

Salivary Antioxidants in Patients With Type 1 or 2 Diabetes and Inflammatory Periodontal Disease: A Case-Control Study

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Background: The purpose of this study was to evaluate and compare salivary concentrations of reduced, oxidized glutathione, uric acid, ascorbic acid, and total antioxidant capacity in subjects with diabetes and systemically healthy subjects with inflammatory periodontal disease.

Methods: Sixteen patients with type 1 diabetes mellitus (DM), 25 patients with type 2 DM, and 24 systemically healthy patients, all with inflammatory periodontal disease, were recruited. Whole-saliva samples were obtained, and full-mouth clinical periodontal measurements, including plaque index, probing depth, gingival recession, clinical attachment level, and bleeding on probing, were recorded at six sites per tooth. Saliva flow rate and salivary levels of reduced and oxidized glutathione, vitamin C, uric acid, and total antioxidant capacity were determined. Data were analyzed statistically by non-parametric tests.

Results: The subjects with type 2 DM had fewer teeth and more sites with probing depths >4 mm than the patients with type 1 DM (both $P < 0.01$). The mean salivary reduced-glutathione concentration was lower in patients with type 1 DM than in the other two groups (both $P < 0.05$). No significant differences in the salivary concentrations of the other antioxidants measured were found among the groups ($P > 0.05$). Oxidized glutathione levels in the patients with type 1 DM were significantly lower than in the systemically healthy group ($P = 0.007$). In both groups with diabetes, salivary reduced-glutathione levels correlated positively with probing depth, and total antioxidant capacity correlated with salivary flow rate ($P < 0.01$).

Conclusion: The decrease in salivary reduced-glutathione levels in patients with type 1 DM may have a role in periodontal tissue destruction by predisposing tissues to oxidative stress. *J Periodontol* 2009;80:1440-1446.

KEY WORDS

Ascorbic acid; diabetes mellitus; glutathione; periodontal disease; uric acid.

Diabetes mellitus (DM), a complex metabolic disorder characterized by prolonged hyperglycemia, has long been recognized as one of the leading causes of morbidity and mortality globally.¹ DM is caused by a deficiency in insulin production or an impaired use of insulin. Type 1 DM is caused by progressive autoimmune destruction of pancreatic insulin-producing β cells. Type 2 DM describes a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia with disturbances in carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion (β -cell dysfunction), insulin action (insulin resistance), or both.² Subjects with type 2 DM are 2.8 times more likely to have destructive periodontal disease³ and 4.2 times more likely to have significant alveolar bone loss⁴ compared to systemically healthy subjects. Periodontal disease was proposed to be the sixth complication of DM,⁵ with evidence showing a correlation between poorer glycemic control and worsening periodontal health.⁶⁻⁸

Reactive oxygen species play an important role in cell signaling and metabolic processes but also contribute to pathogenic processes in a variety of inflammatory disorders.⁹ Oxidative stress reflects an increase in the production of

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pro-oxidants and/or a decrease in protective antioxidants.¹⁰ Unbalanced radical and non-radical reactive oxygen species can damage cells by a variety of mechanisms, including peroxidation of lipid membranes, protein inactivation, and induction of DNA damage, in addition to stimulating specific signaling pathways that lead to cytokine-induced tissue damage.¹¹ Large amounts of pro-oxidants are produced in prolonged inflammatory responses, which occur in gingivitis and periodontitis.¹² Therefore, inflammation is a source of reactive oxygen species and can compromise the antioxidant capacity of serum and tissues.

