic glycation of proteins and monosaccharide auto-oxidation, may mediate this process in diabetes mellitus patients, gradually building up advanced glycation endproducts (AGEs) (4). A strong positive correlation among the status of metabolism control and the duration of diabetes mellitus and the severity of induced oxidative stress has been reported (16). Other studies have shown that the serum level of malondialdehyde (MDA), a fatty chain peroxidation marker, significantly increased in diabetic patients (5), which may in turn cause atherosclerosis. Additional studies have shown that various serum antioxidants are decreased in diabetes mellitus patients, including superoxide dismutase (SOD), ascorbic acid (vitamin C), uric acid (UA), and glutathione, etc. (5, 6, 15, 18). Furthermore, one study reported that defective nerve conduction in diabetic subjects with mild to moderate peripheral neuropathy may be improved by pharmacological doses of vitamin E (19). Therefore, beyond an improved understanding of the pathogenesis of the disease, the accumulated data may suggest a further exploration of oxidative stress markers for diagnosis and prognosis and for developing new therapeutic strategies.

DEMONSTRATED SALIVARY GLAND INVOLVEMENT AND OXIDATIVE STRESS

Examination of salivary involvement in diabetes mellitus research may assist in the development of new diagnostic tools for this illness, based on salivary analysis. This also may

[1]Endocrine Department and Diabetes in Pregnancy Clinic, [2]Department of Anatomy and Cell Biology, [3]Oral and Maxillofacial Surgery Department, Biochemistry Laboratory and Salivary Clinic, Rambam Medical Center, Haifa, Israel.
[4]Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

contribute to better understanding of an important clinical problem related to xerostomia, periodontitis, and increased rate of caries (7). There have been few studies analyzing the salivary composition in diabetes mellitus patients and not even one examining the salivary antioxidant profile. Therefore, the purpose of the current study was to analyze both serum and salivary composition in diabetes mellitus patients, focusing on free radical-related effects and antioxidants, with respect to the severity of the disease and its accompanying complications.

Accordingly, the sialochemistry analysis currently performed (see Appendix, note 2) included total protein (TP), calcium (Ca), magnesium (Mg), and phosphate (P) concentrations, as well as LDH and amylase activities. Peroxidase activity (see Appendix, note 3) was measured both in the patients' serum and saliva. The total salivary activity of the superoxide dismutase (SOD) enzyme (see Appendix, note 4) was measured, and UA concentration (see Appendix, note 5) was measured both in patient serum and saliva. Photon emission during heating was measured by a thermochemiluminescence (TCL) Photometer (see Appendix, note 6). Protein carbonyl determination was performed in the serum, and carbonyl and malondialdehyde (MDA) production were assessed (see Appendix, note 7).

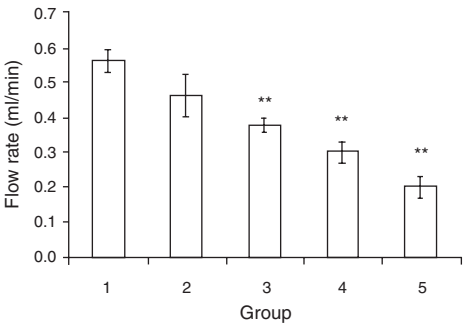

FIG. 1. Mean salivary flow rates (+ standard deviations) of consenting patients with disease (2–5) *versus* healthy controls (1). The study groups (n = 8–12 in each) were composed of the following: (**A**) Groups 2 and 3: those with controlled diabetes mellitus (HbA1c ≤ 7.5%) and Groups 4 and 5: those with uncontrolled diabetes mellitus (HbA1c > 7.5%), and (**B**) those with (Groups 3 and 5) or without (Groups 2 and 4) the following clinical complications: nephropathy, neuropathy, or retinopathy. In Group 1, there were 20 healthy controls. $^{**}p < 0.01$. The mean salivary flow rates of the five subgroups were compared using the Kruskal–Wallis test (a multiple comparison nonparametric test), demonstrating a significant difference where $p = 0.0001$.

SALIVARY FREE RADICAL AND ANTIOXIDANT COMPOSITION AND FLOW RATE ARE ALTERED IN DIABETES MELLITUS PATIENTS

The mean salivary flow rate value of the healthy controls was 0.56 ± 0.14 ml/min and was lower in diabetes mellitus patients. Thus, whereas the flow-rate reduction in the diabetes mellitus-controlled patients without complications did not reach statistical significance, the reduction in the three other, more severe diabetes mellitus groups was significant and correlated with the severity of the disease. Hence, the flow-rate reduction in the diabetes mellitus-controlled group with complications was reduced by 33% ($p < 0.01$) and in the diabetes mellitus-uncontrolled groups with and without complications it was reduced by 47% ($p < 0.01$) and by 55% ($p < 0.01$), respectively (Fig. 1).

