Microvascular involvement in systemic sclerosis: laser Doppler evaluation of reactivity to acetylcholine and sodium nitroprusside by iontophoresis

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Abstract

Objectives—To investigate the skin vasodilatory response to iontophoretically applied acetylcholine (Ach), an endothelium dependent vasodilator, and to sodium nitroprusside (SNP), an endothelium independent vasodilator, in patients with systemic sclerosis (SSc).

Methods—Eleven SSc patients were preliminarily studied (10 females, mean age 40.5; mean disease duration 6.5 years), and 16 age and sex matched control subjects. By means of laser Doppler flowmetry skin blood flow was evaluated at third finger, at baseline, and after post-ischaemic hyperaemia test and during iontophoretically transcutaneous application of 1% solution of Ach and SNP.

Results—No significant differences in basal skin blood flow were detected between SSc patients and controls. Cutaneous vasodilatory response to ischaemia, Ach, and SNP was significantly less pronounced in SSc patients compared with controls ($p < 0.001$). Moreover, among SSc patients a lower ($p < 0.05$) vasodilatory response to Ach compared with ischaemia and SNP was recorded.

Conclusions—These data confirm a reduction of skin digital vasodilatory reserve in SSc patients and suggest a defect of both endothelial dependent arteriolar relaxation and wall compliance in the pathogenesis of this dysfunction.

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Systemic sclerosis (SSc) is a connective tissue disease characterised by diffuse microvasculature changes and progressive fibrosis of the skin and visceral organs, chiefly, lung, heart, gastrointestinal tract, and kidney.¹ The aetiopathogenesis of SSc is still unknown. However, various pathogenetic hypotheses have been suggested; they include a collagen overproduction by fibroblasts and/or endothelial abnormalities, and/or immunological changes.¹⁻³ The microcirculatory abnormalities in SSc patients could be caused by the endothelial dysfunction/damage as suggested by the following clinicoserological manifestations: Raynaud's phenomenon, often preceding the SSc onset, in over 90% of patients, ischaemic skin lesions, nailfold capillaroscopic abnormalities, increased plasma endothelin-1 and tissue plas-

minogen activator, low concentrations of nitric oxide (NO).⁴⁻¹⁰ In addition, an impairment of arteriolar wall compliance has been demonstrated by a reduced post-ischaemic hyperaemia in the digital skin at laser Doppler flowmetry in SSc patients.¹¹ However, the pathogenetic relevance of microvascular abnormalities in the SSc still remains to be defined.

The recent use of iontophoresis, a non-invasive method able to deliver in the skin various deionised drugs, including some vasodilators, has permitted a more accurate assessment by laser Doppler flowmetry of the microvascular endothelial cell function in normal and pathological conditions.¹²⁻¹⁶

To verify the role of both endothelial cell dysfunction and reduced microvascular compliance in the scleroderma microangiopathy, we preliminarily investigated in SSc patients the response of skin microvasculature to the iontophoretic application of acetylcholine (Ach), an endothelium dependent vasodilator, and sodium nitroprusside (SNP), an endothelium independent vasodilator, compared with the vasodilatory effect resulting from ischaemia.

Methods

PATIENTS

Eleven consecutive SSc outpatients (10 females; mean (SD) age 40.5 (10.4), range 21-57; disease duration 6.5 (5.8) years, range 1-18) followed up at the Rheumatology Unit of the University of Pisa, and 16 healthy control subjects, age and sex matched, were enrolled for the study. Patients with SSc were classified according to the American Rheumatism Association Subcommittee for Scleroderma Criteria.¹⁷ In addition, patients were divided into three cutaneous subsets on the basis of the extent of skin sclerosis, namely limited, intermediate, and diffuse SSc.^{5, 18} Exclusion criteria were: age ≥ 60 years, smokers, presence of heart and renal failure, severe digital skin ulcers, arterial hypertension, diabetes mellitus, and any other disease possibly responsible for microvasculopathy; moreover, people taking vasodilator or anti-inflammatory treatment were studied after a two week washout period. Patients were asked not to consume caffeine containing drinks during the 24 hours preceding the study.

All subjects gave their informed consent before entering in the trial, which was approved by our Institutional Ethical Committee.

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Figure 1 Apparatus of the laser Doppler iontophoresis.

At the entry, a careful clinicoserological assessment was carried out in all patients, as previously described.^{5,6} Nailfold capillary examination was performed using a Leitz microscope and interpreted according to the criteria of Maricq *et al.*¹⁹

MICROCIRCULATORY ASSESSMENT

The skin blood flow evaluation was carried out on the patient in seated position after 30 minutes of acclimatisation at a temperature of $22 \pm 1^\circ\text{C}$. Digital blood flow was measured by a laser Doppler flowmeter (Periflux PF4001, standard probe PF408, Perimed, Sweden). The laser Doppler apparatus (fig 1) was connected via RS232 interface with a PC Notebook (Zenith Data Systems, Taiwan), which analysed on line the information on the skin blood flow by a Perisoft program.