Saliva possesses a wide range of antioxidant defense molecules, including uric acid, vitamin C (ascorbic acid), reduced glutathione (GSH), oxidized glutathione (GSSG), and others.¹³ Such antioxidants work in concert, and total antioxidant capacity may be the most relevant parameter for assessing defense capabilities.¹⁴

Enhanced oxidative stress was observed in patients with diabetes, as indicated by increased free radical production,¹⁵ lipid peroxidation, and diminished antioxidant status.¹⁶ The relationship among DM, periodontal disease, and salivary antioxidant status has not been clarified. We hypothesized that the diabetic state would reduce the salivary antioxidant capacity of subjects and, further, that this antioxidant impairment may help to explain the association between diabetes and inflammatory periodontal disease. Thus, the aim of this study was to comparatively evaluate salivary concentrations of GSH, GSSG, uric acid, ascorbic acid, and total antioxidant capacity in subjects with type 1 or 2 diabetes and inflammatory periodontal disease as well as systemically healthy counterparts.

MATERIALS AND METHODS

Study Population

Forty-one patients with diabetes and no other known systemic diseases that could influence periodontal status participated in this cross-sectional study. Sixteen patients with type 1 diabetes (five males and 11 females; age range: 17 to 73 years) and 25 patients with type 2 diabetes (11 males and 14 females; age range: 42 to 69 years), and all with plaque-induced inflammatory periodontal disease, were recruited. Patients with diabetes were those consecutively referred during routine medical care visits from an outpatient diabetes clinic (Department of Metabolic Diseases and Endocrinology, School of Medicine, Ege University). All patients with diabetes were diagnosed as having DM (type 1 or 2) ≥ 1 year prior to the study using American Diabetes Association diagnostic criteria¹⁷ and were being treated with stable doses of oral hypoglycemic agents and/or insulin by the same physician (ŞÇ). The control group consisted of 24 systemically healthy subjects with plaque-induced

inflammatory periodontal disease (10 males and 14 females; age range: 22 to 60 years) who were recruited from those patients seeking dental treatment at the School of Dentistry, Ege University. Patients were excluded if they had aggressive periodontitis, <14 teeth present, or a history of antibiotic therapy within the preceding 3 months or periodontal treatment within the last 6 months. Smoking history was recorded, but smokers were not excluded. Subjects were enrolled from May 2004 to November 2006. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki. The study protocol was explained, and informed written consent was received from each individual before his/her enrollment in the study.

Saliva Sampling

Saliva samples were obtained in the morning after an overnight fast, during which subjects were requested not to drink (except water) or chew gum. Whole-saliva samples were obtained by expectoration into polypropylene tubes prior to clinical measurements; the time period for sample collection was recorded in minutes. The saliva samples were weighed and wet weights were recorded because patients with DM often have xerostomia. The collection time was five minutes, and the flow rate was calculated as ml/min. Saliva samples were immediately frozen at -40°C and stored until the sample collection period was completed. The samples were lyophilized and stored at -20°C until subsequent biochemical analyses, which were performed at the Oral Health and Systemic Disease Research Group, School of Dentistry, University of Louisville.

Clinical Measurements

Subsequent to saliva sampling, clinical periodontal parameters, including the dichotomous plaque index (+/-), probing depth (PD), and the presence of bleeding on probing (BOP; +/-), were assessed at six sites for each tooth present, except third molars, using a Williams probe. All clinical examinations were carried out by a single examiner (PG), who was trained, calibrated, and masked to the systemic condition of the patient.

Laboratory Analyses

Systemic markers of DM. Venous blood samples were taken from each patient with DM and analyzed for fasting plasma glucose, glycosylated hemoglobin (HbA1c), total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol. Moreover, the duration of type 1 or 2 DM and the presence or absence of diabetes complications were recorded for each patient.

Measurement of total antioxidant capacity. The total antioxidant capacity of saliva samples was

measured using an enzyme immuno assay microplate kit.[§] Absorbances were read at 560 nm on a multilabel counter.^{||} Antioxidant capacity was related to a water-soluble vitamin E analog, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and, therefore, was expressed as Trolox equivalents (μM).

Determination of GSH content. GSH content was determined by a colorimetric microplate assay,[¶] with reaction kinetics measured by determining the rate of color change at 405 nm using a multilabel counter. The kit relies on the oxidation of GSH by 5,5"-dithiobis(2-nitrobenzoic acid).

