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The Study of Oxidative Stress in Fibrotic and Non-Fibrotic Skin of Patients with Systemic Sclerosis

Direttore della Scuola : Ch.mo Prof. Gaetano Thiene

Coordinatore d'indirizzo: Ch.mo Prof. Leonardo Punzi

Supervisore :Ch.mo Prof. Franco Cozzi

Dottorando : Dott. Khalil Ibrahim Bourji

Abstract

Introduzione

La sclerosi sistemica (SSc) è una malattia multisistemica cronica del tessuto connettivo caratterizzata dalla fibrosi progressiva della cute e degli organi interni. Sebbene i meccanismi patogenetici coinvolti nella malattia sono poco chiari, è stato dimostrato che i pazienti affetti da SSc presentano alti livelli di stress ossidativo (Reactive Oxygen Species [ROS]) tale da suggerire un ruolo nella fibrogenesi. In questo studio valutiamo i livelli di ROS sia nella cute indurita che quella apparentemente sana dei pazienti affetti da SSc e li confrontiamo con dei controlli sani.

Metodi

Sono stati arruolati 9 pazienti (4 uomini e 5 donne) con dcSSc soddisfacendo i criteri diagnostici (EULAR/ACR) e 7 controlli sani abbinati per età e sesso. L'età media dei pazienti era 46 ± 10 anni e la durata media della malattia era 7.5 ± 5 anni. Tutti i pazienti erano ANA (Scl-70) positivi. Il coinvolgimento cutaneo è stato valutato con lo score di Rodnan modificato (mRSS). Al momento dello studio, da ciascun paziente sono state prelevate 2 biopsie cutanei (punch biopsy 4 mm), una da pelle indurita (con mRSS 2-3) e un'altra da pelle apparentemente sana (con mRSS 0). Contemporaneamente, sono stati prelevati campioni di sangue per l'analisi dello stress nel siero. Per l'analisi dei ROS nella cute è stata utilizzata la metodica spettrometrica altamente sensibile EPR (Electron Paramagnetic Resonance). Invece, diverse metodiche spettrofotometriche sono stati utilizzati per l'analisi dello stress nel siero.

Risultati

I livelli dei ROS (mediana e range, in nmol/l/min/mg di peso secco) erano 24.7(10.9-47.0) nella cute indurita, 18.7(7.3-34.0) nella cute apparentemente sana e 7.7(3.5-13.6) nella cute dei controlli sani. Lo stress era significativamente più alto nei pazienti (sia cute indurita che cute apparentemente sana) rispetto ai controlli ($p < 0.05$). Inoltre, la cute indurita mostrava livelli di stress più alti della cute apparentemente sana dello stesso paziente

($p=0.01$). lo stress nella cute indurita si correlava con la capacità vitale forzata (FVC) ($r= -0.75$, $p=0.02$) e la velocità di eritrosedimentazione (VES) ($r=0.70$, $p=0.04$). Rispetto ai controlli, il siero dei pazienti affetti da SSc ha mostrato livelli di acido ascorbico più basso (8 [3.8-9.8] vs. 10.5 [9-19.1] mg/L, $p=0.004$) e livelli di perossidi lipidici più alti (873.5 [342-1973] vs. 422 [105-576] $\mu\text{mol/L}$, $p=0.004$).

Conclusioni

I risultati del nostro studio indicano la presenza di alto stress ossidativo sia nella cute indurita che in quella apparentemente sana dei pazienti affetti di SSc, con una tendenza più alta nella cute indurita. La presenza di alto stress nella cute apparentemente sana può indicare un precoce ruolo dello stress nella fibrogenesi. Per approvare quest'ipotesi necessitiamo di studi longitudinali prospettici.

Abstract

Background and Objectives

Systemic Sclerosis (SSc) is a chronic multisystemic connective tissue disease characterized by progressive fibrosis affecting skin and internal organs. Despite serious efforts to unveil the pathogenic mechanisms of SSc, they are still unclear. High levels of Reactive Oxygen Species (ROS) in affected patients have been shown, and ROS are suggested to play a role in fibrosis pathogenesis. In this study we evaluate ROS levels in non-fibrotic and fibrotic skin of patients with SSc and we compare them with those obtained from healthy controls.

Patients and Methods

We enrolled 9 SSc patients fulfilling the EULAR/ACR classification criteria and 7 healthy controls. Patients included were 4 men and 5 women with mean age of 46 ± 10 yrs. Controls were matched by sex and age. All patients were affected by diffuse cutaneous form of SSc and the ANA pattern anti-Sc170. Mean disease duration was 7.5 ± 5 yrs. Skin involvement was evaluated by modified Rodnan Skin Score (mRSS). Skin samples (4mm punch biopsy) were taken from fibrotic skin and non-fibrotic skin of patients and from healthy controls as well. To detect ROS, specimens were analyzed immediately after sampling by electron paramagnetic resonance spectroscopy. Blood samples have been drawn from all patients and controls to assess oxidative stress biomarkers.

Results

ROS levels (expressed as median and range, unit of measurement was nmol/l/min/mg of dry weight) were 24.7 (10.9– 47.0) in fibrotic skin, 18.7 (7.3–34.0) in non-fibrotic skin and 7.7 (3.5–13.6) in healthy controls skin. ROS levels in Fibrotic and Non-fibrotic skin of SSc patients were significantly higher than in Healthy Controls ($p=0.002$ and $p=0.009$, respectively). ROS levels in fibrotic skin were raised in comparison to non-fibrotic skin, when samples related to each patient were compared ($p=0.01$). ROS levels in fibrotic skin were correlated with forced vital capacity ($r= -0.75$, $p=0.02$) and erythrocyte sedimentation rate ($r=0.70$, $p=0.04$). All other clinical and lab parameters showed no significant

correlation. When compared to controls, blood from SSc patients showed lower ascorbate (vitamin C) levels (8 [3.8-9.8] vs. 10.5 [9-19.1] mg/L, $p=0.004$) and higher lipid peroxides (873.5 [342-1973] vs. 422 [105-576] $\mu\text{mol/L}$, $p=0.004$).

