Altered Salivary Redox Homeostasis in Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** Oxidative stress has been implicated in the pathogenesis of systemic sclerosis (SSc). Our objective was to determine whether SSc is associated with altered redox homeostasis in human saliva.

Methods. Study participants were 70 women with SSc and 120 female controls. 8-hydroxy-2'-deoxyguanosine (8-OHdG), 8-epi-prostaglandin F 2α (8-epi-PGF2α), and total protein carbonyls were assayed by ELISA to quantify oxidative damage to nucleic acids, lipids, and proteins, respectively, in whole nonstimulated saliva.

Results. We observed a significantly positive association between salivary log protein carbonyls and SSc in a crude statistic (OR 9.06, $p < 0.0001$), and multivariable model adjusted for log 8-OHdG, log 8-epi-PGF2α, and antioxidant exposure (OR 9.26, $p < 0.0001$). No significant association was noted between SSc and salivary log 8-epi-PGF2α or log 8-OHdG.

Conclusion. Salivary redox homeostasis is perturbed in patients with SSc and may inform on the pathophysiology and presence of the disease (biomarkers) and efficacy of therapeutic interventions. (J Rheumatol First Release July 1 2010; doi:10.3899/jrheum.091451)

Key Indexing Terms:
OXIDATIVE STRESS SCLERODERMA SALIVA 8-HYDROXY-2'-DEOXYGUANOSINE 8-EPI-PROSTAGLANDIN F 2α PROTEIN CARBONYLS

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized clinically by thickening and fibrosis of the skin[1], and by the involvement of internal organs, most commonly the gastrointestinal tract, lungs, kidneys, and heart[2,3,4,5]. Prevalence estimates vary from 30 per million to 276 per million population[6,7]. SSc mainly affects women in the prime of life and is associated with significant morbidity and increased mortality. There is currently no disease-modifying drug available to treat this condition.

Numerous studies have demonstrated oxidative stress and altered redox homeostasis in plasma and urine derived from persons with SSc[8,9,10,11,12,13,14,15,16]. Although oxidative stress and reactive oxygen species have been implicated in tissue inflammation and destruction within the human oral cavity[17,18,19], few studies have focused on redox changes in saliva as a potential source of SSc biomarkers. The salivary glands may undergo fibrosis and contribute to xerostomia in patients with SSc which, in turn, may predispose to increased oral cavity oxidative stress[20,21,22]. In an effort to develop oral biomarkers of SSc, our study was designed to ascertain levels of oxidized DNA, lipids, and proteins in whole nonstimulated saliva from large cohorts of women with and without the disease.

MATERIALS AND METHODS

Study design and population. This cross-sectional study was approved by the Research Ethics Committee of the Jewish General Hospital (JGH), Montreal, Quebec, Canada. Written informed consent was obtained from all participants.

Seventy women with SSc were recruited from the rheumatology division of internal medicine at the JGH. Controls consisted of 120 female health professionals and visitors recruited within the hospital. Smokers, pregnant women, diabetics, individuals with cancer, and those with significant chronic systemic inflammatory diseases other than SSc were excluded. Exposure to vitamin E (≥ 400 IU/day), ascorbic acid (≥ 500 mg/day), or

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other antioxidants (at any dosage) was recorded for antioxidant interference analyses.

Saliva collection and preparation. Whole nonstimulated saliva samples were collected to best preserve their natural chemical composition. The collection was done at least 30 min after food or liquid ingestion in sterilized centrifuge tubes either before or after participants had received their routine medical checkup. To minimize temporal fluctuations in salivary redox homeostasis[23], all samples were collected between 9:00 AM and noon. At the end of the collection period, the tube was sealed, kept at 4°C, and conveyed to the laboratory for processing within 1 h. Prior to analysis, the saliva was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was withdrawn and stored in small aliquot tubes at –80°C until analysis.