Determination of GSSG content. The GSSG content of saliva samples was determined using a glutathione assay kit.[#] This kit measures fluorescence generated by the reaction between GSH (not GSSG) and *o*-phthalaldehyde at 340/450 nm.

Determination of ascorbic acid content. Salivary ascorbate concentrations were determined, essentially as we described previously.¹⁸ Ascorbic acid in deproteinated (4% metaphosphoric acid) saliva was oxidized by the nitroxide free radical 2,2,5,5-tetramethyl-4-piperidin-1-oxyl to dehydroascorbic acid, which, in turn, condenses with *o*-phenylenediamine to form a quinoxaline derivative that absorbs light at 340 nm.¹⁹ Ascorbate concentrations were determined by extrapolating from standard curves generated by the same methodology using the principle that change in absorbance at 340 nm is proportional to ascorbate content. Absorbances were read on a multilabel counter.

Determination of uric acid content. Uric acid concentrations were determined by using the amplex red uric acid colorimetric assay.^{**} Absorbances were read at 560 nm on a multilabel counter and background-corrected using the negative control.

Statistical Analysis

The Kruskal-Wallis test followed by the Mann-Whitney U test was used for the group comparisons of the salivary antioxidant levels as well as the clinical periodontal measurements. Spearman correlations were used to examine the relationships among salivary antioxidant levels, diabetes-related systemic parameters, and the clinical periodontal measurements.

RESULTS

Clinical Analyses

The demographic variables and the clinical periodontal findings of the study groups are presented in Table 1. There were no significant differences between the study groups with regard to diabetes duration, gender, or smoker/non-smoker ratio ($P > 0.05$). The mean age of the group with type 1 DM was similar to that of the systemically healthy group, and these two groups were younger than the group with type 2 DM

($P < 0.001$). The group with type 2 diabetes had fewer teeth and more sites with PD > 4 mm than the group with type 1 diabetes ($P < 0.01$).

Biochemical Analyses

Diabetes-related peripheral blood parameters are presented in Table 2. No significant differences were found in these variables between the groups with types 1 and 2 diabetes ($P > 0.05$). No significant correlations were found between the diabetes-related systemic parameters and the salivary antioxidant levels in either of the two groups with diabetes ($P > 0.05$).

Antioxidant data are presented in Table 3. Reduced salivary GSH content in the group with type 1 DM was significantly less than the group with type 2 DM and the systemically healthy control group ($P < 0.01$ and $P < 0.05$, respectively). The GSSG level in the group with type 1 DM was significantly lower than in the systemically healthy group ($P = 0.007$). The saliva flow rates were similar in the groups with types 1 and 2 DM and significantly less than the systemically healthy group with periodontal disease ($P < 0.05$ and $P < 0.001$, respectively). There were no significant differences in the saliva levels of antioxidants between the group with type 2 DM and the systemically healthy group with periodontal disease ($P > 0.05$).

Using the Spearman correlation analysis, salivary GSH levels in the groups with diabetes positively correlated with PD and the number of sites with PD > 4 mm ($P = 0.001$ and $P = 0.02$, respectively; $r = 0.590$ and $r = 0.419$, respectively). The GSSG levels correlated positively with the BOP score ($P = 0.0456$; $r = 0.323$). The total antioxidant capacity in the two groups with diabetes correlated positively with the saliva flow rate and the saliva ascorbic acid level ($P = 0.003$ and $P = 0.005$, respectively; $r = 0.547$ and $r = 0.585$, respectively). The percentage of sites with BOP correlated positively with the duration of DM and the presence of diabetic complications ($P = 0.007$ and $P = 0.002$, respectively; $r = 0.576$ and $r = 0.590$, respectively). The systemically healthy group with periodontal disease exhibited a positive correlation between saliva flow rate and plaque index ($P = 0.02$; $r = 0.419$).

