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Abnormal exhaled ethane concentrations in scleroderma

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Abstract

Scleroderma (systemic sclerosis) is a chronic multisystem autoimmune disease in which oxidative stress is suspected to play a role in the pathophysiology. Therefore, it was postulated that patients with scleroderma would have abnormally high breath ethane concentrations, which is a volatile product of free-radical-mediated lipid peroxidation, compared with a group of controls. There was a significant difference ($p < 0.05$) between the mean exhaled ethane concentration of 5.27 pmol ml^{-1} CO_2 (SEM = 0.76) in the scleroderma patients ($n = 36$) versus the mean exhaled concentration of 2.72 pmol ml^{-1} CO_2 (SEM = 0.71) in a group of healthy controls ($n = 21$). Within the scleroderma group, those subjects taking a calcium channel blocker had lower ethane concentrations compared with patients who were not taking these drugs ($p = 0.05$). There was a significant inverse association between lung diffusion capacity for carbon monoxide (per cent of predicted) and ethane concentration ($b = -2.8$, $p = 0.026$, CI = −5.2 to −0.35). These data support the presence of increased oxidative stress among patients with scleroderma that is detected by measuring breath ethane concentrations.

Keywords: *Breath analysis, ethane, ethanol, oxidative stress, systemic sclerosis, scleroderma.*

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Introduction

Scleroderma is a chronic multisystem disease characterized by tissue fibrosis, small and medium artery vasculopathy, and autoimmunity. It is classified by the degree of skin involvement into two major subtypes: limited and diffuse cutaneous forms. Limited scleroderma has clinical evidence of skin thickening that is localized below the elbow and knee. These patients generally have a good prognosis but a subset may have severe vascular disease manifest by either pulmonary hypertension or severe Raynaud's phenomenon with digital ischaemia and amputation (Wigley et al. 1992). In the diffuse cutaneous form, the skin is involved above the elbows and arms and on the trunk. These patients are more likely to have multiple internal organ disease, including the gastrointestinal tract, heart, lungs and kidneys (Wigley 1996).

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Survival in patients with diffuse scleroderma is reduced overall compared with the limited form of the disease.

There is evidence of an association between an abnormal oxidative stress and scleroderma from several studies (Stein et al. 1996, Emerit et al. 1997, Simonini et al. 1999, 2000, Cracowski et al. 2001b, Sambo et al. 2001, Allanore et al. 2004). While the specific cause for an increase in oxidative stress status in scleroderma patients remains unclear, multiple factors likely play a role. For example, it known that scleroderma is an inflammatory disease. Stimulated macrophages can infiltrate tissues and release pro-inflammatory cytokines such as TNF-α and other pro-fibrotic proteins such as TGF-β (Scala et al. 2004). Inflammation can play a predominant role in the generation of reactive oxygen species. The respiratory bursts of polymorphonuclear leukocytes can directly damage tissues, especially in the lungs. In addition, the skin and tissues of other targeted organs in scleroderma are subjected to recurrent episodes of ischaemia-reperfusion caused by a widespread vascular disease involving medium and small arteries. Subsequently, haemodynamic changes related to vascular occlusion and endothelial cell activation and dysfunction may contribute to the sustained oxidative stress component (Jimenez & Derk 2004). Furthermore, metal-catalysed oxidation reactions have been shown to mediate specific DNA fragmentation and enhance immunogenicity of the cleaved antigens (Casciola-Rosen et al. 1997).

Raynaud's phenomenon is almost a universal clinical problem among patients with all forms of scleroderma. It is caused by disease of digital and cutaneous vessels causing abnormal prolonged vasoconstriction and then tissue ischaemia. Thermoregulatory and nutritional blood flow to the hands and feet can be completely restricted leading to skin necrosis and ulceration or digit loss (Hummers & Wigley 2003). This same process is thought to occur in the heart, lung, kidney and gastrointestinal tract in scleroderma due to the arterial disease in those organs. It is known that ischaemia-reperfusion-mediated injury causes tissue damage by the release of oxygen-free radicals (Granger et al. 1981). One of the specific mechanisms of tissue damage exerted by ischaemia-reperfusion injury occurs through lipid peroxidation (Kazui et al. 1992). Ethane has been demonstrated to be an *in vivo* biomarker that can be collected non-invasively in exhaled breath. Ethane has been shown to be elevated following ischaemia-reperfusion injury (Kazui et al. 1994). Interestingly, breath ethane is increased in other systemic inflammatory diseases such as Crohn's disease (Aghdassi et al. 2003). These data suggested that ethane might be elevated in the exhaled breath of patients with scleroderma as a consequence of both the inflammatory state and recurrent tissue ischaemia-reperfusion.

