

Digital iontophoresis of vasoactive substances as measured by laser Doppler imaging—a non-invasive technique by which to measure microvascular dysfunction in Raynaud's phenomenon

M. E. Anderson¹, T. L. Moore¹, M. Lunt² and A. L. Herrick^{1,2}

Objective. To test the hypothesis that microvascular vasodilation is impaired in patients with systemic sclerosis (SSc) compared with patients with primary Raynaud's phenomenon (PRP) and healthy controls, using the technique of laser Doppler imaging to quantify blood flow responses to iontophoresis of vasoactive agents.

Methods. Microvascular blood flow was measured by laser Doppler imaging before, during and after 120 s iontophoresis (30 μ A) of 1% acetylcholine chloride (ACh, endothelium-dependent) and 1% sodium nitroprusside (NaNP, endothelium-independent). Two adjacent fingers of the left hand were studied, and the procedure then repeated on the right. Ten patients with limited cutaneous SSc (LCSSc), 10 patients with PRP and 11 healthy control subjects were studied.

Results. Vasodilation in response to both ACh and NaNP iontophoresis, as measured by 'area under the blood flow.time curve' (AUC), normalized for baseline flux, was similar in the control and PRP groups, but was diminished in the LCSSc group compared with both control and PRP groups (ACh results: control vs LCSSc $P=0.028$, PRP vs LCSSc $P=0.005$; NaNP results: control vs LCSSc $P=0.004$, PRP vs LCSSc $P=0.005$). There were no differences between groups in baseline flux values nor in voltages required to drive the 30 μ A current.

Conclusions. Both endothelium-dependent and endothelium-independent vasodilation are impaired in patients with LCSSc. Vasodilatory responses in patients with PRP are similar to those in controls. If reproducibility is confirmed to be satisfactory, then these techniques could be used to examine disease progression over time and responsiveness to vasoactive treatment, thus facilitating clinical trials.

KEY WORDS: Iontophoresis, Laser Doppler imaging, Endothelium, Microvasculature, Raynaud's phenomenon, Systemic sclerosis.

Abnormalities of both microvascular structure and function are key features of systemic sclerosis (SSc) [1], and it has been suggested that SSc is primarily a vascular disease [2]. Structural abnormalities of the microvasculature can readily be identified using the technique of widefield nailfold microscopy [3] and quantified using nailfold video microscopy [4].

One of the challenges facing clinicians and scientists with an interest in SSc is the measurement of microvascular (dys)function. The ability to measure microvascular function at different stages of the disease process, and in response to different forms of therapy, would increase our understanding of the complex pathophysiology of SSc [5]. We know that in SSc, endothelial abnormalities occur early [6] and it therefore seems likely that endothelium-dependent vasodilation becomes impaired early on in the disease process. Endothelium-dependent and endothelium-independent microvascular vasodilation can be studied using the non-invasive process of iontophoresis, whereby vasoactive chemicals are 'driven' into the skin using a small electric charge, with resultant blood flow responses being measured by laser Doppler flowmetry. To date the studies of iontophoresis in patients with primary Raynaud's phenomenon (PRP) and SSc have given conflicting results [7–13]. These studies have all measured blood flow responses with single-probe laser Doppler, one of the limitations of which is poor reproducibility [7, 10].

In this study our aim was to test the hypothesis that microvascular vasodilation is impaired in patients with SSc, and to compare endothelium-dependent and endothelium-independent vasodilation in patients with SSc, PRP and healthy control subjects using the technique of laser Doppler imaging (scanning laser Doppler) to quantify blood flow responses to iontophoresis. Laser Doppler imaging measures blood flow over an area rather than at a single site, and is a non-contact method (as opposed to single-probe flowmetry when the probe contact might in itself alter blood flow). We included patients with PRP as well as patients with SSc because a key issue in the pathophysiology of PRP and SSc is why patients with SSc, but not those with PRP, can progress to irreversible tissue ischaemia. Because patients with the limited cutaneous variant of SSc (LCSSc) have the most marked vascular abnormalities [4, 14], we decided to recruit only patients with this subtype.

Patients and methods

Patients

Ten patients with LCSSc as defined by LeRoy *et al.* [15], 10 patients with PRP and 11 healthy control subjects were included. Their clinical characteristics are shown in Table 1. All 10 SSc

¹University of Manchester Rheumatic Diseases Centre, Hope Hospital, Salford M6 8HD and ²ARC Epidemiology Unit, University of Manchester, Manchester M13 9PT, UK.