Conclusion

Our results indicate the presence of high oxidative stress both in non-fibrotic skin and fibrotic skin of SSc patients, but with higher tendency in the latter. Raised ROS levels in non-fibrotic skin of SSc patients might be a hint of early involvement in skin fibrogenesis. However, a longitudinal prospective study is necessary for such proof.

Highlights

- Skin of patients with dcSSc, either fibrotic or “apparently” normal skin showed higher ROS levels as compared to healthy controls skin.
- ROS levels were higher in Fibrotic skin than in non-fibrotic skin of patients.
- ROS levels in fibrotic skin of dcSSc patients were positively correlated with ESR and negatively correlated with FVC.
- Blood from patients with dcSSc had lower ascorbate levels and higher lipid peroxides levels as compared to healthy controls.

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Introduction

Systemic Sclerosis [SSc] is a chronic multisystem connective tissue disease characterized by progressive fibrosis of many organs of the body [1, 2]. Although skin involvement forms the hallmark of SSc, internal organ damage due to fibrosis results in mortality for this disease [3]. Even though the pathogenesis of SSc is unclear, fibrosis is proposed to be the outcome of a combination of autoimmune and proliferative/obliterative vasculopathy mechanisms [4-6]. Beside these pathogenetic pathways proposed to explain fibrosis, a role of oxidative stress in the pathogenesis of SSc has also been suggested [7, 8].

Under different endogenous or exogenous stresses, atoms with unpaired electrons called Free Radicals are generated. The 2 major types of Free Radicals formed from Oxygen and Nitrogen are, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) [9]. Both ROS and RNS can cause oxidative damage to DNA, lipids, carbohydrates and proteins in cells which leads to structural damage and ultimately results in cellular damage [10, 11]. To counteract the oxidative damage caused by ROS and RNS, the cells have developed a defense mechanism called antioxidants. An imbalance between the elevated levels of ROS/RNS and/or the impaired function of the antioxidant defence system is known as oxidative stress [8]. In SSc, elevation of oxidative stress “markers” or alteration of antioxidant mechanisms have been described by many investigators [8, 12-18].

Oxidative stress markers in plasma were found to be elevated in patients with diffuse cutaneous SSc (dcSSc) compared to controls, and the presence of anti-Scl70 in these patients was associated with higher stress [12]. ROS levels, in particular superoxide levels, were higher in dermal fibroblasts obtained from the skin of patients with dcSSc compared to normal cells [13]. On the other hand, some investigators have shown an abnormal antioxidant status in scleroderma patients, demonstrating either high detoxifying enzymes (Erythrocyte glutathione transferase, eGST) [14] or high Total Antioxidant Power (TAP) [15] in these patients. Conversely, non-enzymatic antioxidants such as ascorbic acid

(vitamin C), α -tocopherol, β -carotene, and selenium were also shown to be reduced in plasma of patients with SSc [8, 16]. A role of anti-detoxifying-enzymes antibodies has also been proposed. The anti-Prx I (Peroxiredoxin I) antibodies were increased in both limited and diffuse SSc and in other autoimmune diseases [17]. The same group also showed that anti-MSRA (Methionine Sulfoxide reductase A) antibodies were raised in SSc patients and were correlated with disease severity [18].

Despite the existence of oxidative stress in patients with SSc, the stage of the disease in which ROS/RNS manifests is unknown. We hypothesize that ROS levels are elevated early in the skin of SSc patients. In this study we assess ROS levels in non-fibrotic and fibrotic skin of patients with SSc.

Materials and Methods

Patients and Populations

In our study we enrolled adult patients, over 18 years old at baseline, with diffuse cutaneous SSc (dcSSc) satisfying the recent ACR/EULAR criteria for Systemic Sclerosis [19]. All patients were diagnosed for SSc and subsequently followed in outpatient regime at the Division of Rheumatology in Hôpital de Hautepierre -Les Hôpitaux Universitaires de Strasbourg (France). Patients with limited cutaneous SSc, scleroderma-like conditions, localized Scleroderma or overlapping syndromes (SSc associated rheumatoid arthritis, inflammatory myopathies and/or systemic lupus erythematosus) have been excluded. Patients and controls with a recent history of smoking (in the last 3 months) or infections (in the last 4 weeks) were excluded as well. The protocol of our study was approved by our institutional review board. After a detailed explanation of study endpoints, study procedures and possible risks, informed consents were obtained from all patients and healthy volunteers according to the declaration of Helsinki.