F_2-isoprostanes, another biomarker of lipid peroxidation, is increased in the urine of scleroderma patients compared with normal control subjects (Stein et al. 1996). Other researchers find that concentrations of urinary F_2-isoprostanes are increased in scleroderma patients compared with patients with primary Raynaud's phenomenon, and those with undifferentiated connective tissue diseases (Cracowski et al. 2002). Therefore, it is hypothesized here that (1) scleroderma patients would have elevated breath ethane compared with healthy controls and (2) patient characteristics, e.g. disease activity and/or the use of particular medications (e.g. antioxidants, anti-inflammatory drugs or vasoactive drugs such as calcium channel blockers), would have a measurable impact on breath ethane concentrations in subgroups of scleroderma patients.

Gastrointestinal dysmotility and gastro-oesophageal reflux disease are common in both limited and diffuse scleroderma. In the gastrointestinal tract, manifestations include oropharyngeal dysphagia, gastro-oesophageal reflux disease (GERD), gastric and intestinal dysmotility, and bacterial overgrowth (Sjogren 1994). A secondary goal of the present study was to quantify breath ethanol in scleroderma patients. Breath ethanol is a product of fermentation by bacteria residing in the gut and colon (Leclerc et al. 1997). Consequently, breath ethanol is proposed to be a possible marker of intestinal bacterial overgrowth and is associated with predisposing conditions, such as obesity and intestinal dysmotility (Cope et al. 2000). Since gastrointestinal dysmotility is common in scleroderma patients, it was hypothesized that scleroderma patients would have elevated breath ethanol concentrations.

Materials and methods

The protocol for breath collection was approved as a minimal risk protocol by the Committee for Human Research at The Johns Hopkins University Bloomberg School of Public Health. Informed verbal consent was obtained from all study participants before collecting breath samples. Healthy, non-smoking control subjects ($n = 21$) were recruited from a group participating in a nutrition research study at The Johns Hopkins University. Scleroderma patients ($n = 46$) were recruited for study from The Johns Hopkins Scleroderma Center. All patients had the diagnosis of scleroderma (limited or diffuse) confirmed by an expert rheumatologist (F. M. W) using standard criteria (American Rheumatism Association 1980). Limited disease was classified as skin thickening localized distal to the metacarpal pharyngeal joints or below the knee and elbow joints. Patients were classified as diffuse if the skin was involved above the elbows or knees or on the trunk. Breath ethane, ethanol, methanol, acetone and isoprene concentrations were determined in all patients. Samples were not used in subsequent analysis if the samples contained low concentrations of carbon dioxide (< 20 mmHg), indicating an inadequate breath sample. Patients who were current smokers were excluded from the scleroderma group.

Indices for Raynaud's phenomenon, skin severity, pulmonary severity, gastrointestinal severity, cardiac severity, renal severity and general disease severity were defined as described (Medsger et al. 1999). If a score was not recorded at the same time that the breath was sampled, then the closest severity score at the time of the breath collection was used. Lung-diffusing capacity was determined by a carbon monoxide single-breath test (DLCO). Pulmonary function was assessed by forced vital capacity (FVC). Pulmonary function tests (PFTs) were performed during the same clinical visit when the breath sample was obtained, or else the closest measurement to that date was used in data analysis. Ninety per cent of PFTs were recorded within 1 year of breath collection (median = 0 days, range = 3.5 years). Echocardiographic (ECHO) studies were performed to estimate right ventricular systolic pressure (RVSP). Ninety per cent of these echocardiographic examinations were performed within 2 years of breath collection (median = 9 days, range = 4 years). Both PFTs and ECHO examinations were conducted as a part of the standard of care for patients in the Scleroderma Center. Serological data were obtained from the Scleroderma Center database to evaluate the presence of anti-topoisomerase and anti-centromere antibodies.

The protocol used for breath collection and analysis has been previously described in detail (Cope et al. 2004). Subjects were asked to breathe tidally through a mouth filter attached to a two-way non-rebreathing valve (NRV; Hans Rudolph, Inc., Kansas City, MO, USA). Most subjects were present at the clinic for more than 30 min before the collection of breath samples, and all subjects were seated at rest for breath collection. Breath was collected for 1 min on thermal desorption tubes containing a triple bed of adsorbents (Risby 2002). A room air sample was collected before breath sampling. The first adsorbent was graphitized carbon (carbopack X); the second and third absorbent beds consisted of carbon molecular sieves, carboxen-1018 and carboxen-1021, respectively (Supelco Corp., Bellefonte, PA, USA). Breath was collected onto duplicate thermal desorption tubes using an automated constant flow pump at a flow rate of 80 ml min^{-1}. During breath collection a computerized breath collection system was used to monitor tidal breathing. Tidal volume, respiration rate, average exhaled carbon dioxide, end-tidal carbon dioxide and mouth pressure were all monitored for each breath collected.

