

## Iloprost therapy acutely decreases oxidative stress in patients affected by systemic sclerosis

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## ABSTRACT

**Background.** Oxidative stress has been considered a leading factor in the pathogenesis of systemic sclerosis (SSc). Consistently with this hypothesis the determination of urinary isoprostanes, a reliable method for evaluation of oxidative stress, has recently showed increased levels of isoprostanes in SSc patients. Data about the effect on oxidative stress of accepted therapies for SSc such as iloprost therapy are lacking.

**Objective.** The aim of this prospective study was to verify whether iloprost therapy in patients with SSc acutely reduces oxidative stress assessed by determination of 8-Iso PGF<sub>2α</sub> urinary levels. **Methods:** urine samples were obtained before and after a five-day cycle of iloprost infusion and urinary 8-Iso PGF<sub>2α</sub> levels were determined using a commercially available enzyme immunoassay.

**Results.** Consistent with previous reports, we found an increased level of oxidative stress in SSc patients with respect to healthy controls. Basal urinary 8-iso PGF<sub>2α</sub> levels in SSc patients were significantly higher than those in healthy controls [2002(1122-3575) pg/mg creatinine vs. 334(225.7-441) pg/mg creatinine,  $p<0.001$ ]. Moreover, as expected, urinary 8-iso PGF<sub>2α</sub> levels after iloprost therapy were significantly lower than basal levels [1277.5 pg/mg creatinine (742.7-2017.3) vs. 2002 pg/mg creatinine (1122-3575),  $p=0.001$ ] but persisted significantly elevated respect to the levels of healthy controls ( $p<0.001$ ). The effect of iloprost on oxidative stress appeared significant in patients with early and limited form of disease.

**Conclusions.** This prospective open-label explorative study suggests that standard course of iloprost therapy may acutely reduce oxidative stress in SSc patients. This effect appears to be more consistent in the early phases and in the limited subset of disease. Further larger trials are needed to confirm our results and to explain the pathway of such reduction, its clinical significance and potential therapeutic implications.

## Introduction

Systemic sclerosis (SSc) is an autoimmune mediated disorder of connective

tissue characterized by fibrosis of skin and internal organs and dysregulation of microvasculature function. Collagen overproduction by activated stromal fibroblasts, autoantibodies production and acral vasospasm known as Raynaud's phenomenon (RP) are the hallmarks of disease.

Although the pathogenesis of SSc remains largely speculative oxidative stress has been reported to be involved in fibroblast activation (1) autoantibodies generation (2) and RP (3). Isoprostanes, a family of eicosanoids produced predominantly by free radical oxidation of arachidonic acid esterified to phospholipids, are considered the best available markers of oxidative stress *in vivo* (4). Measurement of F<sub>2</sub>-isoprostanes in the early events in several chronic diseases, such as asthma and hepatic cirrhosis, has also suggested a role of those compounds as mediators of disease.

Similarly an increased level of urinary F<sub>2</sub>-isoprostanes, has been demonstrated in patients affected by SSc (5), mainly in the early steps of disease (6), supporting the hypothesis that oxidative stress may play a pathogenic role.

Iloprost, a synthetic analogue of prostacycline (PgI<sub>2</sub>), is widely used for the treatment of vascular features of SSc such as RP due to its well known vasodilator and antiaggregant effect. Moreover besides their vascular effects, iloprost is thought to act as a disease-modifying agent (7) regulating immune responses, influencing fibrous tissue formation and promoting neoangiogenesis (8). Insufficient data are available in literature on the effect of iloprost infusion on oxidative stress in SSc patients.

Therefore, the aim of the study was to verify whether in SSc patients iloprost therapy reduces oxidative stress, assessed by determination of urinary isoprostane 8-iso PGF<sub>2α</sub>.

## Methods

### Patients

Twenty-eight consecutive adult SSc patients, all fulfilling the American College of Rheumatology criteria for the classification of SSc, referred to the Istituto di Patologia Medica e Metodologia Clinica (Sassari-Italy) from

Competing interests: none declared.