Clinical and Laboratory data

Paper and digital records of all patients have been reviewed retrospectively at the moment of the study and only last follow-up values have been considered. Clinical data gathered for this study included, history of SSc in terms of date of first Raynaud phenomenon (RP) and date of first non-Raynaud sign of disease, actual presence or absence of RP, digital ulcers (DU), dysphagia, gastro-esophageal reflux symptoms (GERD), dyspnea (NYHA classification). Echocardiographic assessment for all patients has been performed, looking for the left ventricle ejection fraction (EF), right ventricle dilation, systolic pulmonary artery pressure (PAPs) and pulmonary arterial hypertension (PAH) that was defined as a mean pulmonary artery pressure greater than 25 mmHg, with a pulmonary capillary wedge pressure of less than 15 mmHg, and a pulmonary vascular resistance greater than three Wood units [20]. Pulmonary functional tests (PFTs), forced vital capacity (FVC), diffusion capacity to carbon monoxide (DLCO), forced expiratory volume during the first second (FEV1), were evaluated as well. Standard laboratory parameters in study included, antinuclear antibody pattern (ANA), markers of inflammation (ESR - erythrocyte sedimentation rate, CRP - C reactive protein), serum creatinine levels (Cr).

Skin Biopsy

Skin punch biopsies of 4mm diameter have been performed in this study [21]. Based on the modified rodnan skin score (mRSS) that assesses the thickness of the skin by palpation of 17 body areas on a 0 to 3 scale, sites of biopsies were chosen [22]. Chosen biopsy sites were marked by surgical marker then skin antiseptic and local anesthetic (Lidocaine 1%) were applied. In patients with dcSSc, first punch biopsy was taken from apparently normal skin (mRSS 0) and a second sample from a fibrotic skin (mRSS 2-3). According to the skin score, all samples from normal skin have been sampled from patient's arms (right or left) while samples from fibrotic skin were taken from their forearms (right or left). On the other hand, healthy volunteers had only one biopsy taken from the arm or the thigh, considering patient's will. Each skin punch extended into subcutaneous fat in order to

include all cutaneous layers. Each specimen was put in an individual tube containing Krebs HEPES Buffer (see below) placed on ice, and each tube was labeled by patient's name, date of birth, date, time of sampling and site of sampling. Finally, simple non-absorbable suture was performed for closure.

Determination of ROS formation by Electron Paramagnetic Resonance [23]

Unpaired electrons can absorb electromagnetic radiation energy and move between energy levels when stimulated by an electromagnetic field. This absorption can be recorded by electron paramagnetic resonance (EPR) spectrometry. While unpaired electrons of ROS are very short lived, and therefore difficult to detect, they can form stable adducts with 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine hydrochloride (CMH), a specific spin probe which act as a "spin trap". These adducts yield specific spectra when subjected to EPR spectroscopy.

Skin biopsies were prepared under biomicroscope optical inspection aided by using petri dishes with black bottoms. When present, hypodermal tissue was removed, samples were cut in ten pieces and washed twice with the Krebs HEPES Buffer (NaCl 99 mM; KCl 4.69 mM; CaCl₂ 2.5 mM; MgSO₄ 1.2 mM; NaHCO₃ 25 mM; KH₂PO₄ 1.03mM; D(+)-Glucose 5.6 mM; Na-hEPES 20 mM; pH 7.4) containing 25 µmol/L deferoxamine and 5 µmol/L diethyldithiocarbamate (DETC) to minimize CMH auto-oxidation. Following which, the samples were incubated in a plate at 37°C with the spin probe CMH (200µM) for 30 minutes under 20 mmHg of oxygen partial pressure (in order to be under physiological conditions) using Gas-Controller (Noxygen Sciences Transfer, Elzach, Germany). The reaction was stopped by placing the plate on ice. A volume of 40 µl of supernatant was injected in a disposable capillary tube, and placed in Bench Top EPR spectrometer E-SCAN (Bruker, Germany) at 15°C. Detection of ROS unpaired electron was conducted under the following EPR settings: centre field 3461.144g, microwave power 21.85mW, modulation amplitude 2.40g, sweep time 5.24 sec (10 scans), sweep width 60g, number of lag curve points 1. The amplitude of the signal was measured, and the concentration of

CM-radical was determined by calibration with standard concentrations of the radicals CM. After the measurement, the samples were dried for 15 minutes at 150°C, and O2-. Production was expressed in nM per minute per milligram of dry weight (nM/min/mg).

Blood redox imbalance biomarkers

Venous blood samples were drawn on EDTA or Na-heparin as anticoagulant or clot activating gel according to the investigated parameter. Blood samples were immediately centrifuged on site and plasma or sera were frozen as aliquots on ice packs coming from -80°C freezer and placed in a refrigerating box. For ascorbate (vitamin C), 0.5 ml plasma was immediately transferred to ice-cold tubes containing 0.5 ml of 10% metaphosphoric acid. The whole mixture was frozen on dry ice. Analyses were performed by a spectrophotometric method using the reduction of 2,6-dichlorophenolindophenol (Perkin Elmer Lambda 40, Norwalk, USA) [24]. Plasma vitamin E (α - and γ -tocopherols), β -carotene, and ubiquinone were assayed by HPLC procedure (Alliance, Waters, USA) coupled with a diode array detector (PDA 2996, Waters, USA) [25] using Chromsystems kits (32 000, 34 000, and 68 000). Thiol proteins were detected according to Ellman's method [26]. The GSH/GSSG ratio (GSH = reduced glutathione and GSSG = oxidized glutathione), as marker of oxidative stress, was determined in whole EDTA blood by the GSH/GSSG-412 kit (Bioxytech, Oxis International Inc., Portland, WA, USA).

The analysis of lipid peroxides (ROOH) as marker of oxidative damage to lipids was performed in plasma (EDTA blood) with the commercial kit (Oxystat, Biomedica Gruppe, Wien, Austria). Briefly, the peroxide (-OOH) concentration was determined spectrophotometrically by reaction of the biological peroxides with peroxidase and a subsequent colour reaction using 3,3',5,5'-tetramethylbenzidine as substrate. The titre in free antibodies (IgG) against oxidized lowdensity lipoprotein (Ab-Ox-LDL) was assessed with a commercial enzyme immunoassay (Biomedica Gruppe) using Cu²⁺ oxidized LDL as antigen.