The collected breath sample was analysed by two-stage automated thermal desorption capillary gas chromatography and flame ionization detection (GC-FID). Ultra-high purity helium was used to purge the tubes and was the carrier gas. Chromatographic separations were carried out on a 60-m fused silica open tubular column (0.32 mm) coated with a thick film (5 μm) of dimethyl silicone stationary phase (Rtx-1, Restek, Bellefonte, PA, USA). The chromatographic temperature protocol was as follows: isothermal at 35°C for 10 min, 35–200°C at 5°C min^{-1}, and isothermal at 200°C for 10 min.

Breath molecule concentrations were corrected for the presence of molecules in room air, and steady-state carbon dioxide concentrations were used to normalize breath molecule concentrations as described (Cope et al. 2004). For quantification, breath ethane and ethanol concentrations are expressed in units of pmol ml^{-1} CO_2. To compare patients and controls, a value of zero was assigned for samples where room air concentrations exceeded the concentration determined in the exhaled breath samples. The number of zero values in both the control ($n = 9$) and scleroderma group ($n = 9$) was the same. The zero values were dropped for analysis within the scleroderma group. A two-sample Student's t-test was used to compare two groups of normally distributed data. Pair-wise correlations were evaluated by Spearman's coefficient. Multiple linear regression analysis was performed to examine associations between independent predictor variables and a dependent variable. The model took the form:

$$y_{\text{fit}} = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3$$

where y_{fit} is the predicted fit of the regression line, b_0 is the y-intercept, b_1, b_2 and b_3 are the regression coefficients, and x_1, x_2 and x_3 are the individual measurements for each variable. For every regression model, a plot of the residuals ($y_{\text{obs}} - y_{\text{fit}}$) versus each explanatory variable (x) and y_{fit} was constructed to assess linearity and normality (Altman 1991). Variance inflation factors were used to assess multicollinearity between predictor variables. Wilcoxon's rank-sum test was used to evaluate statistically significant differences between discrete groups if data were non-parametrically distributed. A Fisher's exact test was used to test for significant difference in proportions of observed versus expected frequencies. For all statistical tests, $p \leq 0.05$ was considered as being statistically significant. All statistical analyses were performed

using the Stata 8.2 computerized statistical package (Stata Corp., College Station, TX, USA).

Results

Breath ethane

Eight samples were not used in subsequent analysis because they contained low concentrations of carbon dioxide (<20 mmHg), indicating an inadequate breath sample. Two additional patients who were smokers were excluded from the scleroderma group. Table I shows the physiological breathing parameters for scleroderma and control subjects. There were significant differences between the two groups in age and body mass index (BMI). The age of the scleroderma group was significantly higher than that of the controls subjects, but the BMI was significantly lower. End-tidal and steady-state carbon dioxide were both significantly higher in the control group compared with scleroderma patients, but the minute ventilation was similar between the groups.

It was found that scleroderma patients generated ethane at a level twice greater than the concentration of the control group. The null hypothesis that there was no difference in exhaled ethane concentrations between the two groups was rejected ($p = 0.04$). Figure 1 shows the mean ($\pm$SEM) ethane concentration from the scleroderma patients and the group of controls. The exhaled ethane concentration was 5.27 pmol ml^{-1} CO_2 (SEM $=0.76$) in the scleroderma patients ($n=36$) versus the mean exhaled concentration of 2.72 pmol ml^{-1} CO_2 (SEM $=0.71$) in the control group ($n=21$).

Twenty-seven scleroderma subjects had breath ethane concentrations exceeding the room air concentration. In this group of ethane producers, 12 patients were taking a calcium channel blocker to treat Raynaud's phenomenon, but 15 patients were not. A comparison between the two groups is shown in Figure 2. Calcium channel blocker users had a significantly lower mean ethane concentration (5.58 pmol ml^{-1} CO_2, SEM $=1.28$, $n=12$) compared with non-users (8.24 pmol ml^{-1} CO_2, SEM $=0.96$, $n=15$, $p=0.05$). Eight of the 36 scleroderma patients were taking non-steroidal anti-inflammatory drugs for pain relief, and 11 of 36 patients were taking prednisone to control inflammation. There was no significant effect on breath ethane in subgroups taking anti-inflammatory medications (either steroids or non-steroidal anti-inflammatory drugs) compared with scleroderma patients who were not.