DISCUSSION

Diabetes-associated oxidative stress is a consequence of the production of free radicals and a reduced antioxidative defense capacity. Few studies^{15,16} have evaluated the salivary antioxidants in patients

§ Cayman Chemical, Ann Arbor, MI; kit includes Trolox, Hoffmann-La Roche, Basel, Switzerland.

|| Victor³ 1420 Multilabel Counter, Perkin Elmer Life and Analytical Sciences, Shelton, CT.

¶ Oxford Biomedical Research, Oxford, MI.

BioVision Research Products, Mountain View, CA.

** Invitrogen, Carlsbad, CA.

Table 1.
Clinical Characteristics of Study Groups

Clinical Parameters	Group 1 (type 1 DM)	Group 2 (type 2 DM)	Group 3 (systemically healthy group)
n	16	25	24
Males/females (n)	5/11	11/14	10/14
Smokers/non-smokers (n)	4/12	5/20	9/15
Age (years; mean \pm SD)	35.1 \pm 15.8	54.4 \pm 8.2*	37.1 \pm 13.3
Teeth present (n; mean \pm SD)	24.8 \pm 4.8	18.0 \pm 5.8†	24.5 \pm 4.2
PD (mm; mean \pm SD)	1.4 \pm 0.5	1.9 \pm 0.8	1.5 \pm 0.7
Gingival recession (mm; mean \pm SD)	1.1 \pm 1.5	2.0 \pm 1.1‡	1.1 \pm 0.9
Clinical attachment level (mm; mean \pm SD)	2.3 \pm 1.7	3.3 \pm 1.3	2.2 \pm 1.1
BOP (% sites; mean \pm SD)	48.9 \pm 27.9	57.9 \pm 23.4	55.7 \pm 29.9
Plaque index (%; mean \pm SD)	71.7 \pm 27.4	86.12 \pm 23.4	79.6 \pm 32.5
Sites with PD >4 mm (n; mean \pm SD)	0.13 \pm 0.5	3.4 \pm 7.4§	0.79 \pm 2.2

Data were compared among the study groups using the Kruskal-Wallis test, followed by the post hoc Mann-Whitney U test.

* Significantly higher than the other groups ($P < 0.01$).

† Significantly lower than the other groups ($P < 0.01$).

‡ Significantly higher than the group with type 1 DM ($P < 0.05$).

§ Significantly higher than the other groups ($P < 0.05$).

Table 2.
**Diabetes-Related Systemic Parameters
(mean \pm SD) in Groups With Diabetes**

Biochemical Parameters	Group 1 (type 1 DM)	Group 2 (type 2 DM)
HbA1c (%)	7.72 \pm 1.6	7.16 \pm 2.0
Fasting plasma glucose (mg/dl)	144.2 \pm 62.3	143.2 \pm 78.6
Total cholesterol (mg/dl)	220.7 \pm 136.2	192.9 \pm 35.1
Triglycerides (mg/dl)	366.1 \pm 973.0	180.0 \pm 101.9
HDL cholesterol (mg/dl)	50.4 \pm 16.8	45.7 \pm 9.5
LDL cholesterol (mg/dl)	125.0 \pm 61.9	113.4 \pm 34.7

Data were compared between the study groups using the Kruskal-Wallis test, followed by the post hoc Mann-Whitney U test. There were no significant differences between the two diabetic groups ($P > 0.05$).

with diabetes, and conflicting data were reported. The present study was conducted to evaluate the influence of type 1 or 2 DM on the salivary levels of some major antioxidants independently from the periodontal status. Diabetes-related systemic parameters that reflect the metabolic control status of DM were not different in our groups with type 1 or 2 DM. The present finding that the group with type 1 DM had more teeth and fewer sites with PD >4 mm than the group with type 2 DM might be explained by the age difference between these two groups; the patients with type 2 DM were significantly older than the patients with type 1 DM and the systemically healthy controls. The group with type 2 DM exhibited the worst clinical periodontal findings. This may be explained by the additive effects of age and DM negatively influencing the defense system of the patient, which also includes the antioxidative defense.