Statistical analysis

Mean \pm SD, median (range) or percentages were used to report results. Analysis of variance (ANOVA) was performed using Kruskal Wallis test. Wilcoxon signed rank test was used to compare two related samples. Correlations between variables were evaluated by either Spearman test or Mann Witney test depending on variable type (nominal or continuous). Results with p values < 0.05 were considered statistically significant.

Results

Clinical and laboratory assessment

Patients group included 9 patients with dcSSc, 5 females (55%) and 4 males (45%) with mean age of 46 ± 10 years. Controls group included 7 healthy volunteers matched by age and sex.

Among patients' group, mean duration of the disease (time from first non-Raynaud sign) was 7.5 ± 5 years while mean duration of Raynaud (time from first Raynaud episode) was 9.7 ± 6.7 years. At the moment of biopsies, five patients (55%) out of 9 had active digital ulcers, 4 of them had less than 3 ulcers and one patient had 5 ulcers. Pitting scars were present in 8 patients (89%). Median mRSS of SSc patients was 18 (11-40).

Among the 9 patients, only 1 patient had severe dyspnea (NYHA class IV) and 1 patient had a moderate dyspnea (NYHA class III), the rest of patients had milder dyspnea (2 patients were in NYHA class I, 5 patients were in class II). On transthoracic echocardiogram assessment, the patient with severe dyspnea had PAH, with PAPs of 87 mmHg with moderate to severe right ventricular dilation, the remainder 8 patients had normal sPAP values with mean sPAP of 25.1 ± 3.2 mmHg. Where the patient with moderate dyspnea but without PAH had heart failure with left ventricular ejection fraction of 40%, the remainder 8 patients had normal EF% with mean EF% of 66.1 ± 6.4 %. PFTs showed abnormal reduction of DLCO% in all patients, FVC was reduced in 4 patients and FEV1 was reduced in 2 patients (80% predicted has been used as cut off for identifying

abnormal results). Means of % DLCO, VC and FEV1 were 51.1 ± 22.9 , 77.7 ± 23.2 , 75.6 ± 19.4 ; respectively. Four patients (45%) referred to have mild to moderate dysphagia, and 8 patients (89%) had GERD symptoms.

All patients had positive ANA with anti-Scl70 specificity, evaluated by indirect immunofluorescence (IIF) on HEp-2 cells. Serum creatinine levels were normal in all patients. Seven patients (77%) had increased ESR and 5 patients (55%) had increased CRP levels. Means of serum Creatinine, ESR and CRP were $60.6 \pm 12 \mu\text{mol/l}$ (n.v. 45-111), $26.3 \pm 15.5 \text{ mm/h}$ (n.v. <9, first hour), $14 \pm 12.5 \text{ mg/l}$ (n.v. <4); respectively. (table 1)

Pt.	age (yrs)	Sex	disease duration (yrs)	RP	DU (nb)	pitting scars	total mRSS	NYHA class (I-IV)	LVEF (%)	sPAP (mmHg)	RV dilation (grade)	dysphagia (grade)	GERD	DLCo (%)	FVC (%)	FEV1 (%)	Creat	ESR	CRP
1	68	M	1	yes	No	no	11	2	73	27	no	no	no	33	71	83	82	40	32
2	39	M	8	yes	No	yes	15	1	68	23	no	no	yes	45	69	81	56	34	3
3	43	F	2	yes	yes (1)	yes	15	2	68	27	no	no	yes	76	107	80	61	2	2
4	45	F	12	yes	yes (2)	yes	14	2	62	27	no	no	yes	73	98	87	64	20	2
5	49	M	3	yes	yes (5)	yes	40	3	40	18	yes (2)	yes (1)	yes	33	48	52	55	51	32
6	52	M	10	yes	yes (2)	yes	19	4	75	87	yes (3)	yes (1)	yes	11	42	40	74	25	24
7	35	F	6	yes	yes (1)	yes	20	2	60	25	no	yes (1)	yes	55	83	80	62	17	4,2
8	48	F	6	yes	No	yes	16	2	56	28	no	yes	yes	55,7	77	72	42	37	16
9	39	F	20	yes	No	yes	12	1	67	26	no	no	yes	79	105	106	50	11	11,5

Table. 1 Demographic, clinical, instrumental and laboratory findings

Abbreviations: RP, Raynaud phenomena; DU, digital ulcers; total mRSS, total modified Rodnan Skin Score; NYHA class, New York Heart Association functional classification of heart failure; LVEF, left ventricular ejection fraction; sPAP, systolic pulmonary artery pressure; RV, right ventricle; GERD, gastroesophageal reflux disease; DLCO, diffusing lung capacity for carbon monoxide; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; Creat, serum creatinine (n.v 45-1 $\mu\text{mol/l}$); ESR, erythrocyte sedimentation rate (n.v < 9 mm/h; 1st hour); CRP, C-reactive protein (n.v < 4 mg/l). Grade: mild (1), moderate (2), severe (3).