The salivary composition of the diabetes mellitus patients was altered in most of the parameters examined as compared with controls and more so in the diabetes mellitus-uncontrolled groups, although it did not reach statistical significance in all cases. For example, in the diabetes mellitus-uncontrolled group without complications, the total protein, amylase, and magnesium levels were increased by 47% (p = 0.05), 28% (p = 0.05), 50% (p = 0.04), while those of LDH and calcium dropped by 59% (p = 0.04) and 63% (p = 0.0001), respectively. In the diabetes mellitus-controlled group of patients with complications, the LDH and calcium values dropped by 60% (p = 0.05) and 72% (p = 0.0004), respectively.

The mean salivary-peroxidase activity value, normalized to volume, of healthy controls was 450 ± 132 U/100 μl and was significantly higher in all four diabetes mellitus groups: 31%–55%. The mean serum-peroxidase activity value, normalized to volume, of healthy controls was 699 ± 174 U/100 μl and was also significantly higher in all four diabetes mellitus groups: 25%–30% (Fig. 2). Thus, the salivary peroxidase values in the diabetes mellitus-controlled groups with and without complications and in the diabetes mellitus-uncontrolled groups with and without complications were increased by 39%, 50%, 55%, and 31%, respectively. The serum peroxidase values were increased by 25%, 25%, 27%, and 30%, respectively.

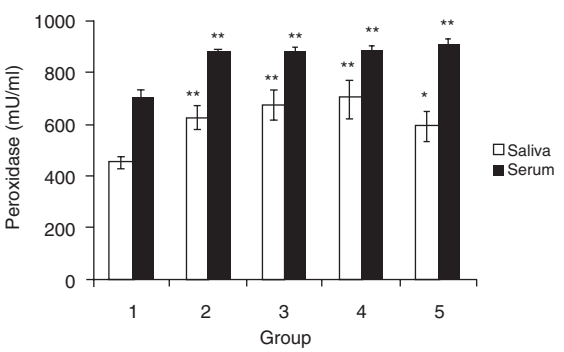

FIG. 2. Mean salivary and serum pereoxidase activity rates (+ standard deviations) of consenting patients with disease (2–5) versus healthy controls (1). Conditions as in Fig. 1. In Group 1, there were 20 healthy controls. $^{**}p < 0.01$, $^{*}p < 0.05$. The mean salivary and serum peroxidase activity rates among the five subgroups were compared using the Kruskal–Wallis test (a multiple comparison nonparametric test), demonstrating a significant difference where $p = 0.0002$ for saliva and $p = 0.0001$ for serum.

TABLE 1. CORRELATION AMONG SALIVARY SUPEROXIDE DISMUTASE (SOD) ACTIVITY AND NEUROPATHY (**A**), SERUM THERMOCHEMILUMINESCENCE (TCL) AND RETINOPATHY (**B**), AND SERUM CARBONYLS AND NEPHROPATHY (**C**)

A

Neuropathy		*SOD (saliva)*
Yes	*n*	5
	Range	0.14–6.8
	Median	4.37
No	*n*	16
	Range	0.01–4.56
	Median	2.17
	Significance	$p = 0.069$

B

Retinopathy		*TCL (serum)*
Yes	*n*	10
	Range	146–247
	Median	174
No	*n*	30
	Range	112–240
	Median	154
	Significance	$p = 0.012$ **

C

Nephropathy		*Carbonyls (serum)*
Yes	*n*	12
	Range	1.17–2.7
	Median	1.58
No	*n*	28
	Range	0.87–2.98
	Median	1.29
	Significance	$p = 0.076$

The mean salivary SOD activity value, normalized to volume, of healthy controls was 0.66 ± 0.17 U/ml and was significantly higher in all four diabetes mellitus groups. In the diabetes mellitus-controlled groups with and without complications (Groups 2 and 3) SOD was 4.0 and 5.6 times higher, respectively, and in the diabetes mellitus-uncontrolled groups (Groups 4 and 5) it was 3.6 and 4.5 times higher, respectively. The salivary SOD values in patients with neuropathy were substantially higher (100%) than in patients without neuropathy ($p = 0.069$) (Table 1A).