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Correspondence to: A. L. Herrick. E-mail: aherrick@fs1.ho.man.ac.uk

TABLE 1. Clinical features of patients with LCSSc and PRP and control subjects

	Controls (n = 11)	PRP (n = 10)	LCSSc (n = 10)
Male:Female	4:7	2:8	2:8
Age (yr) ^a	45 (19–54)	41 (19–54)	52 (43–58)
Duration of Raynaud's (yr) ^a	NA	10 (2–23)	13 (6–26)
Number of smokers	0	2	2

^aResults are median (range).
NA, not applicable.

patients fulfilled the American College of Rheumatology (ACR, formerly the American Rheumatism Association) criteria for disease [16] and experienced Raynaud's phenomenon. Six of the LCSSc patients had mild digital skin involvement (involved, but able to pinch), two patients had moderate involvement (unable to pinch, able to move), and two patients had severe involvement (unable to move). In all of these patients, skin involvement of the fingers of both hands was similar.

Patients in the PRP group all had Raynaud's phenomenon for at least 2 yr, with neither clinical nor serological evidence of connective tissue disease. One PRP patient and two LCSSc patients were normally on vasodilator therapy for Raynaud's phenomenon (nifedipine in all three cases). Nifedipine was stopped for the 2 weeks prior to the study. The study was approved by the Salford and Trafford Ethics Committee and all patients gave written consent.

Iontophoresis and laser Doppler imaging

Acclimatization. Iontophoresis and laser Doppler imaging were performed after 20 min acclimatization at 23°C in a temperature-controlled room. Patients and controls were asked not to smoke and not to consume caffeine-containing beverages for at least 4 h prior to the study.

Equipment. Acetylcholine chloride (ACh) and sodium nitroprusside (NaNP) were used to investigate endothelium-dependent and endothelium-independent vasodilation respectively. For each

subject, two Perspex digital iontophoresis chambers (8 mm diameter circular aperture, with a platinum wire electrode at the circumference, contoured to fit neatly over the fingers) were attached using double-sided adhesive discs, one to each of two adjacent fingers of the left hand (dorsum of middle phalanx). Different sizes of digital iontophoresis chamber were available. Therefore a snug fit to the finger could always be obtained, preventing leakage from around the base of the chamber but without constricting the finger.

One chamber was filled with 1% ACh (Aldrich, Gillingham, UK) and the other with 1% NaNP (David Bull Laboratories Pty, Victoria, Australia) solution (dissolved in double-distilled water) (Fig. 1). The ACh-containing chamber was attached to the positive electrode and the NaNP-containing chamber was attached to the negative electrode of the iontophoresis controller (DRT4, Moor Instruments Ltd, Axminster, UK). In order to optimize laser beam passage through the solutions and prevent artefacts caused by optical glare generated from the menisci, a glass cover-slip was placed over each of the solutions, thus creating an optically neutral interface between the solution and air.

Protocol. Baseline skin microvascular blood flow within the area of the apertures was measured using a scanning 633 nm red laser Doppler (LD) imager (Moor LDI-VR scanner, Moor Instruments Ltd, Axminster) prior to iontophoresis. At commencement of iontophoresis at 30 μ A for 120 s (two chambers simultaneously), the LD imager initiated recording of a set of 25 serial scans measuring skin blood flow at the areas of iontophoresis. Each scan was of 17 s duration, with a 20 s interval between the beginning of consecutive scans, resulting in a total scanning time of 497 s per protocol. Fig. 2 illustrates the response of a control subject to the protocol.

The region of interest (ROI) was defined on the black and white 'd.c.' image. Although the ROIs should have been the same for all tests and all subjects (same size aperture and same distance from the scanner in all subjects), due to minor unavoidable differences in angulation of the iontophoresis chamber on different fingers, the ROIs varied slightly between individuals, although the same ROIs were used in each of the individual serial scans.