ROS in skin and clinical correlations

Skin specimen of each subject in this study was analysed for ROS expression by using electron paramagnetic resonance (EPR) spectrometry (figure 1). Results expressed as ROS levels per milligram of dry weight of specimen. In patients with dcSSc, median ROS production in fibrotic skin was 24.7 (10.9 - 47.0) nM/min/mg and median ROS production in non-fibrotic skin was 7.7 (3.5–13.6) nM/min/mg. In control group of healthy volunteers, median skin ROS production was 7.7 (3.5–13.6) nM/min/mg. (table 2) By comparing

ROS levels between fibrotic skin of scleroderma patients and skin from healthy controls, these levels were significantly higher among fibrotic skin ($p=0.002$). Similarly, non-fibrotic skin of scleroderma patients also showed significantly higher levels of ROS when compared to controls ($p=0.009$) (figure 2). Moreover, comparing samples from fibrotic skin to samples from non-fibrotic skin of each patient, ROS levels were higher in fibrotic skin ($p=0.01$) (figure 3). Correlations between ROS levels and every clinical, instrumental and laboratory variable have been assessed. However, only FVC and ESR were found to correlate with ROS levels in fibrotic skin of SSc patients ($r= -0.75$, $p=0.02$) and ($r=0.70$, $p=0.04$); respectively. (figures 4,5). No correlations were found between ROS levels in non-fibrotic skin of patients and clinical, instrumental or laboratory variables.

Table. 2 ROS levels in skin biopsies

patients	Non-fibrotic skin (mRSS 0)	Fibrotic skin (mRSS 2-3)	controls	Control skin
1	14,4	24,7	1	13,6
2	33	47	2	10,1
3	7,3	11	3	7,7
4	10,9	10,9	4	8,7
5	30,9	37,2	5	6,8
6	12,8	31,2	6	7,1
7	18,7	20,5	7	3,5
8	34	38,3	-	-
9	20,5	19,7	-	-
Median (range)	18.7 (7.3 – 34)	24 (10.9 – 47)		7.7 (3.5 – 13.6)

Data are in nM per minute per milligram of dry weight (nM/min/mg).

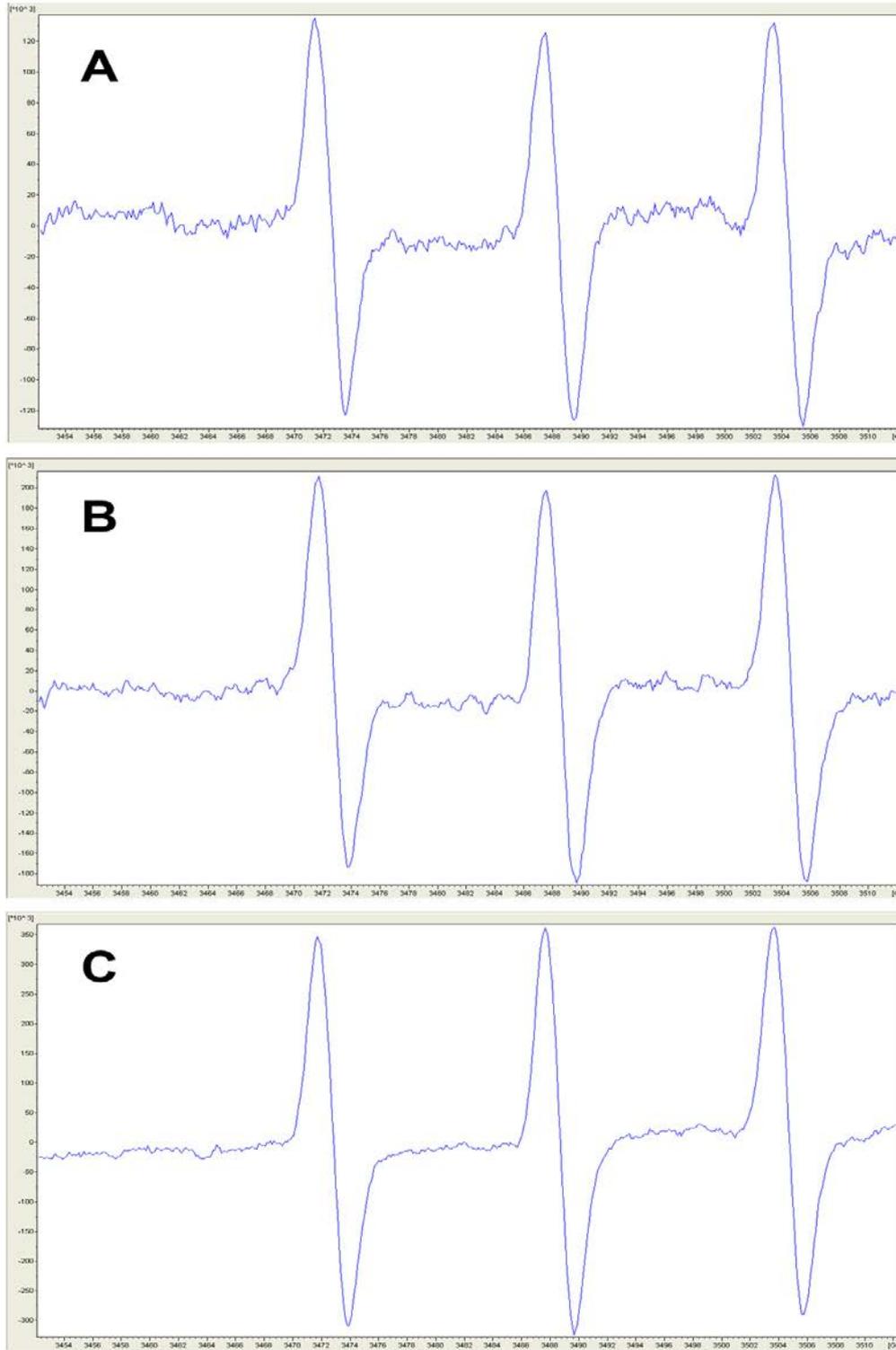


Fig.1 Representative EPR signal in control (A), SSc non fibrotic skin (B) and SSc fibrotic skin (C).