Table I. Anthropometric and physiological characteristics of scleroderma and controls subjects.

	Controls ($n=21$)	Scleroderma ($n=46$)
Age (years)	46.8 (1.1)	54.0 (1.9)†
BMI (kg m^{-2})	32.8 (1.8)	24.2 (1.0)⋆
Minute ventilation (l min^{-1})	8.9 (1.2)	7.2 (0.5)
End-tidal CO_2 (mmHg)	37.5 (1.0)	33.3 (0.8)‡
Steady-state CO_2 (mmHg)	32.1 (1.1)	28.2 (0.7)‡

All statistics are expressed as means (SE).
Significant difference of ⋆$p<0.0001$, †$p<0.02$ and ‡$p<0.003$.

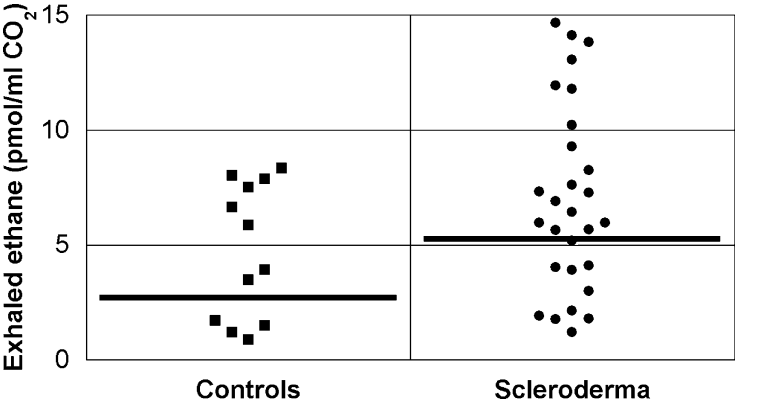

Figure 1. Increased ethane production in scleroderma patients versus control subjects. Breath samples from patients with scleroderma (solid dots, $n=27$) are compared with breath samples collected from non-diseased control (solid squares, $n=12$) subjects. Nine subjects in each group with ethane concentrations below the room air concentration are not shown. Ethane concentrations are expressed in carbon dioxide-normalized units. The horizontal line represents the mean. The difference between the groups was evaluated using the Wilcoxon rank-sum test. There was a statistically significant difference between scleroderma and control groups at the 95% confidence level ($p=0.05$).

Exhaled ethane concentration was inversely associated with DLCO. Figure 3 shows the regression plot for the correlation between ethane concentration and DLCO. The simple linear regression model predicts that a 2.8% decrease in DLCO (per cent predicted) is associated with each unit increase in exhaled ethane concentration ($b=-2.76$, SE $=1.16$, $R^2=0.18$, 95% CI $=-5.2$ to -0.35, $p=0.026$, $n=27$).

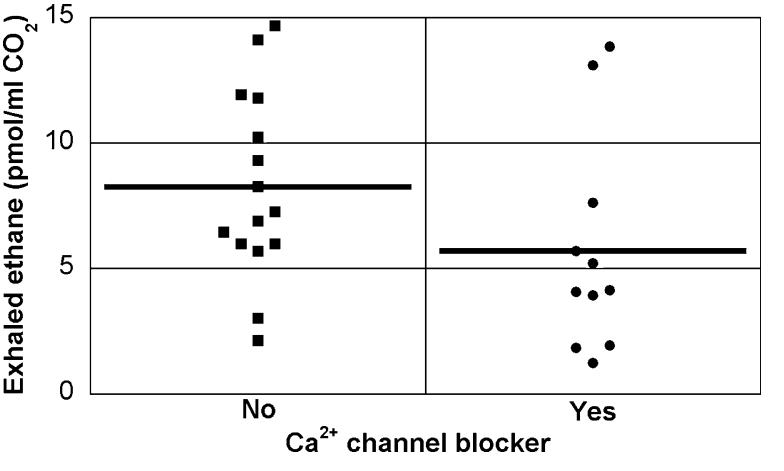

Figure 2. Ethane generation was reduced in the group of scleroderma patients receiving a Ca^{2+} channel inhibitor (solid dots, $n=11$) compared with scleroderma not prescribed this class of medications (solid squares, $n=15$). Ethane concentration (y-axis) is expressed in carbon dioxide-normalized units. The horizontal line represents the mean. Only patients who had ethane concentrations greater than the room air were included in this analysis. The difference between the groups was evaluated using the Wilcoxon rank-sum test. There was a statistically significant difference between scleroderma and control groups at the 95% confidence level ($p=0.05$).