The mean salivary and serum UA concentrations, normalized to volume, of healthy controls were 2.72 ± 0.26 and 4.25 ± 0.43 mg/dl, respectively. The UA concentrations in all four diabetes mellitus groups were increased both in the serum and the saliva. Regarding serum, the increase was statistically significant in all four diabetes mellitus-controlled and uncontrolled groups, while in saliva it was significant only in the diabetes mellitus-uncontrolled groups: The salivary UA concentrations in the diabetes mellitus-uncontrolled groups with and without complications were increased by 65% ($p = 0.03$) and by 147% ($p = 0.037$), respectively. The UA concentrations in the serum of both these groups were similarly increased by 72% ($p = 0.005$) and by 40% ($p = 0.02$), respectively (Fig. 3).

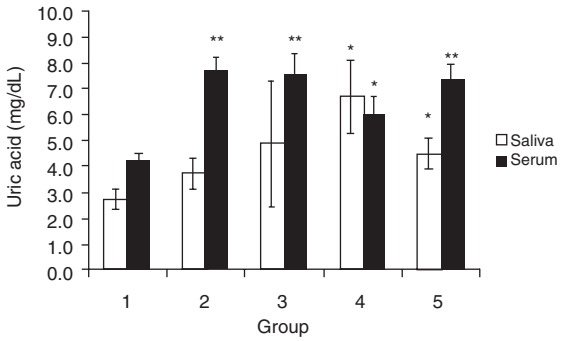

FIG. 3. Mean salivary and serum uric acid concentrations (+ standard deviations) of consenting patients with disease (2–5) versus healthy controls (1). Conditions as in Fig. 1. In Group 1, there were 20 healthy controls. $**p < 0.01$, $*p < 0.05$. The mean salivary and serum uric acid concentrations of the five subgroups were compared using the Kruskal–Wallis test (a multiple comparison nonparametric test), demonstrating a significant difference where $p = 0.038$ for saliva and $p = 0.002$ for serum.

Interestingly, the values of salivary peroxidase, SOD, and UA, as well as the values of serum peroxidase and UA per protein (Table 2A–E) presented a similar pattern of change as these antioxidants calculated per volume (Figs. 3 and 4).

The serum TCL values of the diabetes mellitus patients were significantly higher than for controls except for the diabetes mellitus-controlled group without complications. The serum TCL values in the diabetes mellitus-controlled group with complications were increased by 14% ($p = 0.01$) and in the diabetes mellitus-uncontrolled groups with and without complications they were increased by 34% ($p < 0.01$) and by 5% ($p < 0.05$), respectively (Fig. 4). The TCL values in patients with retinopathy were significantly higher (13%) than for those without retinopathy ($p = 0.012$) (Table 1B).

The mean serum carbonyl concentration of the control group was 0.75 mmol/mg and in all four diabetes mellitus groups it was significantly higher (more so for the diabetes mellitus-uncontrolled groups). The serum carbonyl concentrations in the diabetes mellitus-controlled groups with and without complications were increased by 36% ($p = 0.0001$) and 66% ($p = 0.0001$), respectively, while in the diabetes mellitus-uncontrolled groups they were increased by 92% ($p = 0.0001$) and 170% ($p = 0.0001$), respectively (Fig. 5). The carbonyl values in patients with nephropathy were substantially higher (22%) than in those without nephropathy ($p = 0.076$), (Table 1C).

The Spearman correlation analysis revealed significantly high correlation rates between salivary flow rate and diabetes mellitus severity ($r = –0.75$) and HbA1c levels ($r = –0.55$). Moderate correlation rates were found between salivary peroxidase, SOD and UA when compared with the severity of the disease ($r = 0.40–0.45$). High correlation rates were also found between HbA1c levels and serum carbonyls ($r = 0.60$) and between the severity of the diabetes mellitus and the

TABLE 2. SALIVA AND SERUM ANTIOXIDANT LEVELS IN CONTROL AND DIABETIC GROUPS WITH VARIOUS COMPLICATIONS

A. SALIVA UA PER PROTEIN CONCENTRATIONS PRESENTED BY STUDY GROUPS

Group/UA/protein in saliva	*1*	*2*	*3*	*4*	*5*
Subjects	20	7	11	8	10
Range	0.007–0.348	0.026–0.603	0.032–1.393	0.018–0.232	0.034–0.277
Median	0.044	0.051	0.053	0.060	0.065
Mean	0.064	0.199	0.193	0.091	0.090
SER	0.016	0.100	0.120	0.030	0.022

(Kwallis: $p = 0.567$)