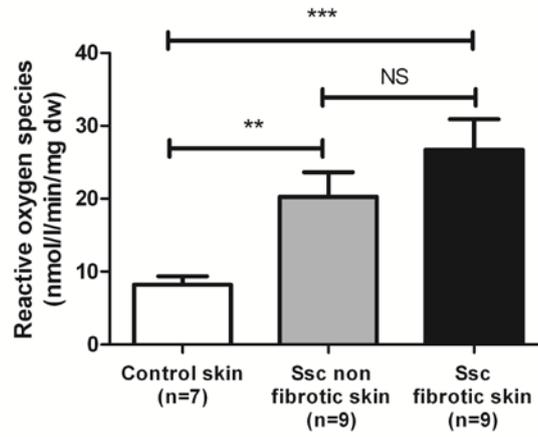


Fig.2 Reactive Oxygen Species (ROS) levels in controls skin and in patients skin (fibrotic skin and non-fibrotic skin), ** $p=0.009$, *** $p=0.002$.

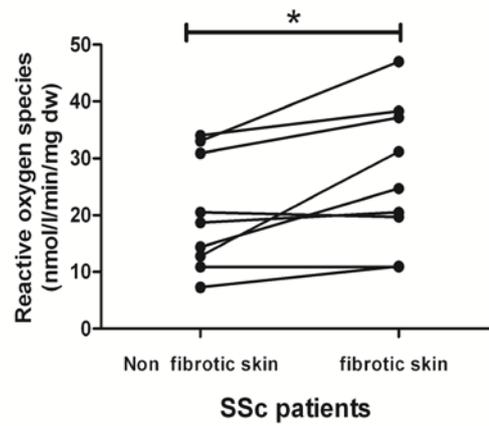


Fig.3 Comparison of ROS levels between matched samples (Wilcoxon signed rank test), * $p=0.01$.

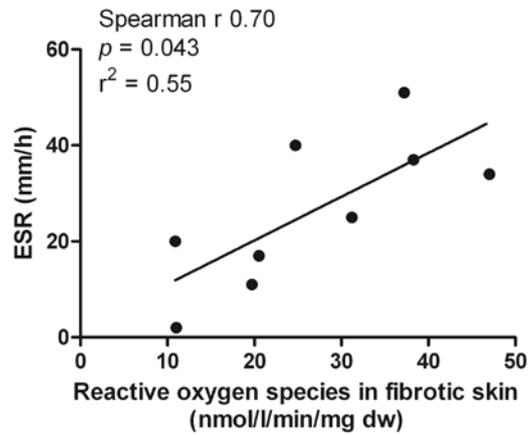


Fig.4 Correlation between Erythrocyte Sedimentation Rate (ESR) and ROS levels in fibrotic skin of patients.

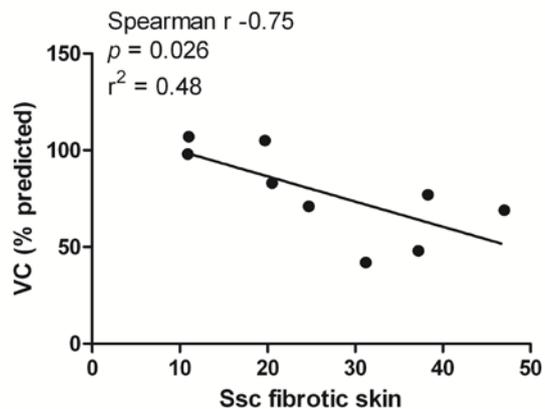


Fig.5 Correlation between forced vital capacity (FVC) and ROS levels in fibrotic skin of patients.

Blood redox imbalance biomarkers

Figure 6 shows the comparison of the levels of the different redox imbalance biomarkers assessed in this study between SSc patients and controls. Among the antioxidants, vitamin C levels were significantly lower in SSc patients blood compared to controls (8 [3.8-9.8] vs. 10.5 [9-19.1] mg/L, $p=0.004$). Lipid peroxides, markers of oxidative damage to lipids, were significantly higher among SSc patients (873.5 [342-1973] vs. 422 [105-576] $\mu\text{mol/L}$,

p=0.004). Although lacking a strong significance, GSH/GSSG ratio was lower in SSc patients (182 [0.9-466] vs. 430 [0.9-506], p=0.17).

Moreover, the correlation between ROS levels in the skin and blood redox imbalance biomarkers has been studied (figures 7, 8). Vitamin C levels in the blood showed a significant negative correlation with ROS levels, whether considering fibrotic (r= -0.74, p=0.002) or non fibrotic (r= -0.82, p=0.0003) skin of SSc patients. Another significant correlation was found between lipid peroxides and ROS levels, whether considering fibrotic (r= 0.76, p=0.001) or non fibrotic (r= 0.77, p=0.001) skin of SSc patients.