POST-ISCHAEMIC HYPERAEMIA TEST

The laser probe was placed on the dorsal surface of the right third finger by a double sided adhesive disc. After the recording of digital basal blood flow for five minutes, a micro-cuff was applied around the finger and inflated up to 250 mm Hg for a three minute period; then, it was instantaneously deflated and digital blood flow was again recorded until the flow was comparable with the baseline one. The value of basal digital blood flow was expressed as conventional perfusion units (PU), while post-ischaemic blood flow as "area under curve" (PU in function of the time) recorded during the last two minutes preceding and at the end of ischaemia, respectively. The ratio of these values expressed in percentages indicated the vasodilatory response to ischaemia.

IONTOPHORETIC STUDY

After a five hour interval from the post-ischaemic hyperaemia test, patients underwent iontophoresis procedure (Periont, Perimed, Sweden) to assess their vasodilatory response to Ach (acetylcholine HCl, Farmigee, Pisa, Italy) and SNP (sodium nitroprusside, Malesci, Milan, Italy) application. Before the ion-

Table 1 Clinicoserological features of 11 systemic sclerosis (SSc) patients

	%
Female-male	10-1
Age, mean (SD) (range) (y)	40.5 (10.4) (21-57)
SSc duration, mean (SD) (range) (y)	6.5 (5.8) (1-18)
SSc subsets	
Limited	36
Intermediate	18
Diffuse	46
Raynaud's phenomenon	100
Telangiectasias	100
Skin digital pitting	72
Cutaneous ulcers	18
Calcinosis	18
Oesophageal dysmotility	72
Lung fibrosis	91
Pulmonary hypertension	27
Heart involvement	55
Autoantibodies*	
Anti-nuclear	82
Anti-ENA	46
Anti-centromere	9
Anti-Scl70	72

*Anti-ENA: anti-extractable nuclear antigen; anti-Scl70: anti-topoisomerase I antibody.

tophoretic application, the above drugs were freshly diluted at the concentration of 1% in sterile deionised water. By means of a silver chloride electrode, supporting a sponge (12 mm in diameter) of an absolutely ion free material, with a high absorption and without any effects on the drug, 100 microlitre of Ach and SNP were used for the test. Both drugs were randomly introduced, with an interval of two hours, into the cutaneous tissue using an anodal (for Ach) and cathodal (for SNP) charge ($0.30 \text{ m}^2\text{A}$) for 20, 30, and 40 seconds, at constant time intervals of 180 seconds. Through a pocket sited in the middle of drug electrode a small laser diode lighted a limited area of digital skin. The "drug electrode" was fixed to a thermostated probe allowing a constant temperature of 36°C .

Both basal and maximum blood flow during the Ach and SNP application were respectively expressed as the value of the "area under curve", measured two minutes before the iontophoresis and during the first two minutes subsequent to each application. The per cent of increment of such values defined the maximum vasodilatory response to each drug.

The intra-subject reproducibility of this protocol has been evaluated in six healthy volunteers. The coefficient of variation of the maximum vasodilatory response to Ach and SNP was 49 ± 21 and $27 \pm 14\%$, respectively. Day to day reproducibility to the same substances, determined in four subjects on three occasions, was 36 ± 17 and $21 \pm 13\%$, respectively.

DATA ANALYSIS

The basal skin blood flow, the maximum vasodilatory response to ischaemia, Ach, and SNP were compared between SSc patients and control subjects by Student's *t* test. In each group the variability in response to Ach and SNP stimulation was assessed using two way analysis of variance for repeated measures (ANOVA). In both tested groups, the maximum vasodilatory response to Ach and SNP application were compared by using Student's *t* test for two tails.

Table 2 Per cent increment of maximum vasodilatory response (area under curve) to ischaemia, acetylcholine (Ach), and sodium nitroprusside (SNP) in SSc patients and control subjects

	Ischaemia	p Value	Ach	p Value	SNP
SSc patients (11)	119.9 (73.1)	0.05	49.2 (41.8)	0.05	112 (82.3)
p Value	0.001		0.001		0.001
Controls (16)	461.6 (168.3)	NS	454.2 (361.4)	NS	528 (172.2)

Data shown as mean (SD).

The results are expressed as mean (SD); p values < 0.05 were considered significant.

Results

Table 1 summarises the main epidemiological and clinicoserological findings of 11 SSc patients. In all subjects Raynaud's phenomenon was present at the time of the diagnosis of SSc; in seven cases this manifestation preceded, from 1 to 15 years, the clinical onset of the disease. Other microvascular complications, including teleangiectasias (100%), fingertip pitting lesions (72%), and cutaneous ulcers on the lower limbs (18%), were also recorded. Among visceral organ involvement interstitial lung fibrosis was demonstrated in most patients. In all cases nailfold capillary microscopy examination showed a typical scleroderma pattern—that is, reduced capillary number and severely enlarged loops (table 1).