B. SALIVA SOD PER PROTEIN PRESENTED BY STUDY GROUPS

Group/SOD/protein in saliva	*1*	*2*	*3*	*4*	*5*
Subjects	18	4	9	5	3
Range	0.003–0.084	0.029–0.344	0.001–0.30	0.014–0.078	0.001–0.070
Median	0.013	0.119	0.042	0.025	0.028
Mean	0.018	0.153	0.076	0.038	0.033
SER	0.004	0.076	0.032	0.013	0.020

(Kwallis: $p = 0.022$ Sig)

C. SALIVA PEROXIDASE PER PROTEIN PRESENTED BY STUDY GROUPS

Group/peroxidase/ protein in saliva	*1*	*2*	*3*	*4*	*5*
Subjects	20	7	11	8	9
Range	0.002–0.022	0.003–0.108	0.001–0.037	0.001–0.012	0.004–0.011
Median	0.007	0.012	0.009	0.006	0.007
Mean	0.007	0.038	0.012	0.007	0.007
SER	0.001	0.016	0.003	0.001	0.001

(Kwallis: $p = 0.395$)

D. SERUM UA PER PROTEIN PRESENTED BY STUDY GROUPS

Group/UA/protein in serum	*1*	*2*	*3*	*4*	*5*
Subjects	20	8	12	9	11
Range	0.337–1.45	0.947–1.51	0.616–1.95	0.682–1.803	0.545–1.506
Mean	0.716	1.173	1.125	1.078	1.013
SER	0.054	0.211	0.425	0.372	0.337

(Oneway: $p = 0.0018$ Sig)

E. SERUM PEROXIDASE PER PROTEIN PRESENTED BY STUDY GROUPS

Group/peroxidase/protein in serum	*1*	*2*	*3*	*4*	*5*
Subjects	19	8	12	9	11
Range	0.010–0.146	0.10–0.135	0.08–0.134	0.104–0.142	0.10–0.117
Mean	0.088	0.117	0.116	0.126	0.110
SER	0.006	0.004	0.005	0.004	0.002

(Oneway: $p = 0.0001$ Sig)

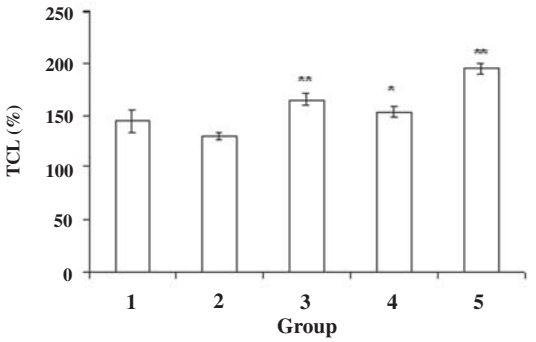

FIG. 4. Mean serum thermochemiluminescence (TCL) levels (+ standard deviations) of consenting patients with disease (2–5) versus healthy controls (1). Conditions as in Figure 1. In Group 1, there were 20 healthy controls. $^{**}p < 0.01$, $^{*}p < 0.05$. The mean serum TCL levels of the five subgroups were compared using the Kruskal–Wallis test (a multiple comparison nonparametric test), demonstrating a significant difference where $p = 0.0001$.

following serum parameters: peroxidase ($r = 0.70$), carbonyls ($r = 0.92$), TCL ($r = 0.60$) and MDA 1–3 ($r = 0.55$–0.65) (Table 3). Correlation rates between the three different clinical complications and the various salivary/serum parameters were analyzed. High rates were found between neuropathy and salivary SOD ($p = 0.069$), nephropathy and serum carbonyls ($p = 0.076$), retinopathy and MDA-1 ($p = 0.047$), and retinopathy and TCL ($p = 0.012$) (Table 4). The MDA concentrations of healthy controls in the pre-incubation (MDA-1) and two post-incubation time points analyzed (MDA-2,3) were 1.95 ± 0.35 n*M*/ml, 2.78 ± 0.47 n*M*/ml, and 8.20 ± 2.4 n*M*/ml, respectively. In comparison, the MDA concentrations of all four diabetes mellitus groups at all three time-points evaluated were 1.3–2.0 times higher ($p < 0.002$) (Table 4). Sensitivity, specificity, and positive and negative predictive values were calculated for the parameters demonstrating the highest correlations rates with the severity of the diabetes mellitus ($r \leq$ l0.70, $r < -0.70$) (Table 5). The cutoff values were calculated according to mean control values ± one STD. The values for the salivary flow rate ranged from 77% to 95%, and for the serum carbonyls and serum peroxidase from 74% to 100% (Table 5).