Total protein carbonyls. Total protein carbonyls (a measure of protein oxidation) in saliva were quantified in individual samples using a sensitive ELISA method and oxidized bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) as standard[24,25,26]. The standard curve was constructed by mixing varying proportions of oxidized BSA and fully reduced BSA at a constant total protein concentration (4 mg/ml). Salivary protein levels were measured using Lowry's method (Bio-Rad Laboratories Inc., Hercules, CA, USA) and adjusted to 4 mg protein/ml by either dilution or speed vacuum concentration. The standards and protein samples were incubated with 3 volumes of 10 mM 2,4-dinitro-phenylhydrazine (2,4-DNP) in 6 M guanidine-HCl, 0.5 M potassium phosphate, pH 2.5, for 45 min at room temperature (mixing every 10–15 min). DNP-derived proteins of 5 μl aliquots were diluted in 1 ml phosphate buffer solution and 200 μl were absorbed to a 96-well immunoplate by incubation overnight at 4°C. The plate was incubated with primary biotinylated anti-DNP antibody (1:1000 dilution in 0.1% Tween-20/PBS; Molecular Probes, Eugene, OR, USA) for 1 h at 37°C. A 6-point standard curve was generated for each plate analysis. Specific absorbance for each sample was calculated by subtracting basal absorbance of the DNP reagent from the total absorbance. Protein carbonyl levels were expressed as nmol per mg of total protein in saliva[27]. Intraassay and interassay coefficients of variation for the procedure were 4.93%–6.53% and 9.97%, respectively, over a 2 year period.

8-hydroxy-2'-deoxyguanosine (8-OHdG). Competitive ELISA assays for salivary 8-OHdG were performed in duplicate according to the manufacturer's instructions (Assay Designs Inc., Ann Arbor, MI, USA)[28]. The 8-OHdG monoclonal antibody and the 1:4 diluted saliva samples were added to ELISA plates precoated with 8-OHdG. Anti-8-OHdG captured by the immobilized 8-OHdG was detected with secondary antibody: horseradish peroxidase conjugate. The assay was developed with tetramethylbenzidine substrate and the absorbance was measured in a microplate reader at 450 nm. Standard 8-OHdG was assayed over a concentration range of 0.94 to 60 ng/ml in duplicate for each experimental batch. The data were expressed as 8-OHdG ng/ml saliva. The sensitivity of the method was 0.59 ng/ml. Intraassay and interassay (13.42%) coefficients of variation for the procedure were 2.40%–7.33% and 13.42%, respectively, over a 2 year period.

8-epi-prostaglandin F 2α (8-epi-PGF2α). 8-epi-PGF2α, a sensitive and specific index of lipid peroxidation, was measured using a competitive ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). This assay was based on competition between 8-epi-PGF2α and an 8-epi-PGF2α-acetylcholinesterase conjugate (8-epi-PGF2α tracer) for a limited number of 8-epi-PGF2α-specific rabbit antiserum binding sites[26,27,29]. Saliva, 0.5 ml, was diluted with 6 ml of ultrapure water and acidified to pH 3.0 with 30% acetic acid. The sample was then passed through a C_{18} solid-phase extraction cartridge (Supelco Inc., Bellefonte, PA, USA) preactivated with methanol (5 ml) and ultrapure water (5 ml). The cartridges were rinsed with 5 ml ultrapure water, followed by 5 ml hexane. The 8-epi-PGF2α was eluted with 5 ml ethyl acetate containing 1% methanol. The eluate was dried under low flow rate nitrogen and reconstituted in 0.5 ml enzyme immunoassay buffer from the kit. A 50-μl reconstituted sample was used for ELISA analysis in duplicate. An 8-point standard curve (varying amounts of 8-epi-PGF2α), nonspecific binding, and maximum binding were set up in duplicate for each batch of assay following the manufacturer's instruction. The 8-epi-PGF2α levels were calculated and expressed in picogram/ml saliva. The intraassay and interassay coefficients of variation were 6.87%–9.89% and 13.68%, respectively, over a 2 year period. The recovery of 8-epi-PGF2α after extraction averaged 92%.