At baseline, skin blood flow did not show any significant difference between SSc patients (16.3 (6.7) PU) and controls (21.8 (12.3) PU); however, one patient with cutaneous ulcers on the legs showed a basal blood flow considerably lower than the mean value.

After the post-ischaemic hyperaemia test the per cent increment of vasodilatory response was significantly lower in SSc patients than in controls (119 (73.1%) compared with 461.6 (168.3%); $p < 0.001$) (table 2).

The maximum vasodilatory effect to Ach and SNP stimulation was recorded at the third application of the drug in both SSc patients and controls. However, a significant dose related increment in vasodilatory response to the drugs was detected only in controls ($p < 0.001$, ANOVA test). In SSc patients a significant change in maximum vasodilatory effect was recorded when compared with controls ($p < 0.001$; table 2). With regard to the maximum vasodilatory response to the different stimuli (ischaemia, Ach, and SNP), SSc patients showed a lower response to Ach ($p < 0.05$, table 2) than SNP and ischaemia. In contrast, no significant variations were seen in control subjects.

In SSc patients no correlations were observed between the microvascular involvement and different cutaneous subsets or capillaroscopic abnormalities.

Discussion

This study represents the first attempt to use the iontophoretic technique together with laser Doppler flowmetry to evaluate the digital microcirculatory involvement in SSc patients. Although the pathogenesis of scleroderma microangiopathy is still uncertain, abnormally high values of endothelin-1 and reduced plasma concentrations of NO in SSc patients

suggest a dysfunction or damage to the endothelium, or both.⁵⁻¹⁰ On the other hand, some investigations with laser Doppler flowmetry have shown a reduced vasodilatory response to ischaemia stimulation in cutaneous microvasculature of SSc patients, which may reflect structural changes in digital arteriolar walls associated with a low vessel compliance.¹¹⁻²⁰

Given the above findings we preliminarily investigated the microvasculature in a limited number of SSc patients by using two different methodologies—that is, the post-ischaemic hyperaemia test and iontophoresis. The second method is a well known non-invasive technique that allows the introduction through the skin, by means of a small electric current, of different ionised substances.¹²

Iontophoresis, initially used for local application of therapeutic agents,²¹ is now available to test the effects of some vasodilatory agents on cutaneous microvasculature dysfunction resulting from endothelial cell or muscle wall injury, or both.¹³⁻¹⁶

According to previous findings,¹¹⁻²⁰ our study confirms that SSc patients show a reduced vasodilatory reserve of the skin microvasculature in response to ischaemia. Moreover, the vasodilatation by iontophoretically Ach and SNP application is considerably impaired in SSc patients compared with age and sex matched controls. On the basis of these findings, we could hypothesise that digital cutaneous sclerosis reduces the iontophoretic transfer of vasoactive agents across the skin. However, after different stimuli (Ach, SNP, and ischaemia) SSc patients showed a variable vasodilatory response; in fact, the maximum blood flow caused by Ach was significantly lower than that seen with SNP and ischaemia. It is possible to hypothesise that the reduction in vasodilatory response to Ach and SNP could be related to a defective absorption of two drugs because of the cutaneous fibrosis in SSc patients. The vasodilatation secondary to SNP did not show a difference compared with hyperaemia after the ischaemia test, which represents the maximum stimulus for vasodilatation, in both control subjects and SSc patients; this suggests an adequate iontophoretic diffusion of these drugs across the normal skin and the absence of a "railing effect" of cutaneous sclerosis. In our SSc patients a low vasodilatory response caused by SNP and ischaemia might reflect structural or functional abnormalities of the smooth muscle of arteriolar wall. However, the lower vasodilatory effect related to Ach, compared with SNP, suggests a further dysfunction of endothelial cells, as previously hypothesised.⁶⁻⁹⁻¹⁰

In contrast, Anderson *et al*¹⁶ did not find, on the forearm, different vasodilatory responses to Ach and SNP iontophoresis between Raynaud's, SSc patients, and control subjects. These contrasting results might be explained by the choice of different sites for the iontophoretic study; the microvascular involvement is significantly more pronounced in the digital district.

On the whole, these preliminary findings further support the pathogenetic role of both endothelial and arteriolar wall dysfunction in the microangiopathy of SSc patients. These abnormalities can be evaluated by means of iontophoresis, a non-invasive and easily reliable method. Further studies on a larger patient population could better define the relative role of endothelial and arteriolar wall dysfunction on the severity of organ damage at different stages of the disease.

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