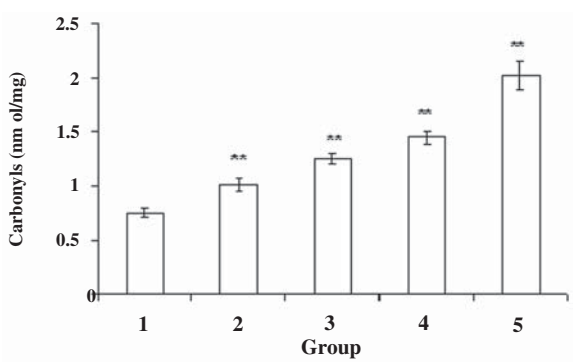

FIG. 5. Mean serum carbonyls concentrations (+ standard deviations) of consenting patients with disease (2–5) versus healthy controls (1). Conditions as in Figure 1. In Group 1, there were 20 healthy controls. $^{**}p < 0.01$. The mean serum carbonyl concentrations of the five subgroups were compared using the Kruskal–Wallis test (a multiple comparison nonparametric test), demonstrating a significant difference where $p = 0.0001$.

TABLE 3. CORRELATION RATES BETWEEN SALIVA AND SERUM PARAMETERS WITH SEVERITY AND HEMOGLOBIN A1C (HBA1C) LEVELS

		Severity (r)	*HbA1c (r)*
Saliva	Flow rate	–0.75	–0.55
	Peroxidase	0.40	0.04
	SOD	0.45	0.15
	Uric Acid	0.43	0.35
Serum	Peroxidase	0.70	0.18
	Uric acid	0.40	–0.04
	Carbonyls	0.92	0.60
	TCL	0.60	0.18
	MDA-1	0.65	–0.03
	MDA-2	0.55	–0.20
	MDA-3	0.62	0.62

Superoxide dismutase (SOD), thermochemiluminescence (TCL), malondialdehyde (MDA).

The correlation between altered salivary parameters and the severity of the disease may indicate that evaluation of the salivary status of diabetes mellitus patients as part of diagnosis and assessment of the disease's activity and severity is warranted. Additionally, the sensitivity, specificity, and positive and negative predictive values of salivary flow rate and peroxidase activity levels were found to be rather high. Salivary analysis is simple, noninvasive, and can easily be performed by the patient himself.

SIGNIFICANCE OF MEASURING SALIVARY ANTIOXIDANTS, COMPOSITION, AND FLOW RATE IN DIAGNOSING DIABETES MELLITUS

The evidence obtained in the current study seems to suggest a role for both salivary involvement and oxidative stress in the pathogenesis of diabetes mellitus. This is based on the salivary flow rate reduction and its accompanying compositional alterations. The increase in the serum antioxidant capacity (both by peroxidase and UA) seems unable to cope with diabetes mellitus-induced oxidative stress and thus the induced lipid peroxidation is not fully prevented, nor are the protein structure alterations. The high correlation rates among various salivary and serum oxidative stress-related parameters and diabetes mellitus severity and associated clinical complications and HbA1c levels, seem to further suggest this notion.

The increase in various antioxidants in both serum and saliva is in accord with a previously published study (2). The significant reduction currently reported in the salivary gland flow rates correlated with the severity of the disease and

Table 4. Correlation Rates among Saliva and Serum Oxidative Stress-Related Parameters and Salivary Flow Rates and Clinical Complications

	Complications/parameter	*Neuropathy p*	*Nephropathy p*	*Retinopathy p*
Saliva	Flow rate	0.40	0.75	0.98
Saliva	Peroxidase	0.36	0.33	0.97
Saliva	SOD	0.069 [a]	0.48	0.59
Saliva	Uric acid	0.91	0.56	0.88
Serum	Peroxidase	0.66	0.79	0.35
Serum	Uric acid	0.92	0.15	0.38
Serum	Carbonyls	0.12	0.076 [a]	0.53
Serum	TCL	0.40	0.12	0.012 [b]
Serum	MDA-1	0.98	0.60	0.047 [a]
Serum	MDA-2	0.10	0.09	0.61
Serum	MDA-3	0.66	0.79	0.74

Superoxide dismutase (SOD), thermochemiluminescence (TCL), malondialdehyde (MDA).
[a]$p < 0.05$; [b]$p < 0.01$.