Statistical analysis. Descriptive analyses were performed to evaluate the distribution of each biomarker among SSc and controls. A logarithmic transformation was applied to 8 OHdG (ng/ml), 8-epi-PGF2α (pg/ml), and protein carbonyls (nmol/mg protein). These transformations were applied to follow fundamental statistical principles of normal distribution. Chi-squared and Student's t-test were used to test statistical differences between SSc and controls relative to antioxidant use (no/yes) and age, respectively. Univariate and multivariable conditional age-matched logistic regression analyses (Proc Phreg, SAS) were used to estimate the association between SSc as the dependent variable (no/yes) with each specific biomarker (independent variables). The multivariable model was adjusted for antioxidant use (no/yes). For all analyses, the 2-tailed alpha significance level was 5%. All analyses were performed with SAS (version 9.1).

RESULTS

Demographics and antioxidant ingestion. Clinical and demographic characteristics of the SSc and control groups are listed in Table 1. The mean ages of the SSc (54.44 ± 11.45 yrs) and control group (42.53 ± 17.75 yrs) were significantly different ($p < 0.0001$). SSc subjects reported significantly more antioxidant ingestion (44%) than controls (18%; $p < 0.0001$). Log 8-OHdG, log protein carbonyls, and log 8-epi-PGF2α between the users and nonusers of antioxidants were similar (Table 2).

Oxidative protein damage. SSc subjects exhibited higher levels of salivary log protein carbonyls (1.24 ± 0.43 nmol/mg protein) than controls (0.76 ± 0.46 nmol/mg protein; $p < 0.0001$; Figure 1A). In a crude statistics model, a positive association was noted between SSc and salivary log protein carbonyls (OR 9.06, 95% CI 3.90–21.98, $p < 0.0001$). This association was not modified when the multivariate model was adjusted for log 8-OHdG, log 8-epi-PGF2α levels, and antioxidant exposure (OR 9.26, 95% CI 3.90–21.98, $p < 0.0001$). The positive association remained in the multivariable conditional model including only no-antioxidant (OR 11.61, 95% CI 3.39–39.72, $p < 0.0001$) or antioxidant exposure (OR 14.21, 95% CI 2.35–86.09, $p = 0.004$).

Oxidative DNA damage. The SSc group and controls showed similar salivary log 8-OHdG concentrations (3.35 ± 0.74 ng/ml and 3.22 ± 0.73 ng/ml, respectively; $p = 0.22$; Figure 1B). A nonsignificant association was noted between salivary log 8-OHdG and SSc (OR 1.19, 95% CI 0.76–1.85,

Table 1. Demographics and antioxidant ingestion of patients with systemic sclerosis and controls.

Characteristics	Systemic Sclerosis	Control	Total
Sample size (%)	70 (37)	120 (63)	190 (100)
Age, yrs, mean ± SD	54.44 ± 11.45*	42.53 ± 17.75	46.92 ± 16.71
Antioxidant user (%)	31 (44)*	21 (18)	52 (27)

* $p < 0.0001$, SSc vs control.

Table 2. Mean (SD) of biomarkers between scleroderma cases and controls.

Oxidative Damage Marker	Systemic Sclerosis		Controls	
	Nonantioxidant, User, n = 39	Antioxidant User, n = 31	Nonantioxidant, User, n = 99	Antioxidant User, n = 21
Log protein carbonyls, nmol/mg protein	1.24 (0.39)	1.25 (0.49)	0.75 (0.42)	0.81 (0.63)
Log 8-OHdG, ng/ml	3.28 (0.79)	3.43 (0.67)	3.22 (0.72)	3.21 (0.76)
Log 8-epi-PGF 2α, pg/ml	3.43 (0.95)	3.12 (0.82)	3.40 (0.73)	3.25 (0.86)

No significant difference between groups ($p > 0.05$).