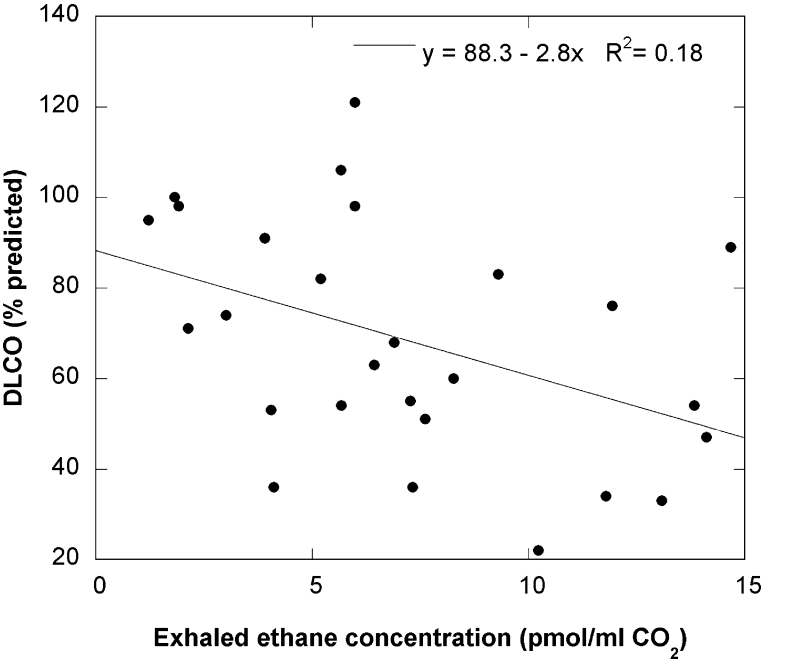

Figure 3. Linear regression of ethane concentration versus DLCO ($n=27$). All subjects were ethane producers. The significance of the regression slope was $p=0.026$.

Breath ethane concentration explained only 18% of variability in the simple regression model. Therefore, other variables were investigated as predictors of DLCO. The results of pair-wise correlation tests indicated that FVC, race, ethane and Raynaud's phenomenon severity index were significantly correlated with DLCO (data not shown). Multiple regression analysis was used to construct a model of the most significant variables. As shown in Table II, FVC, ethane concentration and Raynaud's severity were significantly associated with DLCO. Other variables, such as the type of scleroderma (limited or diffuse), RVSP, age, the presence of anti-centromere and anti-topoisomease antibodies, the use of calcium channel blockers or other severity score indices, were examined, but none showed a significant contribution to the model. However, an inverse association was found between ethane concentration and RVSP ($b=-1.09$, SE $=0.48$, $R^2=0.19$, 95% CI $=-2.02$ to -0.01, $p<0.047$, $n=21$). Interestingly, when ethane concentration was adjusted for RVSP, the association between DLCO and ethane was more significant (Table III).

Table II. Multiple regression analysis of significant predictors of DLCO[†].

Variable	Coefficient	SE	t-Statistic	p	95% CI
FVC (% predicted)	0.65	0.14	4.56	0.0001	0.36 to 0.95
Ethane (pmol ml^{-1} CO_2)	−2.09	0.79	−2.66	0.014	−3.73 to −0.47
RP severity index (0–3, mean score)	−9.53	4.09	−2.33	0.029	−18.01 to −1.05

[†]Dependent variable is DLCO ($n=27$) in patients with endogenous ethane concentration that exceeded exogenous room air ethane concentrations.
Adjusted $R^2=0.62$, root MSE $=15.87$; $F(3, 23)=15.42$.

Table III. Multiple regression analysis of ethane and RVSP on DLCO[‡].

Variable	Coefficient	SE	*t*-Statistic	*p*	95% CI
Ethane (pmol ml^{-1} CO_2)	−4.03	1.32	−3.05	0.007	−6.8 to −1.26
RVSP (mmHg)⋆	−1.10	0.56	−1.94	0.069	−2.29 to 0.093

[‡]Dependent variable is DLCO ($n=21$).
⋆ECHO studies were conducted in 21 of 27 patients in which endogenous ethane exceeded exogenous room air ethane concentrations.
Adjusted $R^2=0.28$, root MSE = 20.987, $F(2, 18)=4.88$.