reached a nadir in the most severe diabetes mellitus group (uncontrolled diabetes mellitus patients who already manifested clinical complications) (by 65%, $p = 0.0001$). This result may point to the salivary glands as major diabetes mellitus target organs. The increase in salivary protein concentrations, as well as the decrease in calcium levels, is in accord with a previous study (8), in which salivary flow rate reduction in diabetic patients was reported. The increase in salivary parameters may be explained by the mutual effects of a reduction in the watery salivary component and/or the upregulation of salivary enzymatic production on the one hand and/or the leaking of serum components into the saliva on the other (which may explain, for example, the increases in protein and amylase). Conversely, glycation and/or increase in free radicals by glycated proteins is probably the explanation for the significant reduction in enzyme activity such as LDH, which is similar to the profound reduction in LDH activity following exposure to free radicals originating in cigarette smoke, on which we reported recently (10). The oral cavity in diabetes mellitus patients should receive special attention, given the increased possibility of oral inflammation and reduced healing capacity. An impaired balance between oxidants and antioxidants has been found in diabetes mellitus-free individuals with periodontitis, accompanied by evidence for oxidative damage to both their salivary proteins and DNA (3, 13). Thus, in the serum and oral cavity of diabetes mellitus patients, it seems that the increase induced in antioxidant capacity cannot cope with the oxidative damage induced and more so in severe diabetes mellitus patients.

Table 5. Sensitivity, Specificity, Positive and Negative Predictive Values of Salivary Flow Rate and Peroxidase Activity Values and of Serum Carbonyls Concentrations

	Salivary flow rate (%)	*Serum carbonyls (%)*	*Salivary peroxidase (%)*
Sensitivity	91	100	100
Specifity	85	80	79
Positive predictive value	77	74	74
Negative predictive value	95	100	100

Cut-off values were calculated according to the controls mean and standard deviation (STD) values.

Salivary flow rate: mean 5.59; STD 1.42; Cut-off: 5.59 – 1.42 = 4.17.

Serum carbonyls: mean 0.768; STD 0.195; Cut-off: 0.768 + 0.195 = 0.963.

Salivary peroxidase: mean 0.58; STD 0.027; Cut-off: 0.58 – 0.027 = 0.607.

The overall salivary antioxidant increase in diabetes mellitus patients may result from a state of systemic oxidative stress and a general increase in serum antioxidants, but it may also reflect a specific response of the salivary glands to diabetes mellitus (*i.e.*, the upregulation of the production of salivary antioxidant enzymes such as salivary peroxidase and SOD). It seems that both mechanisms act concurrently in diabetes mellitus pathogenesis, in light of the fact that salivary peroxidase and SOD are produced specifically in the parotid gland, while UA is serum-borne (9, 11). The fact that the increase in UA was higher (as high as 147%), while for the salivary peroxidase and SOD it was limited (ranging from 25% to 55%) may suggest that the active role played by the salivary glands themselves in fighting oxidative stress-related pathology in the oral cavity of diabetes mellitus patients is relatively moderate, while the salivary changes may better reflect the systemic oxidative state. In any case, as the reduction in salivary flow rate is extensive (65% in the diabetes mellitus-uncontrolled group), it seems that the overall moderate increase in salivary antioxidants is insufficient and presumably explains the limited salivary capacity for coping with diabetes mellitus-induced oral inflammatory diseases such as periodontitis.

FUTURE POTENTIAL OF THE HYPOTHESIS AND LIMITATIONS OF THE APPROACH

From the clinical standpoint, it may be reasonable to conclude that diabetes mellitus patients, and especially severely affected patients, should be treated with both systemic and oral antioxidants, free radical scavengers, and saliva substitutes. Hence, we suggest that extra care should be given to the oral cavity health of diabetes mellitus patients and further research should be devoted to specific diabetes mellitus-related injurious effects on salivary glands. The significant effects of diabetes mellitus on salivary gland flow rates and antioxidant parameters may also be important in diagnosing and evaluating the disease. Salivary antioxidant level is dependent on very few components, mostly the uric acid molecule and the peroxidase enzyme, while the role of fatty soluble antioxidants such as vitamin E is nil since there are almost no fatty acids in saliva. This and the fact that measuring both uric acid and peroxidase is a simple process, make the salivary antioxidant examination a relatively easy and attractive analysis.

Moreover, the correlation between altered salivary parameters and the status of the disease may be useful in improving the evaluation of diabetes mellitus patient salivary status in the clinical set up, and the reported high sensitivity, specificity, and positive and negative predictive values of salivary flow rate and peroxidase activity levels might be seriously considered in this respect.

Salivary analysis is simple, noninvasive, and can easily be performed by the patient at home. Salivary collection can be done at the patient's convenience without involving the assistance of a medical professional or the need for a medical clinic. Not only is salivary analysis substantially less expensive than blood harvesting, it also carries no risk of infection spread.

