Evaluation of endothelial function in patients with limited systemic sclerosis by use of brachial artery Doppler ultrasound

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ABSTRACT

Objectives: The aim of this study was to compare the brachial artery endothelium-dependent and endothelium-independent dilating responses in patients with limited systemic sclerosis (LSSc) with those of healthy subjects of the same gender, age and color. **Methods:** Twenty adult, non-obese, non-smoker, non-diabetic, non-dyslipidemic, and non-hypertensive women, who fulfilled the American College of Rheumatology criteria for the diagnosis of SSc, were submitted to right brachial artery Doppler ultrasound. The vasodilating responses were analyzed as follows: the endothelium-dependent dilating response, after a 5-minute ischemia in the right arm; and the endothelium-independent dilating response, after administering 300 mcg of nitroglycerin (NTG) sublingually. The results were compared with the response obtained in healthy subjects. **Results:** Brachial artery longitudinal diameter was significantly low at baseline 1: 3.57 ± 0.52 mm and 3.93 ± 0.39 mm for the LSSc group and the control group, respectively, P = 0.005. The vascular reactivity after the ischemia/reactive hyperemia and the NTG showed no significant difference between the groups (8.60 ± 5.45 mm *vs.* 9.26 ± 5.91 mm and 25.01 ± 12.55 mm *vs.* 19.59 ± 7.94 mm for the LSSc and control groups, respectively). Also, no statistically significant difference was found between red blood cell velocity (RBCV) after reactive hyperemia and NTG (110.2 ± 43.86 cm/s *vs.* 102.0 ± 25.89 cm/s and 63.80 ± 17.69 cm/s *vs.* 65.4 ± 12.90 cm/s in the LSSc and control groups, respectively). **Conclusion:** Although the LSSc group showed lower brachial artery diameter, the endothelium-dependent and the endothelium-independent dilating responses were preserved in both groups.

Keywords: limited scleroderma, vascular endothelium, brachial artery, ultrasound, Doppler effect.

INTRODUCTION

Systemic sclerosis (SSc) is a chronic connective tissue disease characterized by thickening and fibrosis of the skin and internal organs, due to deposition of collagen, glycosaminoglycans and other proteins of the extracellular matrix, in addition to vascular damage resulting from proliferative endarteritis. The triggering factor for endothelial injury is not known. However, the initial lesion in genetically predisposed individuals is estimated to be triggered by the following: the injury caused by the ischemia/reperfusion process; the presence of cytotoxic autoantibodies in the blood; infectious agents; and environmental factors.

Endothelial lesion due to reactive oxygen species is responsible for a decrease in the synthesis of prostacyclins, nitric oxide (NO), tissue plasminogen activating factor and heparan sulfate, and for an increase in the synthesis of endothelin-1,

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leading to an unbalance in the vasodilation/vasoconstriction ratio favoring constriction, with permanent damage to blood vessel walls. That unbalance contributes to vascular hypoxia and endothelial injury, resulting in the release of cytokines by macrophages, platelets, mast cells and activated T cells. Thus, a vicious cycle of endothelial injury is maintained, associated with fibrosis resulting from the stimulation of fibroblasts, which are responsible for the synthesis of cell matrix and collagen.[1]

Although ischemia stimulates neoangiogenesis and the levels of angiogenic factors, such as vascular endothelial growth factor (VEGF), are increased, the skin of patients with SSc has large avascular areas. A dysregulation in the angiogenesis process in SSc is believed to occur.[2] The presence of autoantibodies directed against endothelial cells in SSc can induce apoptosis of those cells. In those patients, endothelial precursor cells have been shown to be reduced in number and have a lower differentiation potential.[3] In addition, a change in the sympathetic neural control of the vascular tonus,[4] as well as in some intrinsic aspects of microcirculation, has been shown in SSc. There is an increase in platelet aggregation and activation associated with a reduction in the deformability of red blood cells and in fibrin deposition in the vascular wall,[5] culminating in an elevated risk for the formation of thrombi in the microcirculation, with a higher expression of adhesion molecules, which aggregate neutrophils and platelets in the vascular wall and reduce the vascular lumen.[6]

The endothelium continually releases NO, and this release increases when the membrane receptors of endothelial cells are activated by soluble stimuli (acetylcholine, bradykinin, adenosine phosphate, substance P, and serotonin) or when calcium channels are opened by an increase in shear stress generated by turbulent blood flow.[7] The NO target in the vascular wall is the guanylate cyclase enzyme, found in smooth muscle cells, whose activation generates accumulation of cyclic guanosine monophosphate (cyclic GMP), triggering relaxation of the smooth musculature and vasodilation, with consequent increase in local blood flow.[8]