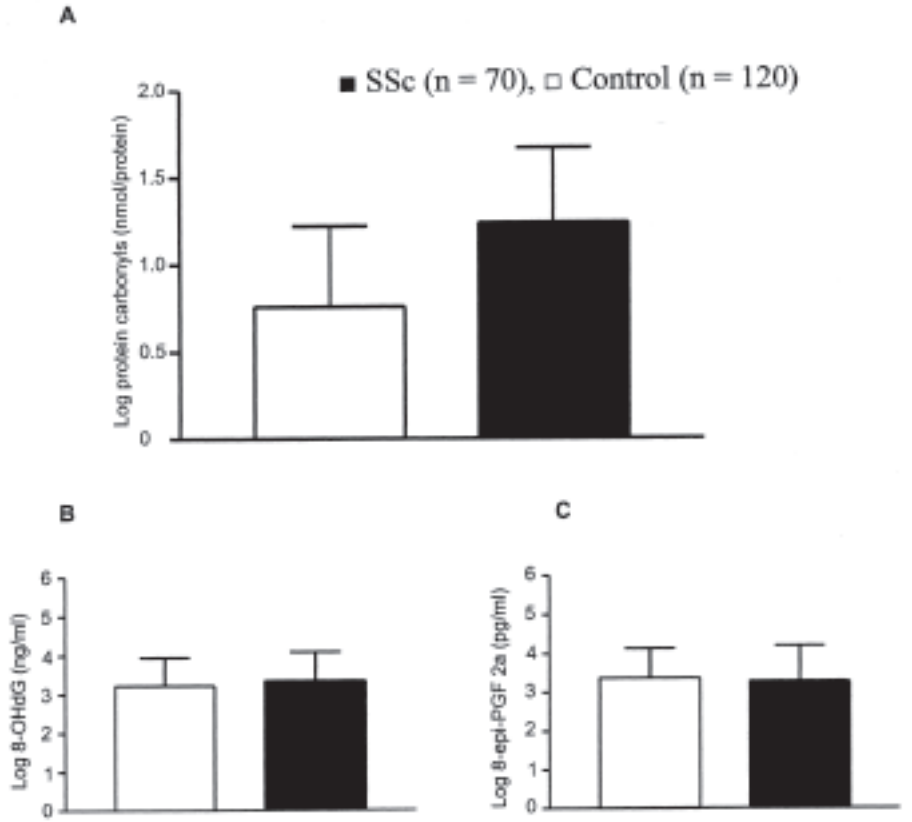

Figure 1. Markers of oxdative stress in saliva among 70 patients with systemic sclerosis and 120 controls. A. Log protein carbonyls. B. Log 8-OHdG. C. Log 8-epi-PGF2α. Data were shown as mean ± SD.

p = 0.45) in univariate conditional logistic regression analysis. This result remained in the conditional model adjusted for salivary log protein carbonyls, log 8-epi-PGF2α, and antioxidant exposure (OR 0.95, 95% CI 0.55–1.64, p = 0.86). Nonsignificant associations were also noted among antioxidant users (OR 1.44, 95% CI 0.35–5.90, p = 0.62) and nonusers (OR 1.01, 95% CI 0.52–1.97, p = 0.97).

Lipid peroxidation. The levels of salivary log 8-epi-PGF2α among SSc subjects (3.29 ± 0.90 pg/ml) were similar to controls (3.37 ± 0.76 pg/ml; p = 0.51; Figure 1C). No statistically significant association was noted between salivary log 8-epi-PGF2α levels and SSc in the crude statistics model (OR 0.91, 95% CI 0.60–1.36, p = 0.63). The multivariable conditional model adjusted for salivary log 8-OHdG, log protein carbonyls, and antioxidant exposure was not significant (OR 0.87, 95% CI 0.54–1.41, p = 0.56). Further, nonsignificant associations were noted among antioxidant users (OR 0.47, 95% CI 0.11–2.10, p = 0.32) and nonusers (OR 0.69, 95% CI 0.37–1.30, p = 0.25).