In addition, the voltage required to generate the 30 μ A current was recorded by an attached voltmeter. The rationale behind

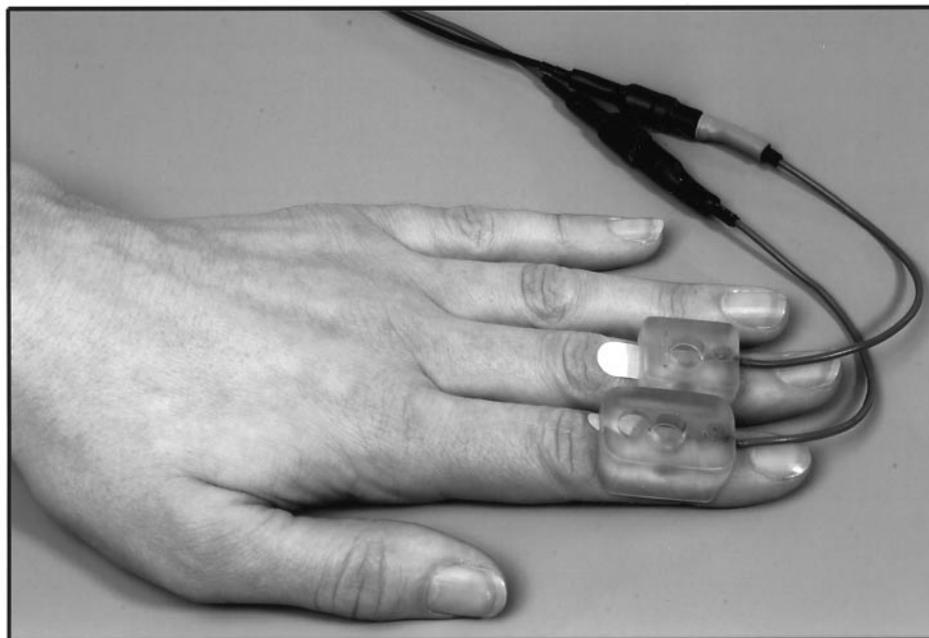


FIG. 1. Digital iontophoresis chambers attached to study subject.

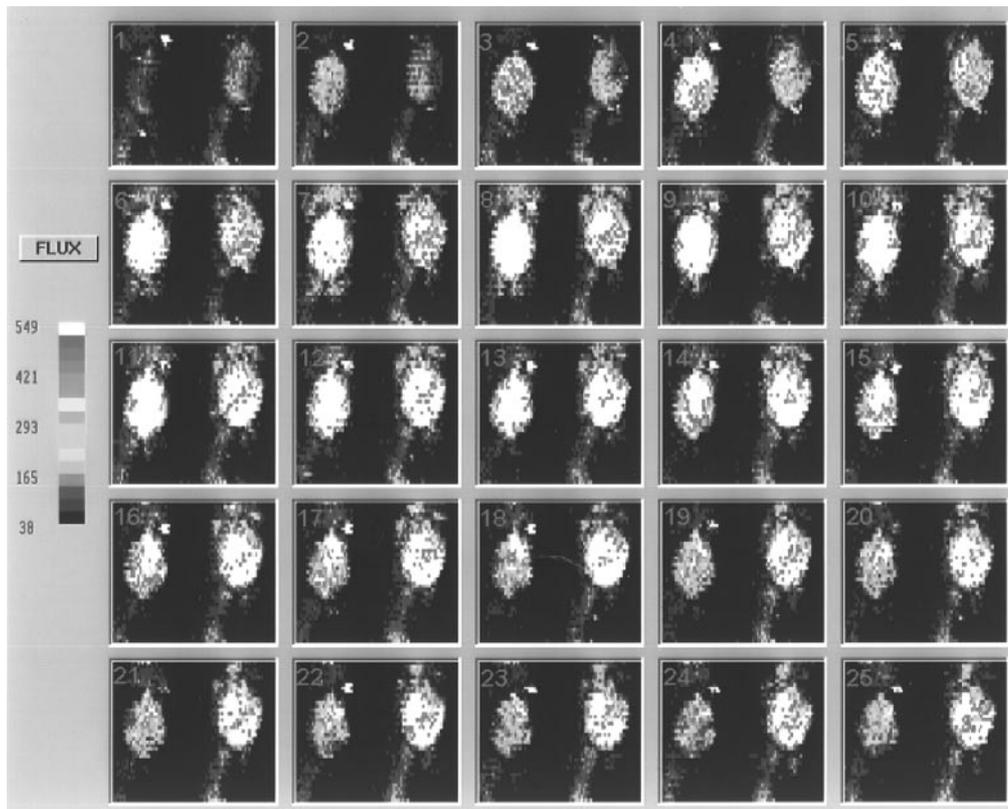


FIG. 2. Healthy control subject response to iontophoresis of ACh (left aperture on each scan) and NaNP (right aperture on each scan), as measured by serial LD imaging. Flux is represented on a colour scale (38–549 perfusion units) from dark blue (low flux) to red/white (high flux). This series of scans illustrates the typically faster onset of vasodilator response to ACh compared with NaNP iontophoresis, and the more sustained vasodilation after NaNP.