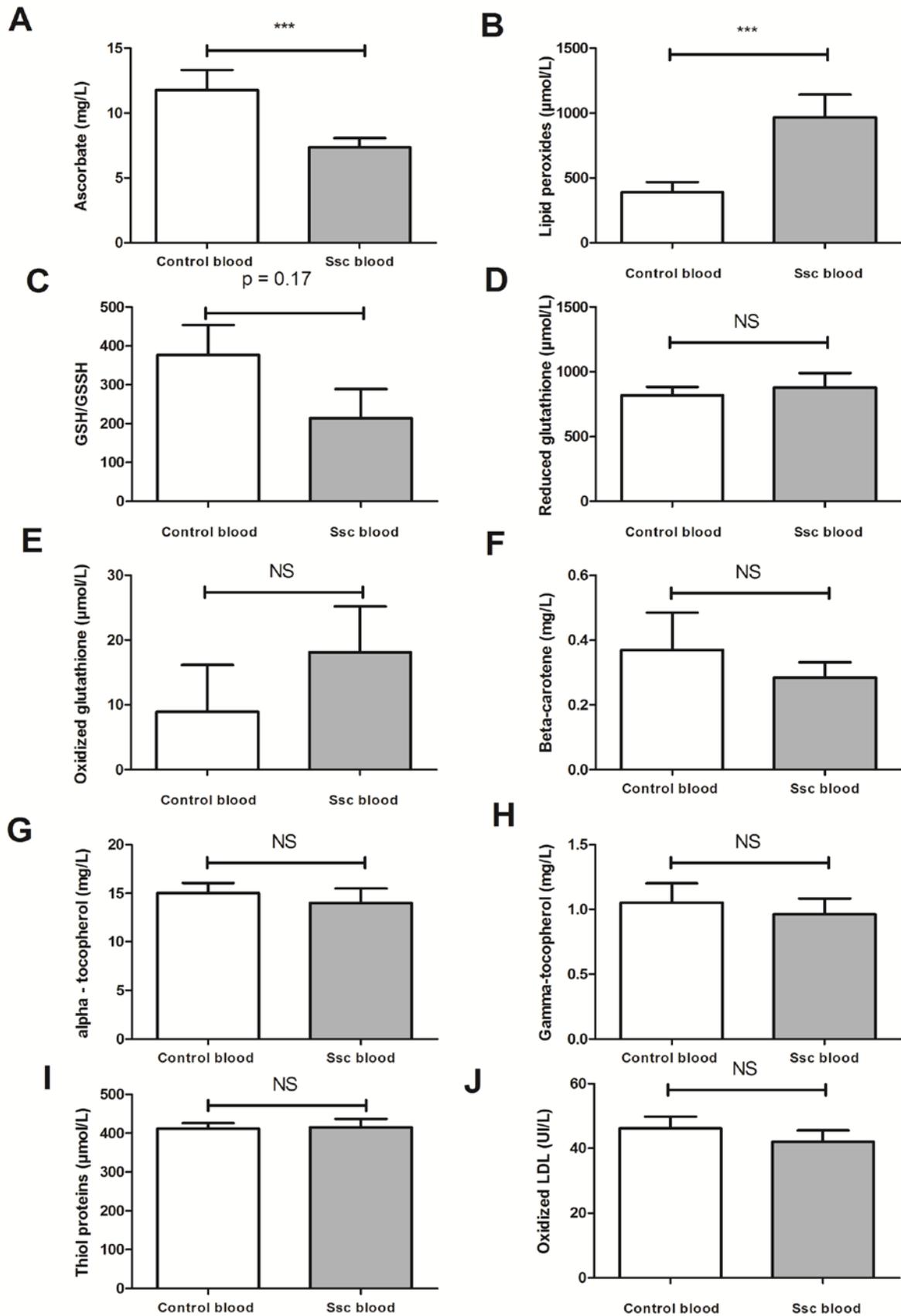


Fig. 6 Comparison of redox imbalance biomarkers between SSc patients and controls. NS non significant; *** $p < 0.05$

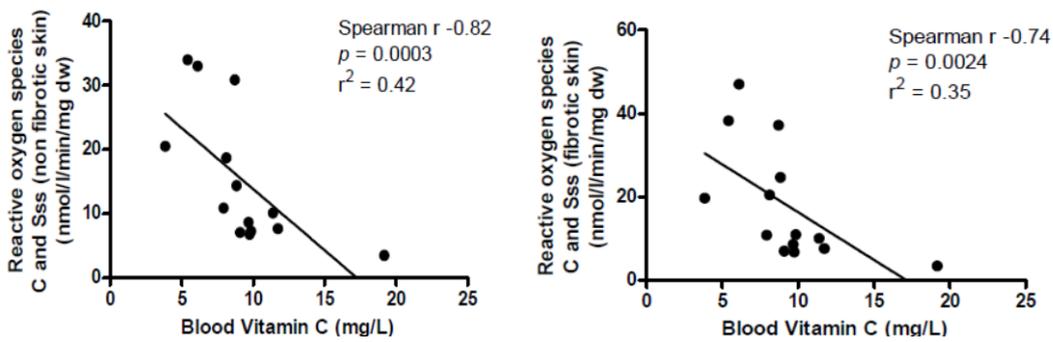


Fig. 7 Correlation between vitamin C levels and ROS levels in non fibrotic skin (left) and fibrotic skin (right) of patients with SSc and controls.

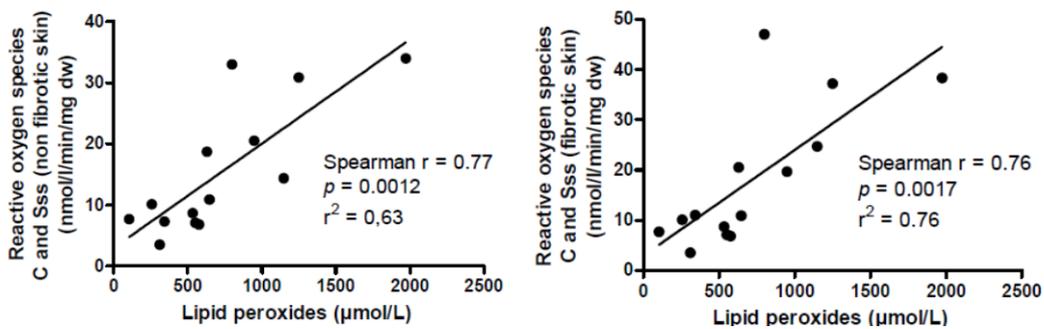


Fig. 8 Correlation between lipid peroxides levels and ROS levels in non fibrotic skin (left) and fibrotic skin (right) of patients with SSc and controls.