DISCUSSION

In recent years, an increasing number of articles proposed analysis of saliva as an alternative to serum for diagnostic purposes[30,31,32,33]. Saliva is an eminently accessible biofluid with enormous biomarker potential[34]. An array of oxidative damage products (e.g., protein carbonyls, 8-OHdG,

4-hydroxyalkenals, malondialdehyde) and antioxidant enzymes activity (e.g., superoxide dismutase, glutathione peroxidase, catalase) have been detected in human saliva. Changes in salivary redox homeostasis as manifested by deviations in absolute levels or expression patterns of these indices may reflect the presence and severity of various oral and systemic conditions (e.g., periodontal disease, diabetes, and inflammatory bowel disease)[27,28,35,36,37,38,39,40,41]. Our results indicate significantly higher levels of log protein carbonyls in whole nonstimulated saliva of patients with SSc relative to control values, and no significant association of the disease with salivary log 8-OHdG and log 8-epi-PGF2α levels.

Reports suggest that oxidative stress might contribute to the clinical and pathological manifestations of SSc, such as vascular damage[42], fibrosis[43], and production of autoantibodies[10,44]. The current data set extends these observations to saliva and provides strong evidence of perturbed redox homeostasis, i.e., augmented protein oxidation, in the oral cavity of patients with SSc. The augmented levels of salivary protein carbonyls observed are commensurate with previous reports of elevated protein carbonyl levels in serum and bronchoalveolar lavage of patients with SSc[45,46,47]. Our findings raise the possibility that salivary protein carbonyls may serve as a useful biological marker of SSc, a potential prognosticator of disease progression, and an index of successful therapeutic intervention. The reason that proteins exhibit selective susceptibility to oxidation modification in SSc relative to other chemical constituents (lipids, nucleic acids) remains incompletely understood. In contradistinction to protein carbonyls, concentrations of 8-OHdG and 8-epi-PGF2α remained unaffected in SSc saliva[48]. This observation contrasts with that of Ogawa, *et al*[14], who demonstrated elevated levels of 8-epi-PGF2α in the serum of patients with SSc. Severe or protracted oxidative stress may inevitably deplete antioxidant defenses, possibly reflected in SSc serum[14], culminating in cell injury, apoptosis, or necrosis[49,50]. The results of our current and previous studies[27] suggest that oxidative substrate modifications in saliva may provide clinically useful biomarkers for the diagnosis, staging, and prognosis of both local (oral) and systemic human diseases. Any single redox biomarker in isolation may be of limited value in this regard given the fairly ubiquitous role of oxidative stress in the pathogenesis of diverse human disorders. However, the spectrum of pro-oxidant chemical species generated and their target substrates is wide, and it may be the pattern of aberrant redox homeostasis that differentiates 1 disease from another. For example, in a previous study, we reported augmented levels of log lipid (OR 4.07) and log protein oxidation (OR 4.1) in nonsmokers with periodontal disease[27]. Periodontal disease may be prevalent in SSc due to reduced salivary content and narrowed oral opening, making oral hygiene difficult[51,52]. However, there have been no definitive studies showing increased periodontal disease in patients with SSc over controls. The salivary redox profile in periodontal disease differs from that of our study, in which we have found a much greater increment in log protein oxidation (OR 9.06, $p < 0.0001$) and no change in log lipid peroxidation (OR 0.91, $p = 0.63$). Thus, it is unlikely that our current data set was confounded by concurrent periodontal disease.

There are some limitations to our study. The co-occurrence of Sjögren's syndrome with SSc may feature inflammation or fibrosis of the salivary glands, which may have contributed to the current findings. Oxidative stress has been noted in Sjögren's syndrome *per se*[39,53,54]. We did not perform routine salivary gland biopsies in our patients and thus it is not possible to know exactly how many patients may have a coexisting Sjögren's syndrome.

Questions concerning the accuracy of the ELISA assay for oxidative DNA damage in saliva and urine have been raised[55]. In the case of saliva, however, a greater agreement between ELISA and LC-MS/MS is achieved if the primary antibody step is performed at 4°C overnight as in our study.

Biochemical and immunochemical assays in our study showed enhanced protein oxidation, without concomitant lipid peroxidation and DNA damage, in whole, nonstimulated saliva from a large cohort of women with SSc. Further research will be required to determine the associations between the abnormalities we have detected in SSc saliva and possible coexisting Sjögren's syndrome and other concurrent rheumatoid conditions. The determination of patterns of aberrant redox homeostasis in saliva may assist in the diagnosis of SSc.

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