January 2004 to January 2005 were included in this study. The disease duration was measured from the onset of the first symptom. The patients were classified into two clinical SSc subsets according to LeRoy (9) classification (*i.e.*, limited cutaneous systemic sclerosis, lSSc, and diffuse cutaneous systemic sclerosis, dSSc).

The clinical data were collected by patient's records/interview, physical examination and instrumental investigations according to the following procedures and methods: RP was diagnosed on the basis of clinical examination and/or evocative clinical history; pulmonary fibrosis was defined by chest radiographs and/or high resolution computed tomography; pulmonary arterial hypertension was detected by color doppler echocardiography; reduced forced vital capacity (FVC) was defined as reduction of FVC >75% respect to normal values; oesophagus involvement was defined on the basis of distal oesophageal dysmotility demonstrated by manometry; occurrence of arrhythmias, blocks, congestive heart failure or pericarditis were considered signs of sclerodermic heart involvement; kidney involvement was reported when occurred marked proteinuria, severe hypertension and/or rapidly progressive renal failure.

Patients were considered eligible if a RP or pulmonary arterial hypertension (PAH) susceptible of iloprost therapy occurred. Also nifedipine, as calcium channel blocker, was used to treat RP. Exclusion criteria were smoking habits, diabetes mellitus, hypercholesterolemia and the presence of congestive heart failure, coronary artery disease, cerebrovascular disease, kidney or liver failure.

Previous medications were maintained during the course of the study; no other drugs were started during the study period.

Eligible patients admitted to our inward and outward clinic underwent a monthly intravenous administration by an infusion pump of the prostacyclin analogue iloprost, at the greatest tolerated dose (0.5–2 ng/kg/min, mean dose 1.3 ng/kg/min), for 6 hours/day of 5 consecutive days.

Urine samples were taken at baseline and 2 hours after completing the cycle of iloprost infusion.

#### Controls

Healthy controls, matched for age ( $\pm 5$  years), sex and absence of clinical conditions known to increase oxidative stress, were selected from author's colleagues, friends and relatives to define of basal 8-iso PGF<sub>2 $\alpha$</sub>  urinary levels. Informed consent was obtained from the patients and the controls.

The study was approved by the local ethics committee.

#### Quantification of F2-isoprostanes

The samples were immediately refrigerated after collection and then aliquoted and stored at  $-80^{\circ}\text{C}$  until assay.

The 8-isoPGF<sub>2 $\alpha$</sub>  urinary levels were measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). A purification step was performed by a 8-isoprostane affinity sorbent/column. The assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The assay employs an Ellman's reaction producing an absorbance at 412 nm proportional to the amount of 8-isoprostane tracer bound to the well and inversely proportional to free 8-isoprostane.

Urinary creatinine levels were determined by the alkaline picrate (Jaffe) reaction (Sentinel CH, Milan, Italy), and urinary 8-isoPGF<sub>2 $\alpha$</sub>  levels were expressed as pg/mg creatinine.

#### Statistical analysis

Normality of distribution of the data tested by skewness and kurtosis showed that urinary isoprostanes levels follow a log normal distribution. Analysis was then performed with the Mann Whitney U-test for independent samples and the Wilcoxon signed rank test for related samples. A two-sided *p*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS (release 11.0, Chicago, IL).

#### Results

##### Patients

In this prospective open label explorative trial we enrolled 28 SSc patients (22 women and 6 men) with a mean age of 54.71 years (range 29–72 years) and mean disease duration of 8.82 years (range 1–24 years). Fifteen (53.6%) patients were classified as having dSSc, 13 (46.4%) as having lSSc. Twelve out of forty SSc patients screened had to be excluded according with exclusion criteria (6 patients for smoking habit, 2 patients for hypercholesterolemia, 2 patients for congestive heart failure, 1 patient for diabetes and 1 patient for liver disease). Patients and controls enrolled in the study have normal values of cholesterol, glycaemia and normal hepatic and renal tests. Other detailed demographic and clinical data are provided in Table I.