Discussion

In this study we show the presence of high oxidative stress in the skin of patients with dcSSc. We also show that both fibrotic skin “apparently” normal skin of patients with SSc have had higher ROS levels when compared to normal skin of healthy volunteers.

Our results are in agreement with previous studies that have also suggested high ROS production in patients with SSc using indirect biomarkers. In two separate studies, Ogawa et al. highlighted on the presence of two oxidative stress biomarkers in SSc. 8-isoprostane (an eicosanoid family member of non-enzymatic origin, produced by random oxidation of

tissue phospholipids) was found to be significantly elevated in sera of patients with SSc, either lcSSc or dcSSc, when compared to normal controls. No differences were found in 8-isoprostane levels between lcSSc and dcSSc patients. [27] Hsp70, a member of the heat shock proteins family, is another sensitive biomarker for oxidative stress, cellular stress and tissue injury. This protein was found to be significantly elevated in serum of patients with SSc compared to healthy controls with no differences between lcSSc and dcSSc patients. [28] The same group have also shown that as a result of oxidative stress, in SSc patients with either limited or diffuse form SSc, had elevated total antioxidant power (TAP) when compared with healthy controls, with no significant difference between limited or diffuse forms [15]. A Mexican study [12] on 28 patients with dcSSc also found elevated levels of oxidative stress biomarkers, malondialdehyde, dityrosine and carbonyls in serum of dcSSc patients as compared to healthy controls, with a significant positive correlation with uric acid levels in patients with dcSSc. Moreover, the antioxidant capacity of plasma (ACP) was lower in dcSSc patients compared to healthy controls.

ROS levels in fibrotic skin of our patients were found to be inversely correlated with FVC and positively correlated with ESR in serum, two factors that have been independently linked to poor outcome [29, 30]. The negative correlation ROS/FVC has been previously shown in Japanese patients [27]. Although correlation does not mean causality, these findings corroborated the proposed role of ROS in the development of ILD [31]. Several studies have provided evidence of elevated oxidative stress and its biomarkers in patients with PAH [32, 33]. On the other hand, ESR together with CRP were shown to be elevated in patients with dcSSc with elevated oxidative stress [12], CRP levels were in correlation with TAP serum levels in patients with SSc [15].

Our analysis of blood redox imbalance biomarkers showed a significant decrease in vitamin C levels and a significant increase in peroxide lipids in patients with SSc when compared to healthy controls. These findings are in agreement with earlier studies [8, 16,

34]. We also showed the presence of a significant correlation between vitamin C and peroxide lipids in the serum and ROS levels in the skin regardless of the fibrotic feature of the biopsied skin (fibrotic, non fibrotic or control skin). Hence, this latter finding support the accuracy of the EPR in determining ROS production in skin biopsies.

This is a proof-of-concept study. To our knowledge, it is the first study to directly analyzed ROS production in complete skin samples from patients with dcSSc using EPR protocol that preserved essential interactions of the cells with their microenvironment. The hallmark of our study is the assessment of different skin phenotypes in the same patient by studying fibrotic and non-fibrotic skin zones of patients with dcSSc. However, only two studies have shown an important activation relationship between ROS and fibroblasts isolated from skin of patients with scleroderma, Sambo et al. [36] have shown an increase in-vitro release of ROS from quiescent scleroderma fibroblasts (in absence of any stimulation). Dermal fibroblasts obtained from the forearms of patients with dcSSc were studied by Tsou et al. [13], which showed significant higher levels of superoxide produced by patients fibroblasts as compared to normal fibroblasts, and a decrease in superoxide production in SSc-fibroblasts in presence of NAC (thiol antioxidant) with a higher thiol content in normal fibroblasts as compared to SSc-fibroblasts.

Limitations of the study

The sample size of study groups might be considered small in terms of reliability and validity of our results, however difficulties have been faced to enroll patients with dcSSc, as patients were expressing their awareness for prolonged healing time in biopsy site, their wide experience with digital ulcers and the difficulties in healing they face explain this awareness.

Another limitation of this study was the subjectivity of choosing site of biopsy and its related skin score. For this reason we tried to standardize site of biopsy in patients with SSc by sampling the affected skin from forearms and the normal skin from arms patients,

and the skin score given to those areas was done by the same physician in all patients and controls.

Conclusions

In conclusion, our study confirms the presence of high ROS levels in the skin of patients with dcSSc, either in fibrotic or in “apparently” normal skin. Thus, ROS is suggested to play an important role in the pathogenesis of fibrosis in scleroderma, a role that could be initial or intermediate in terms of disease time line progression considering our finding of high ROS levels in “apparently” normal skin of SSc patients. Higher ROS levels in fibrotic skin compared to non-fibrotic skin, suggests a possible role of other co-factors to play with ROS in fibrosis pathogenesis. However, in order to certify these statements it is necessary to assess larger cohort of patients in a longitudinal prospective design, and to measure skin oxidative stress in these patients at different moments during their follow up considering the clinical progression and skin fibrosis extension in relation to skin biopsy sampling sites, together with further molecular intra- and extracellular investigations.

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