Saliva can be collected easily in contrast to the invasive nature of blood collection. Whole saliva is the most relevant for the purpose of analyzing salivary antioxidant status because it contains gingival crevicular fluid (GCF), immune cells, and tissue metabolites^{20,21} and reflects the predominant intraoral condition most closely.²² Stimulation may increase the flow of GCF, which may result in false increases in the concentration of antioxidants in the saliva.²³ Accordingly,

we used unstimulated whole-saliva samples for the present study. Patients with diabetes commonly present with xerostomia,²⁴ and we found lower salivary flow rates in patients with type 1 or 2 DM compared to the systemically healthy controls. One limitation of our study is the lack of serum and GCF data on antioxidants. However, these were beyond the scope of the present study, and evaluation of the antioxidant profile in saliva was the particular goal. Evaluation of serum and/or GCF samples could be the aim of a separate study.

Antioxidants, by counteracting the harmful effects of free radicals, protect structural and tissue integrity. Imbalances between the levels of free radicals and reactive oxygen species and the levels of antioxidants were suggested to play an important role in the onset and development of several inflammatory oral diseases.^{11,25} Cowan et al.²⁶ established that circulating concentrations of several antioxidants (α -tocopherol, retinol, lycopene, or β -carotene) did not correlate with

Table 3.
Biochemical Analysis (mean \pm SD) of Saliva Samples

Salivary Parameters	Group 1 (type 1 DM)	Group 2 (type 2 DM)	Group 3 (systemically healthy group)
Saliva wet weight (g)	3.3 \pm 1.2	2.6 \pm 1.1	2.9 \pm 0.9
Saliva flow rate (ml/minute)	0.44 \pm 0.30*	0.29 \pm 0.15*	0.62 \pm 0.37
GSH (μ M)	3.1 \pm 1.98 [†]	5.65 \pm 3.55	4.89 \pm 3.03
GSSG (μ M)	19.0 \pm 9.4*	22.2 \pm 10.7	43.0 \pm 23.3
Ascorbic acid (mg/l)	0.68 \pm 0.95	0.77 \pm 1.81	0.48 \pm 1.38
Total antioxidant capacity (μ M)	0.25 \pm 0.12	0.31 \pm 0.18	0.24 \pm 0.13
Uric acid (μ M)	438.6 \pm 302.4	614.2 \pm 376.5	443.4 \pm 239.0

Data were compared among the study groups using the Kruskal-Wallis test, followed by the post hoc Mann-Whitney U test.

* Significantly lower than the systemically healthy group with periodontal disease ($P < 0.05$).

[†] Significantly lower than the other groups ($P < 0.05$).

those in oral tissues, as had been previously implied. However, research on salivary antioxidants remains limited, and conflicting data have been reported. Moore et al.²⁴ measured the antioxidant capacity of saliva in periodontally diseased and healthy individuals and failed to find any significant difference between the groups. Chapple et al.²³ found that the detection of differences in saliva total antioxidant capacity between periodontitis and control subjects depended upon whether salivary flow rates were accounted for in their data analysis. The same group¹⁴ later confirmed this and found no differences in mean saliva total antioxidant capacity between chronic periodontitis and healthy control groups, but they did find a significantly higher total antioxidant delivery in unstimulated samples from healthy subjects relative to periodontitis. Moreover, no significant correlations were found between the total antioxidant capacity of saliva and age or flow rate, regardless of periodontal status. Scully and Langley-Evans²⁷ reported that periodontal disease is associated with reduced salivary antioxidant status and increased oxidative damage within the oral cavity. In a previous study,¹⁸ we found that neither smoking nor gingival inflammation affected glutathione, ascorbic acid content, or total antioxidant capacity in systemically healthy patients with gingivitis. Therefore, we did not exclude smoking patients from the present study. Moreover, our groups with diabetes and the systemically healthy patient group were similar with regard to the ratios of smokers and non-smokers.