##### F2-isoprostanes

Basal levels of urinary 8-isoPGF<sub>2 $\alpha$</sub>  were significantly elevated by 3-fold in SSc patients compared with healthy controls: median value 2002 pg/mg creatinine

**Table I.** Demographic and clinical variables.

Characteristic	HC (n=28)	SSc (n = 28)	dSSc (n=15)	lSSc (n=13)
Sex (F/M)	22/6	22/6	11/4	11/2
Mean age (range), years	55 (28–70)	54.71 (29–72)	55.2 (29–71)	54.15 (38–72)
Mean disease duration (range), years	-	8.82 (1–24)	7.13 (1–17)	10.77 (2–24)
Skin involvement	-	28 (100%)	15 (100%)	13 (100%)
Pulmonary fibrosis	-	9 (22%)	8 (28.6%)	1 (3.8%)
PAH	-	4 (9.8%)	3 (10.7%)	1 (3.8%)
Raynaud's phenomenon	-	28 (100%)	15 (100%)	13 (100%)
Oesophagopathy	-	18 (43.9%)	10 (35.7%)	8 (30.8%)

PAH: pulmonary arterial hypertension

**Table II.** Urinary 8-isoprostane  $F_{2\alpha}$  levels pre and after iloprost infusion.

Group	n	8-isoprostane $F_{2\alpha}$ Median (interquartile range)		p
		Pre-iloprost	Post-iloprost	
SSc	28	2002 (1122-3575)	1277.5 (742.7-2017.3)	<b>0.001</b>
Healthy controls	28	334 (225.7-441)	-	-
dSSc	15	1710 (1240-3193)	1598 (1115-2081)	<b>0.048</b>
ISSc	13	2147 (1136-3800)	1111 (700-1722)	<b>0.003</b>
Dis dur 0-5 years	13	2520 (1710-3800)	1450 (1111-2125)	<b>0.006</b>
Dis dur. >5 yrs	15	1454 (1006-2696.5)	1262.5 (725-1800)	0.055

(1122-3575) vs. 334 pg/mg creatinine (225-441),  $p < 0.001$  (Table II).

After iloprost therapy 8-isoPGF $_{2\alpha}$  urinary concentration in SSc patients resulted significantly reduced respect to basal levels [1277.5 pg/mg creatinine (742.7-2017.3) vs. 2002 pg/mg creatinine (1122-3575),  $p < 0.01$ ] but persisted significantly elevated with respect to control levels [334 pg/mg creatinine (225.7-441),  $p < 0.001$ ].

A post-hoc analysis of the effect of iloprost on urinary 8-isoPGF $_{2\alpha}$  after stratification of whole group for cutaneous subset and disease duration was performed. Reduction of urinary isoprostanes was significant in the limited cutaneous subset ( $p < 0.01$ ) and in the subgroup of patients with early disease ( $p < 0.01$ ). On the contrary just a slight ( $p = 0.048$ ) reduction of oxidative stress was demonstrated in SSc patients affected by the diffuse cutaneous subset.

## Discussion

The pathogenesis of SSc is unknown, despite intensive studies have been carried out. Beginning from Murrel's hypothesis (10), growing evidences have been accumulated about interrelation between oxidative stress and pathogenic mechanisms of disease. Oxidative stress has been reported to be implicated in the pathogenesis of vascular injury (11), autoantibody production (2) and fibroblast activation and proliferation (1, 12). The main source of oxidative stress in SSc patients is considered to be the recurrent cycles of hypoxia/ischemia and reperfusion that lead to formation of oxygen species highly reactive toward the cellular membrane. On the other hand, a concurrent reduction of inner antioxidant potential has been demonstrated in SSc.

On the grounds of such observations it

is likely that an antioxidant treatment might be useful in SSc. Supplementation with several antioxidants (*e.g.*, resveratrol, melatonin, vitamin E, iron chelating agents) has been proposed, but to date any firm conclusion about their clinical effect is lacking. Similarly it still remains to be elucidated the effect of vasodilating treatment on the oxidative stress in SSc. On a clinical point of view indeed the reduction of the frequency of RP may decrease oxidative stress reducing radical production and lipid peroxidation.