A comparison of mean DLCO, FVC, ethane concentration, RVSP and anti-topoisomerase/anti-centromere antibody counts is shown in Table IV for Caucasians and African-Americans. The one patient of Asian descent was not included in this analysis. The group of African-Americans had significantly lower DLCO, lower FVC and increased Raynaud's phenomenon severity scores compared with Caucasians. Ethane concentration tended towards a significant difference ($p=0.088$).

Breath ethanol

Breath ethanol was significantly lower ($p<0.006$) in scleroderma patients compared with the control group. The median (range) was 32.5 (5–318) pmol ml^{-1} CO_2 in the scleroderma group ($n=30$) compared with 76.0 (30–323) pmol ml^{-1} CO_2 in the control group ($n=21$). No significant intra-group differences were found when subgroup analysis was applied for a gastrointestinal symptom severity score, or use of proton-pump inhibitors.

Discussion

The main finding of this study is that patients with scleroderma have evidence of an increased oxidative stress as measured by increased breath ethane concentrations compared with control subjects. The exhaled ethane concentration was inversely associated with DLCO, a measure of gas exchange that can be affected by either interstitial lung disease or cardiopulmonary vascular disease. FVC, a measure of lung fibrosis, race, breath ethane concentration and Raynaud's phenomenon severity index

Table IV. Scleroderma disease markers stratified by race.

Race	FVC (% predicted)	DLCO (% predicted)	RVSP (mmHg)	Ethane (pmol ml^{-1} CO_2)	Topoisomerase antibodies[§]	Centromere antibodies[§]	RP severity index score
Caucasian	87.1 (3.3)	76.1 (3.1)	38.3 (1.7)	6.3 (0.9)	3	7	1.4 (0.1)
Number	37	37	27	20	21	32	37
African-American	64.9 (7.5)[‡]	42.8 (5.3)[†]	42.5 (4.2)	9.3 (1.5)	3	0	2 (0.3)⋆
Number	8	8	6	6	5	6	8
Total	82.7 (3.2)	69.3 (3.3)	38.8 (1.6)	7.16 (0.77)	6	7	1.5 (0.1)
Number	45	45	33	26	26	38	45

Data are means (SEM) unless otherwise indicated.
[§]Antibodies are expressed as the number of sero-positive subjects.
Significant at [‡]$p=0.02$, [†]$p=0.0003$ and ⋆$p=0.04$. Significance is determined by Wilcoxon's rank-sum test.

were significantly correlated with DLCO. Interestingly, the patients on a vasodilator for Raynaud's phenomenon (i.e. calcium channel blocker) had a significantly lower mean ethane concentration (5.58 pmol ml^{-1} CO_2, SEM = 1.28, $n = 12$) compared with non-users (8.24 pmol ml^{-1} CO_2, SEM = 0.96, $n = 15$, $p = 0.05$). Taken together, these data suggest that patients have an increased oxidative stress status that associates with manifestations of lung and vascular disease.

Since increased oxidative stress is thought to have pathological implications, its measurement may provide insight into the activity and pathogenesis of tissue injury. Perhaps the most immediate damage from free radicals occurs at biological membranes. For example, there is evidence in scleroderma that lipid peroxidation of membrane phospholipids and changes in fatty acid ratios that affect membrane fluidity (Solans et al. 2000) occurs. Oxidative stress must be measured indirectly and thus requires the collection of biomarkers that are produced from free radical damage to cellular macromolecules such as lipids, proteins and DNA. Recent reports provide evidence for the relationship between an increase in lipid peroxidation and scleroderma. Similar to our findings, urinary F_2-isoprostanes were recently demonstrated to be elevated in scleroderma patients and to be correlated with a decrease in DLCO (Volpe et al. 2005). Collecting and analysing ethane from exhaled breath of patients is a non-invasive reliable method to assess lipid peroxidation, *in vivo*. This technique can be used to assess the oxidative stress status of patients with greater frequency and may be used to monitor the level of oxidative stress and the response to therapy.

To maintain normal breathing patterns, parameters that affect breath collection must be monitored during breath collection (Cope et al. 2004). In the present study, end-tidal and steady-state carbon dioxide were significantly lower in the scleroderma group compared with controls, even though minute ventilation rates were similar between the groups. It has been recently reported that end-tidal carbon dioxide is reduced in response to pulmonary arterial hypertension (Yasunobu et al. 2005). Although the end-tidal carbon dioxide concentration was significantly reduced in scleroderma patients compared with controls, there was no correlation between the end-tidal carbon dioxide measurements taken during breath collection and the FVC or DLCO measurements; nor was there any correlation found with RVSP. While it is possible that reduced end-tidal carbon dioxide levels among scleroderma patients may be due to reduced gas exchange, another possibility may be that skin hardening around the oral aperture made it difficult for patients to keep a tight seal with the mouthpiece throughout breath collection.