However, the suggested approach harbors its own limitations. One should remember that serum glucose and HbA1c are still considered the proven "gold standard" for diabetes mellitus diagnosis and monitoring, as a great deal of further research is warranted to fully master the potential of salivary analysis in diabetes mellitus. Collection of saliva must be performed in a meticulous manner and the saliva must be preserved in proper conditions for further analysis, in order to avoid various technique-related effects which might affect both flow rate and salivary composition measurements.

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ABBREVIATIONS

AGE, advanced glycation endproduct; DM, diabetes mellitus; DTNB, dithiobis 2-nitrobensoic acid; MDA, malondialdehyde; SO, superoxide anion; SOD, superoxide dismutase; TCL, thermochemiluminescence; TP, total protein; UA, uric acid.

APPENDIX

Notes

1. Forty consenting patients with diabetes mellitus Type II (Groups 2–5) but otherwise healthy and 20 healthy controls (Group 1) participated in the study. None of these patients had diabetes mellitus Type I, nor were their salivary glucose levels statistically different than those of the controls. In general, varying levels of oral infection and glucose-consuming oral bacteria in various individuals regardless of their health or whether or not they have DM, is a well-known phenomenon that explains the varying levels of salivary glucose in different people.

A. Those with controlled diabetes mellitus (HbA1c $\leq$ 7.5%) (Groups 2 and 3), or uncontrolled diabetes mellitus (HbA1c > 7.5%) (Groups 4 and 5); and

B. Those with (Groups 3 and 5) or without (Groups 2 and 4) the following three diabetes mellitus-related clinical complications: nephropathy, neuropathy, or retinopathy. The definition and criteria used for each of them are as follows:

Retinopathy: a degenerative vision failure that occurs in diabetics when high blood sugar levels cause weakening and damage in retinal blood vessels that can lead to vision problems. In background retinopathy, the blood vessels bulge and leak fluids into the retina and may cause blurred vision. Proliferative retinopathy is more serious and can cause vision loss. In this condition, new blood vessels form in the retina and branch out to other areas of the eye. This can cause blood to leak into the clear fluid inside the eye and may also cause the retina to detach.

Neuropathy: a disorder that results from diabetic microvascular injury involving small blood vessels that supply nerves. Relatively common conditions which may be associated with diabetic neuropathy include third nerve palsy; mononeuropathy; mononeuropathy multiplex; diabetic amyotrophy; a painful polyneuropathy; autonomic neuropathy; and thoracoabdominal, vasa nervorum.

Nephropathy: Diabetic nephropathy (also known as intercapillary glomerulonephritis) is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nodular glomerulosclerosis and by increased microalbumin levels in the urine.

The four study groups were not significantly different from one another in respect to age or gender distribution (Table 6).

Unstimulated (resting) whole saliva specimens were obtained in the morning with no oral stimulus permitted for 90 min prior to collection. The saliva was collected for 10 min, as previously described (9). Following the collection, the specimens were frozen at –20°C for further analysis. Salivary gland flow rates were expressed as volume of saliva (ml) secreted per min.

2. The sialochemistry analysis included total protein (TP), calcium (Ca), magnesium (Mg), and phosphate (P) concentrations, as well as LDH and amylase activities, and was performed as previously described (11).

3. Peroxidase activity was measured both in the patients' serum and saliva according to the NBS assay, as previously described (11). Briefly, the calorimetric change induced by the reaction between the enzyme and the substrate, dithiobis 2-nitrobensoic acid (DTNB) in the presence of mercapto-ethanol, was read at a wavelength of 412 nm for 20 sec.

4. The total salivary activity of the SOD enzyme (Cu/Zn- and Mn-SOD) was measured using the xanthine oxidase/XTT method described by Ukeda *et al*. (20). The method is a modification of the NBT assay, in which XTT is reduced by superoxide anion (SO) generated by xanthine oxidase. Formazan is read at 470 nm. The SOD enzyme inhibits this reaction by scavenging SO. One unit of the enzyme is defined as the amount needed for a 50% inhibition of the absorption.

5. Uric acid concentration was measured both in the serum and the saliva of the patients using a kit supplied by Sentinel CH (Milan, Italy) as previously described (9). In this assay, UA is transformed by uricase into allantoin and hydrogen peroxide which, under the catalytic influence of peroxidase, oxidizes the chromogen (4-aminophenazone/*N*-ethyl-methylanilin propan-sulphonate sodic) to form a red compound whose intensity of color is proportional to the amount of UA present in the sample and is read at a wavelength of 546 nm.