voltage measurements was that higher voltages, reflecting higher skin resistance, might in themselves contribute to the vasodilator response. If voltages differed between subject groups then this would need to be taken into account when analysing results.

The procedure was repeated on two adjacent fingers of the right hand in each subject, in order to allow assessment of the within-subject variability in vasodilator response between left and right digits.

Calculation of the AUC. Median blood flow within the area of each aperture was computed for the baseline and 25 serial scans. For each chemical on each hand of every subject, ‘area under the blood flow.time curve’ (AUC), normalized for baseline flux, was calculated: median baseline blood flow was extrapolated to cover the entire duration of the protocol (multiplied by 26 to cover the number of scans included within the protocol), and then subtracted from the sum of the median blood flows for baseline and 25 corresponding serial scans.

Statistical analysis

Statistical analyses were conducted with SPSS for Windows (version 10) software.

For each vasoactive chemical, repeated measures analysis of variance (ANOVA) was used to compare between the three groups: (a) the AUCs, normalized for baseline flux, (b) the baseline flux, and (c) the voltages required to drive the $30\ \mu\text{A}$ current. For each of (a), (b) and (c) there was a nested model for ACh and a separate nested model for NaNP results. In all nested models, right- and left-side results were defined as the within-subject

variables, whilst patient group was defined as the between-subjects factor.

Adjusting for age, sex and smoking did not alter the results obtained, therefore results presented are unadjusted.

Results

These are shown in Table 2 and Figs 3 and 4.

AUC

Comparisons of right and left digital flux responses. Although there was considerable variation between right and left digital responses for both ACh and NaNP within subjects, there was no consistent difference between the right and left digital microvascular responses between subjects for either chemical (Table 2).

Between-group comparisons of digital flux responses. AUC results are shown in Figs 3 and 4. The normalized AUCs for right and left responses for each individual were averaged for both ACh and NaNP, giving a mean AUC for each chemical for each patient. The mean (95% confidence intervals) AUC results for ACh were as follows: controls 6213 (4016, 8411) perfusion units, PRP patients 7331 (4828, 9834) and LCSSc patients 2826 (1049, 4603) (Fig. 3). Corresponding AUC results for NaNP were: controls 7766 (5999, 9532), PRP patients 7690 (4813, 10567) and LCSSc patients 2472 (449, 4494) (Fig. 4). There was a significant difference in vasodilatory response between groups with both ACh ($P=0.014$) and NaNP ($P=0.006$) iontophoresis as measured by LD imaging (Table 2). Vasodilation in response to both ACh and

TABLE 2. Repeated measures ANOVA of AUC (normalized for baseline flux), baseline flux and iontophoresis voltage. Right- and left-sided results = within-subject variables. Patient group = between-subject variables. Results are *P* values (* = significant value)

	ACh AUC	NaNP AUC	ACh baseline	NaNP baseline	Voltage (V)
Within subject					
Side (right or left)	0.242	0.907	0.923	0.969	0.824
Between subject (group)					
All groups	0.014*	0.006*	0.456	0.613	0.287
Control vs LCSSc	0.028*	0.004*	0.214	0.486	0.163
PRP vs LCSSc	0.005*	0.005*	0.489	0.890	0.183
Control vs PRP	0.323	0.958	0.584	0.347	0.948