The exact mechanism for the increased ethane concentrations and oxidative stress cannot be determined from the present study. Although inflammation and increased oxidative stress from activation of the immune system and up-regulation of pro-inflammatory cytokines can increase oxidative stress, there was no affect of anti-inflammatory medications. Previously, investigators have found that inhaled corticosteroids reduced exhaled ethane in patients experiencing severe exacerbations of asthma (Paredi et al. 2000). However, in the present study the only steroid medication monitored was oral prednisone. Thus, it may be that inhaled corticosteroids are more effective at reducing pulmonary inflammation than those administered orally. Moreover, the study was exploratory and not designed to measure the full spectrum of the disease or the early inflammatory stages when oxidative stress may be quite high. It is also possible that polymorphic differences in superoxide dismutase and decreased levels of micronutrient antioxidants might also contribute to increased oxidative stress

in scleroderma (Tikly et al. 2004). In fact, deficiencies in selenium and vitamin C are described for scleroderma patients (Herrick et al. 1996). Vitamin E levels can be increased in scleroderma patients receiving 500–1000 mg vitamin E day^{-1}, indicating that low absorption of micronutrients can be reversed and thus not play a role in decreased antioxidant status (Cracowski et al. 2005).

The present study is consistent with other research that has shown a correlation between total body oxidative stress status and compromised lung function in scleroderma patients (Luczynska et al. 2005). One study demonstrated that lipid peroxidation is increased in the broncheo-alveolar lavage fluid of scleroderma patients with fibrosing alveolitis (Montuschi et al. 1998), while other research has shown that concentrations of F_2-isoprostanes (e.g. 5-oxoeicosatraenoic acid and 8-epi-PGF2alpha) are increased in lung tissue sections and urine samples collected from patients with pulmonary hypertension (Cracowski et al. 2001a, Bowers et al. 2004).

Breath ethane concentration was a significant independent predictor of DLCO. DLCO is known to be a sensitive predictor of early pulmonary fibrosis and/or pulmonary hypertension (Generini et al. 1999). It can be decreased by interstitial lung disease or pulmonary hypertension, but FVC is reduced only by interstitial lung disease. In addition, lung fibrosis may be a predisposing risk factor for the development of pulmonary hypertension (Schachna et al. 2003). In the present study, FVC and DLCO were highly correlated, which indicates that the reduction in diffusing capacity in the present study group occurs mainly from fibrosis. However, the DLCO may reflect interstitial lung disease and pulmonary hypertension; the fact that there was no association seen with ethane and FVC argues that the association with DLCO may be reflective of the pulmonary vascular process. Thus, the significant predictors of DLCO include FVC, ethane concentration and Raynaud's severity scores. Race also had an effect on these parameters and is suspected of being a predisposing risk factor for secondary lung disease in scleroderma patients (Greidinger et al. 1998).

In a group of patients who underwent ECHO, it was found that increased breath ethane concentration was inversely associated with RVSP. Furthermore, there was a more significant inverse association between breath ethane concentrations and DLCO in the RVSP-adjusted model. Since ethane exhalation is perfusion-limited, increased pulmonary artery pressure may actually result in a decrease of the exhaled ethane concentration. However, while the RVSP measured by echocardiogram is highly accurate at elevated pulmonary artery pressures, it may not be as accurate, particularly at lower pressures (mild pulmonary hypertension) (Denton et al. 1997).

Previously, it has been shown that exhaled nitric oxide is increased in scleroderma patients with interstitial lung disease (Paredi et al. 1999), but is reduced in scleroderma patients with pulmonary hypertension (Kharitonov et al. 1997). One recent study found that during controlled exhalation, the exhaled alveolar nitric oxide concentration in scleroderma patients with lung disease correlated with decrements in DLCO (Girgis et al. 2002). Similar to the present findings with ethane, other investigators have shown that exhaled nitric oxide is inversely associated with RVSP (Rolla et al. 2000). This would suggest that nitric oxide plays a role in regulating vessel dilation in the pulmonary vasculature; however, it is conceivable that reduced diffusion of alveolar nitric oxide from the pulmonary arteries to the alveoli also occurs as a result of vascular disease. Thus, the relationship between ethane concentration, DLCO and RVSP may be modulated by changes in ventilation and

perfusion which can be caused by a vascular disease process or an inflammatory process secondary to alveolitis and/or interstitial fibrosis (Ooi et al. 2003). A recent investigation of ethane exhalation in patients with various forms of interstitial lung disease found no correlation between ethane concentration and DLCO (Kanoh et al. 2005). Thus, it would appear that increased pulmonary vascular disease and perhaps pulmonary hypertension is associated with increased ethane concentrations in scleroderma.