TABLE 6. GENERAL AND CLINICAL CHARACTERISTICS OF STUDY GROUPS

A. DISTRIBUTION OF DIABETES MELLITUS (DM) STUDY (2–5) AND CONTROL (1) GROUPS BY AGE

Group/age	*1*	*2*	*3*	*4*	*5*
Range (years)	51–82	54–75	59–80	52–86	58–71
Mean	62	64	70	70	65
SDV	10	8	8	11	4

($p = 0.08$ NS)

B. DISTRIBUTION OF STUDY (2–5) AND CONTROL (1) GROUPS BY GENDER

Group/ gender	*1 Patients (%)*	*2 Patients (%)*	*3 Patients (%)*	*4 Patients (%)*	*5 Patients (%)*
Male	10 (50)	5 (62)	6 (50)	6 (67)	5 (45)
Female	10 (50)	3 (38)	6 (50)	3 (33)	6 (55)
Total	20 (100)	8 (100)	12 (100)	9 (100)	11 (100)

($p = 0.86$ NS)

C. DISTRIBUTION OF MEAN HEMOGLOBIN A1C (HBA1C) VALUES IN THE STUDY GROUPS (2–5)

Group/ HbA1c (%)	*2*	*3*	*4*	*5*
Range	6.3–7.4	6–7.1	9.2–12.2	9–12
Mean	6.88	6.58	10.63	10.3
Standard error	0.14	0.12	0.37	0.30

D. CLINICAL COMPLICATIONS IN STUDY GROUPS (2–5)

		2 (n = 8)	*3 (n = 12)*	*4 (n = 9)*	*5 (n = 11)*
Neuropathy	Yes	–	4 (33)	–	4 (36)
	No	8 (100)	8 (67)	9 (100)	7 (64)
Nephropathy	Yes	–	6 (50)	–	6 (55)
	No	8 (100)	6 (50)	9 (100)	5 (45)
Retinopathy	Yes	–	6 (50)	–	4 (36)
	No	8 (100)	6 (50)	9 (100)	7 (64)

All patients in groups 3, 5 manifested at least one complication.

Forty consenting patients with DM (Groups 2–5) but otherwise healthy and 20 healthy controls (Group 1) participated in the study.

(**A**) Those with controlled DM (HbA1c ≤ 7.5%) (Groups 2 and 3), or uncontrolled DM (HbA1c > 7.5%), (Groups 4 and 5); and (**B**) those with (Groups 3 and 5) or without (Groups 2 and 4) had the following clinical complications: nephropathy, neuropathy, or retinopathy.

Numbers in parentheses represent the percentage of either males or females of all patients.

6. Photon emission during heating was measured by TCL Photometer (manufactured by Lumitest Ltd., Haifa, Israel) in the serum of the patients as previously described (14), using a photomultiplier model R265P (Hamamatsu Photonics Co. Ltd., Shizuoka, Japan) with a spectral response range of 300–650 nm. The TCL device measures the level of photon emission from excited carbonyls in biological macromolecules (*i.e.*, oxidative modifications of lipids and proteins). The serum examined was distributed over the surface of an aluminum tray (a type of miniature Petri dish) inside the sample preparation block and was then vacuum-dried. The dish was then mounted on a constant-temperature heater kept at 80 ± 0.5°C in the analysis block, and the photon emission was measured for 50 sec.

7. Protein carbonyl determination was performed in the serum according to the Reznick–Packer procedure (12), while the Slater–Sawyer method was used for assessing MDA production (17). Briefly, thiobarbituric acid was used during the reaction to assess the level of induced lipid peroxidation, as there is a direct correlation between the two.

8. Data concerning the levels of various parameters evaluated in the saliva and in serum of the five subgroups were observed and calculated. Frequencies, percentages, and distribution were calculated for categorical variables. Due to the small number of patients in each subgroup (fewer then 30), the frequencies between the categorical variables were analyzed by the Fisher–Irwin exact test (a nonparametric test for small sample). Means, standard deviations, standards errors, medians, and ranges were calculated for continuous variables. Due to large variability of salivary components, the "Kruskal–Wallis test" (a multiple-comparison nonparametric test) was used to compare between subgroups of patients (more than 2) and between pairs of subgroups by "2 sample Wilcoxon rank-sum test" (a nonparametric test). The correlation between pairs of parameter levels were analyzed by "Spearman correlation."

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Address reprint requests to:
Professor Rafael M. Nagler
Oral and Maxillofacial Surgery Department
and Oral Biochemistry Laboratory
Rambam Medical Center
Haifa, Israel

E-mail: nagler@tx.technion.ac.il

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