To test this hypothesis we evaluated urinary levels of 8-iso PGF $_{2\alpha}$  before and after an infusional cycle of iloprost. The 8-iso PGF $_{2\alpha}$  is a biochemically stable member of the family of  $F_2$ -isoprostanes, free radical catalyzed products of lipid peroxidation that are considered to be the best reliable markers of oxidative stress *in vivo*.

Several studies (6, 13) have already shown that 8-iso PGF $_{2\alpha}$  concentrations were increased (by 2 to 5-fold) in biological samples from SSc patients respect to general population. Consistently with these observations also in our study urinary basal 8-iso PGF $_{2\alpha}$  levels were markedly elevated in SSc patients compared with healthy controls.

Moreover, as expected, a cycle of five days of iloprost infusion at standard dosage leads to a significant reduction of 8-iso PGF $_{2\alpha}$  urinary levels in our SSc patients.

Recently, a study with a similar design has been published by Volpe and co-workers (14) but unlike us the authors failed to demonstrate a reduction of 8-iso PGF $_{2\alpha}$  urinary levels after iloprost infusion. The study is well conducted but methodological differences in terms of study design could explain such different results. Firstly, the study

of Volpe refers to a smaller sample size of 10 SSc patients. Secondly, differences in the timing of sampling and number of iloprost infusions could be affect study results: we obtained urinary samples immediately after completing a five day infusional cycle while Volpe and co-workers evaluated urinary levels of 8-iso PGF $_{2\alpha}$  three days after a single iloprost infusion. Lastly, is quite surprising that median value of urinary levels of 8-iso PGF $_{2\alpha}$  of SSc patients (400 pg/mg creatinine) reported by Volpe is lower than that reported for general population (1600±600 pg/mg creatinine) (15).

Our results support the role of iloprost as an "antioxidant agent" acting more significantly in the early phase of disease when the microvascular endothelial injury and lipid peroxidation (6) is at maximal levels. Nevertheless is not clear how iloprost might exert its antioxidant activity.

It can be speculated that this antioxidant effect may be related to improvement in vascular function. It could be supposed that correlation analysis between urinary 8-isoPGF $_{2\alpha}$  reduction and clinical change in RP and digital ulcers might help to elucidate this point. Unfortunately an amount of time longer than the five days of the iloprost infusional cycle is likely needed to detect clinically meaningful change in Raynaud's phenomenon severity or digital ulcers healing. Given that, correlation analysis between acute change in urinary isoprostane levels (thought to be iloprost-related) and medium/long-term change in clinical variables (which could be affected by confounding factors occurring during a longer follow-up period) raises some doubts and was not provided by the study protocol.

Furthermore, it should be remarked that some authors failed to demonstrate that in SSc lipid peroxidation is unique and direct consequence of vascular hypoxia (16) showing that  $F_2$ -isoprostanes production is not increased in patients with a primary RP.

Thus, it is conceivable that, in SSc patients, a reduction of 8-iso PGF $_{2\alpha}$  can be related to several mechanism beside just an improvement of vascular hypoxia. The paper published by Babir-Gurman

*et al.* (17) suggests that iloprost infusion reduces lipid peroxidation (assessed by malondialdehyde) in SSc improving the inner antioxidant potential (reflected by levels of native antioxidants such as superoxide dismutase and catalase).

Similarly, in an animal model, the effect of iloprost on protection of brain tissue from oxidative stress during cerebral hypoperfusion has been shown to be related to an improvement of antioxidant system (18).

## Conclusions

Our study further supports the role of oxidative stress in the pathogenesis of SSc and demonstrates for the first time that iloprost, administered at the standard dosage, acutely reduces the oxidative stress "burden" in SSc patients. Quantification of oxidant stress assaying urinary levels of 8-isoPGF<sub>2α</sub> may represent a simple, non-invasive tool to evaluate disease activity and treatment response. Larger prospective studies are needed to evaluate the following points: a) confirm our results on the acute effect of iloprost on oxidative stress; b) evaluate a potential medium/long-term effect of iloprost on oxidative stress; c) clarify the pathways of anti-oxidant effect of iloprost; d) evaluate and quantify the clinical benefit of oxidative stress reduction.

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