Raynaud's phenomenon in scleroderma is associated with a widespread proliferative occlusive vascular disease, thereby increasing production of oxygen-free radicals and promoting tissue injury. The fact that there was no correlation between breath ethane concentration and an index of Raynaud's phenomenon severity in the present study was surprising. It may be that longitudinal observations are important to reveal the oxidative stress in that Raynaud's is highly variable in activity that is very dependent on stress and cold temperatures. For example, it may be that activation of inflammatory cells and biomarkers of oxidative damage to lipids in membranes are increased during early periods of disease when more frequent ischaemic attacks occur, and more healthy tissues are present (Sambo et al. 1999, Simonini et al. 1999, Solans et al. 2000). Vascular disease may activate metabolic pathways to increase oxidative stress. Under conditions of ischaemia-reperfusion, the enzyme xanthine oxidase catalyses the conversion of hypoxanthine to xanthine and generates superoxide anions (Korthuis & Granger 1986). Nevertheless, one study of activated fibroblasts collected from the skin of scleroderma patients did not show a decreased level of oxidant production in response to xanthine oxidase inhibition by allopurinol (Sambo et al. 2001).

There was a significantly lower breath ethane concentration among patients receiving calcium channel blockers. The reduction in ethane concentrations among patients taking this class of drugs may be due to the noted ability of these drugs to improve Raynaud's phenomenon through strong vasodilating activity on endothelial cell membranes, or may reflect the potent antioxidant effects of certain calcium channel blockers, such as mibefradil, which has been shown to protect against *in vitro* lipid peroxidation (Mak et al. 1992, Mason et al. 1998). A recent study suggests that dihydropyridine-type calcium channel blockers exhibit a profound effect in reducing other plasma biomarkers of oxidative stress in scleroderma patients (Allanore et al. 2004). The present study, using breath ethane, corroborates this impression.

The present hypothesis that breath ethanol would be increased in scleroderma subjects was not supported by the results. While breath ethanol is a product of intestinal bacteria, and overgrowth of bacteria often occurs in scleroderma, no positive correlation was found between these two parameters. However, the patients were not selected for severe or symptomatic bowel disease and therefore they may not have the problem of bacterial overgrowth. Nevertheless, it is of interest that rather than no difference there was a significantly lower concentration of ethanol in the scleroderma subjects compared with controls. One reason for this finding may be that the bacteria that colonize the gut in scleroderma patients do not produce ethanol, or that ethanol-producing bacteria do not reside in the small intestine, but are more prevalent in the lower intestine and colon. Another possibility is that variables that were not controlled for such as recent food consumption have affected the difference between the groups (Cope et al. 2004). The low ethanol concentrations might also be related to the lower

BMI measurements in scleroderma subjects compared with the controls in that the controls were overweight (Nair et al. 2001).

Study limitations

The study was limited because it did not include a standard breath test (e.g. hydrogen breath test) as a positive control, or a culture of bacterial strains present in the gut of scleroderma patients. Moreover, the control subjects were not matched according to body mass and age, two factors that may affect ethanol production and oxidative stress status. It is believed that oxidative stress status increases with age, thus it is possible the more advanced age of scleroderma patients may have affected ethane concentrations. Furthermore, it should be recognized that since this preliminary study had a small sample size, it is possible that some of the significant results might be attributable to type I statistical error.

Conclusions

The data demonstrate that collection of breath ethane is a practical, non-invasive, *in vivo* method to analyse the oxidative stress status in scleroderma patients. Scleroderma patients were more oxidatively stressed than control subjects. The correlation of the breath ethane concentration and decreased DLCO suggests there is an increased oxidative stress status among scleroderma patients with severe cardiopulmonary disease. The use of calcium channel blockers was associated with lowered breath ethane in a subgroup of scleroderma patients and suggests that vasodilator therapy may reduce oxidative stress. Therefore, the analysis of breath ethane concentration may be useful in monitoring oxidative stress-related disease pathogenesis in scleroderma patients.

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