Arana et al.²⁸ determined the oxidative stress in 11 patients with type 2 DM and 18 healthy individuals.

Glutathione peroxidase (GPx) and glutathione reductase (GRd) activities, in addition to GSH and glutathione disulfide concentrations, were analyzed in stimulated saliva samples. They found significantly higher GPx and GRd activities in patients with DM than in controls, whereas the mean GSH level was significantly lower, suggesting that saliva may be suitable for determining the prognosis and evolution of DM and its oral manifestations. In the present study, patients with type 1 DM, but not those with type 2 DM, exhibited significantly lower GSH levels than the systemically healthy control group. The oral health status was evaluated by community periodontal index (CPI) in the study by Arana et al.;²⁸ CPI values were similar in the study groups. Our finding that GSH was reduced in type 1 DM provides further support for the hypothesis that chronic hyperglycemia leads to free radical generation and increased GSH oxidation.²⁸⁻³⁰

In contrast to the CPI index used by Arana et al.,²⁸ separate indexes for oral hygiene status, extent of gingival inflammation, and periodontal tissue destruction were used in the present study to evaluate the clinical periodontal status in a more detailed manner. However, we did not categorize the patients according to their clinical periodontal diagnosis. Gingivitis was evident in all patients, whereas few patients had localized chronic periodontitis.

Reznick et al.³¹ analyzed serum and salivary composition and oxidative stress markers in 20 patients with type 1 DM and 12 healthy control subjects. They reported that uric acid content and total antioxidant values in unstimulated whole-saliva samples were increased in patients with DM compared to the healthy controls; however, the differences were significant only for total antioxidant status. The overall salivary antioxidant increase in patients with DM was suggested to result from a state of systemic oxidative stress. One limitation of our study is the lack of serum analysis. However, the serum antioxidant composition was shown to correlate well with saliva.³¹

Recently, Ben-Zvi et al.³² studied uric acid, peroxidase, and total antioxidant status in saliva and serum in chronic renal failure and patients with DM. They reported that DM increased the oxidative stress burden in serum and saliva. Moreover, the same group stated that the redox state of saliva from patients with DM differed from that of normoglycemic control subjects and suggested that non-invasive saliva-based diagnostics may be used to monitor oxidative status in patients with DM.³³ The group with type 2 DM in the

present study was older, had fewer teeth, had more sites with PD >4 mm, and exhibited a higher salivary GSH level than the group with type 1 DM. The higher salivary GSH level could be a sign of increased oxidative stress, which may have a role in the increased PDs in patients with type 2 DM. Thus, the salivary GSH level in the groups with DM was positively correlated with PD. One limitation of this study is the lack of a group with diabetes presenting with clinically healthy periodontium, which would enable us to conclude whether the levels of salivary antioxidants are definitely related to the diabetic status, independently of the clinical periodontal situation. However, compiling such a group is difficult because almost all patients with DM have some degree of inflammatory periodontal disease.

CONCLUSIONS

Our present findings confirmed that saliva flow rate is reduced in patients with type 1 or 2 DM. The reduced salivary glutathione concentrations noted in patients with type 1 DM may indicate that careful follow-up of these patients with regard to periodontal disease is required. A significant correlation between salivary glutathione concentration and PD in patients with diabetes is a novel finding that deserves further investigation. However, total antioxidant capacity, vitamin C, and uric acid concentrations do not seem to play a major role in the pathogenesis of periodontal manifestations of diabetes.

ACKNOWLEDGMENTS

The study was supported solely by the institutions of the authors. The authors report no conflicts of interest related to this study.

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Submitted March 20, 2009; accepted for publication April 28, 2009.