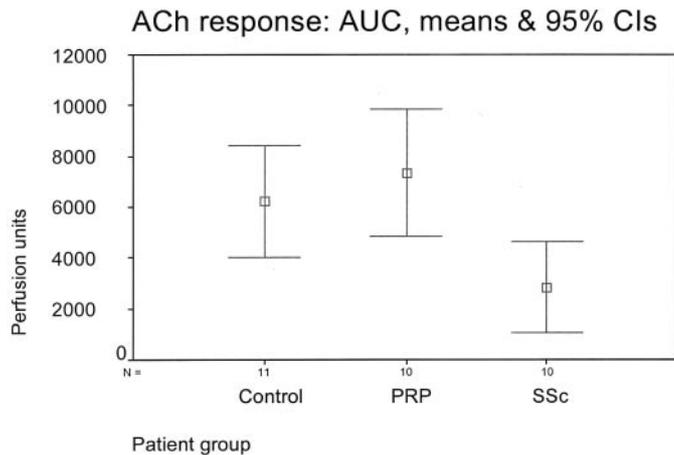


FIG. 3. ACh iontophoresis in control subjects and patients with PRP and LCSSc. Results are mean (95% confidence intervals) AUCs, normalized for baseline flux, in arbitrary perfusion units. Left and right results have been averaged for each subject.

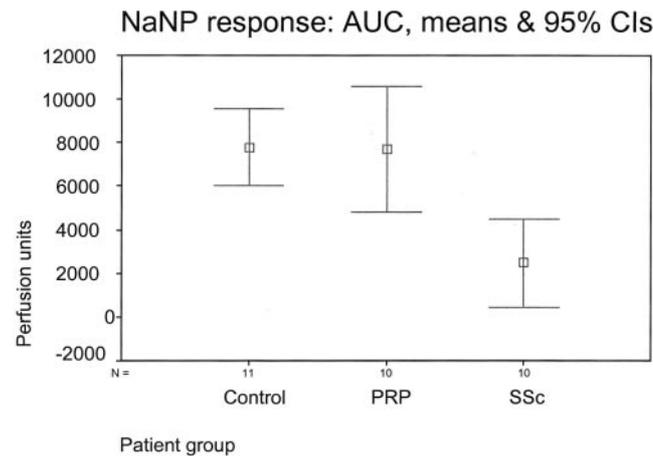


FIG. 4. NaNP iontophoresis in control subjects and patients with PRP and LCSSc. Results are mean (95% confidence intervals) AUCs, normalized for baseline flux, in arbitrary perfusion units. Left and right results have been averaged for each subject.

NaNP iontophoresis was similar in the control and PRP groups, but was diminished in the LCSSc patient group, compared with both controls and with patients with PRP.

Baseline flux

Baseline flux values were similar in all groups for each chemical and there were no differences between right and left sides (Table 2).

Voltages

The voltage required to drive the $30 \mu\text{A}$ current was similar in all groups with no statistically significant differences between right and left (Table 2) on repeated measures ANOVA. However, there was a trend towards higher voltages in the LCSSc group.

Discussion

Our results suggest that in patients with LCSSc there is a defect in both endothelium-dependent vasodilation (ACh response) and in endothelium-independent vasodilation (NaNP response). Our conclusions therefore concur with those of La Civita *et al.* [9], who reported impairment of both endothelium-dependent and endothelium-independent vasodilation in patients with SSc. In our own previous studies using a single probe we initially observed no differences in ACh and NaNP responses between patients with SSc, PRP and healthy controls [7, 10]. However, in our latest single-probe study using a very low-dose iontophoresis protocol (seven 10 s periods at increasing increments of 30, 40, 50, 60, 70, 85, and $100 \mu\text{A}$), we observed impaired endothelium-dependent responses in SSc patients compared with controls, leading us to conclude that the lower 'doses' might be more sensitive in detecting between-group differences [13]. The trend for reduced NaNP responsiveness was not statistically significant [13]. A selective reduction in endothelium-dependent vasodilation, but with preservation of endothelium-independent responses, has been reported by Khan and Belch [11].

In our study, patients with SSc were older than patients with PRP and healthy controls. However, adjustment for age (by adding age to the regression model as a continuous variable) did not alter the results obtained.

Using the imaging technique, we could not replicate our low-dose protocol exactly because this involved frequent small voltage applications, increasing over time. This was because whereas the single probe gives a continuous reading over time, each laser Doppler image takes a finite time to acquire (17 s in our protocol). Therefore we chose a single iontophoresis period of 120 s at $30 \mu\text{A}$. Although not 'real-time imaging' in the strict sense, by rapidly repeating scans of the same small area of the digits, our protocol allowed recording of dynamic responses to ACh/NaNP iontophoresis whilst measuring flux response over an area rather than at a single site, which is likely to give a truer representation of the local dermal microvasculature. None of the subjects found the procedure uncomfortable.

We are not aware of other studies measuring the effects of iontophoresis using laser Doppler imaging in patients with Raynaud's. However, the combined technique has been applied in other conditions, including diabetes [17, 18].

One of the concerns about using responses to iontophoresis to assess microvascular function is the reproducibility of the technique [7, 10]. Although in this study we did not assess reproducibility, other investigators have done so. Kubli *et al.* [19], using laser Doppler imaging, found that day-to-day reproducibility was good, especially for ACh iontophoresis (coefficient of variation

<10%), but stressed the importance of studying exactly the same site on each occasion, due to problems of site-to-site variability. Newton *et al.* [20] reported a coefficient of variation of approximately 17% for ACh (variability assessed over three visits). Site-to-site variability has been confirmed in our own study, as we found considerable variation between right and left digital responses for both ACh and NaNP within subjects. Multiple measurements improve the precision with which different subject groups can be compared, and so ideally (as in our study) more than one site should be examined in cross-sectional studies.

Another potential methodological problem inherent in the iontophoresis/laser Doppler technique is the effect of the electric current itself on blood flow, irrespective of any effect of the charged ions themselves, especially when NaNP is being iontophored [21, 22]. We did not formally test the electrical effects of our protocol, but at the charges used these are likely to be very small. However, to examine for the possibility of an electrical effect, we collected data on the voltages required to drive the electrical current through skin. We found that, as might have been anticipated, a higher voltage was required in the patients with LCSSc, all of whom had sclerodactyly. The most obvious interpretation of these findings is that any electrical effect would have been greatest in the LCSSc group, and would have acted to reduce the observed differences between the SSc and the PRP/control groups. However, Ramsay *et al.* [23], in experiments involving healthy control subjects, reported an inverse correlation between resistance and blood flow. These authors [23] suggested that, even though the constant charge suggests that the same amount of drug is being transferred across the skin, increased skin resistance (reflected by an increase in voltage) might be associated with reduction in effective drug delivery, perhaps as a result of fewer low-resistance channels (e.g. sweat ducts and hair follicles) which are known to be reduced in sclerodermatous skin. From our study it is not possible to state the mechanism for the reduced responsiveness in the LCSSc group. However, the two patients with the most marked sclerodactyly did not require the highest voltages for iontophoresis and did not have the lowest vasodilator responses.

There is now considerable evidence to support an endothelium-dependent defect in SSc in both small and large vessels [11, 13, 24–26]. In addition, we report a defect in the endothelium-independent response. Ours was a small cross-sectional study which included only patients with well-established disease. It seems probable that early on in the disease process endothelium-dependent impairment occurs, progressing over time to a more severe microvascular dysfunction with impairment of endothelium-independent vasodilation. The heterogeneity of the SSc disease process, both between and within individuals, may explain at least in part the differences in results between different iontophoresis studies, most of which include only small numbers of patients. Therefore studies of early disease would be of interest.

We observed no significant differences in responses between patients with PRP and healthy control subjects, contrasting with results of Khan *et al.* [8], who found impaired vasodilation in patients with PRP. It may be that vasodilatory abnormalities in patients with PRP affect larger, more proximal vessels—Smith *et al.* [27], in a study of small arteries dissected from gluteal fat biopsies, reported impairment of endothelium-dependent, but not of endothelium-independent, responses.

In conclusion, we have demonstrated the feasibility of measuring blood flow responses to iontophoresis in patients with SSc and PRP using laser Doppler imaging, and that in patients with established LCSSc, both endothelium-dependent and endothelium-independent vasodilation are impaired. The technique should now be applied to studying pathophysiology in other groups of patients with rheumatic diseases affecting the microvasculature, and in patients with different severities and durations of scleroderma-spectrum disorders including patients with undifferentiated connective tissue disease. Further studies to

examine reproducibility are indicated. If reproducibility is confirmed to be satisfactory, then the technique could be used (together with symptom-based measurements) to examine disease progression over time and responsiveness to vasoactive treatment, thus facilitating clinical trials.

<i>Rheumatology</i>	Key messages
	<ul style="list-style-type: none"> • Laser Doppler imaging can be used to quantify digital microvascular responses to iontophoresis of vasoactive drugs in patients with Raynaud's phenomenon. • Both endothelium-dependent and endothelium-independent vasodilation are impaired in patients with limited cutaneous systemic sclerosis.

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