Structural changes in arteriolar walls (resistant vessels) are well known in SSc. They consist in thickening/proliferation/edema of the intima layer with infiltration of mononuclear cells, hypertrophy of the media and intima layers, rupture of the lamina elastica interna, and presence of fibrotic scars in vessel walls.[9,10] Little is known about the structural changes found in the wall of larger vessels, elastic vessels, such as the brachial, ulnar and radial arteries (conductance vessels), which can be measured by use of non-invasive techniques such as Doppler ultrasound. There is increasing evidence that the macrovasculature is also involved in the SSc disease process, which is speculated to relate to the presence of structural abnormalities, such as thickening and stiffness of the vascular wall.[11] Although the vascular involvement is considered predominantly microvascular in SSc, macrovascular disease can affect more than half of the patients,[12] arterial occlusion being found particularly in the hands of patients with the limited form of SSc (LSSc) and in the presence of the anticentromere antibody.[13]

The ultrasound assessment of the brachial artery (BA) vasodilating response is a non-invasive technique described by Celermajer in 1992,[14] being used as an index to assess the macrovascular function by measuring the BA diameter before and after the ischemic stimulus. The ischemia caused in the forearm determines a marked drop in peripheral vascular resistance, which is followed by an increase in the blood shear force exerted on the arterial wall after its release. The increase in shear stress stimulates the production and release of vasodilating substances by the endothelium, especially NO. The increase observed in the BA diameter is an indirect measure of NO release, and, thus, of endothelium-dependent vasodilation.[15]

A small percentage flow increase mediated by endothelium-dependent vasodilation can be interpreted as low availability of NO, being associated with an increased risk for vascular disease.[16] The deficient endothelium-dependent vasodilation found in SSc can be explained by lack of production and release of vasodilating substances by the chronically injured endothelium, especially NO, while normal vasodilating response to endothelium-independent factors (exogenous nitroglycerin) can be observed. However, macrovascular endothelial dysfunction in SSc has been the object of several studies, whose authors have reported a reduction in the BA flow-mediated dilation (FMD),[17–20] while others have not.[9,21,22]

This study aimed at comparing the endothelium-dependent dilating response obtained after a five-minute ischemia induced by placing a sphygmomanometer cuff in the right upper limb (FMD) and the endothelium-independent dilating response obtained after sublingual nitroglycerin administration (nitroglycerin-mediated dilation - NMD), in patients with LSSc and healthy individuals of matched gender, age and color.

PATIENTS AND METHODS

Study participants

Twenty females aged 25–60 years, diagnosed with SSc according to the 1980 American College of Rheumatology criteria[23], with disease duration over six months and biphasic Raynaud's phenomenon (RP) in the extremities were included in this study, after providing written informed consent and being

assured of the confidentiality of their identities. All patients had LSSc, characterized by cutaneous fibrosis restricted to face, neck and extremities.

This open, observational, non-randomized, prospective study was conducted in the Service of Rheumatology of the Hospital Clementino Fraga Filho of the Universidade Federal do Rio de Janeiro (HUCFF-UFRJ). This study's project was submitted to the Committee on Ethics and Research of the HUCFF-UFRJ and approved on 12/28/2009 (protocol 1120/09).

The exclusion criteria were as follows: overlap syndrome with other collagenoses, myopathies or inflammatory arthropathies; acquired immunodeficiency syndrome; antiphospholipid antibody syndrome; pregnancy and lactation; malignant neoplasias; heart failure; moderate to severe pulmonary artery hypertension; smoking; arterial hypertension; and diabetes.

The control group comprised 20 healthy white females, of the same age group of the patients studied, and who were not on any drugs that could alter endothelial response. They were included in the study after providing written informed consent.

Assessment of the endothelial function

The endothelial function was assessed by measuring FMD and NMD of the BA by use of color flow Doppler ultrasound, which is a non-invasive, high-resolution method that enables detailed assessment of the thoracic circulation, combining the following three components: B mode, pulsed, and color mode Doppler ultrasound.[24] The exam was performed with the individual on the dorsal decubitus position in a controlled temperature (22°C) room.

A sphygmomanometer was positioned on the right arm, approximately 5 cm above the antecubital fossa. Images of the right BA were obtained on a longitudinal view, on the B mode of the Antares (Siemens) ultrasound device, using the linear transducer VFX9-4 with a 10-Mhz frequency positioned 2–3 cm above the antecubital fossa. The BA diameters were measured during the diastole of the cardiac cycle. The flows were obtained by use of spectral analysis of the velocity versus volume curves –1.5-mm window and angle lower than 60º positioned at the center of the vessel. Red blood cell velocity (RBCV) was obtained by use of Doppler ultrasound, with a 1.5-mm window and angle lower than 60º positioned at the center of the vessel.

The exam was initiated after a ten-minute resting period. The BA diameter and RBCV were measured at the baseline 1 phase. Then, the sphygmomanometer placed on the right forearm was inflated at a pressure 50% above the initial systolic pressure, and ischemia was maintained for five minutes.

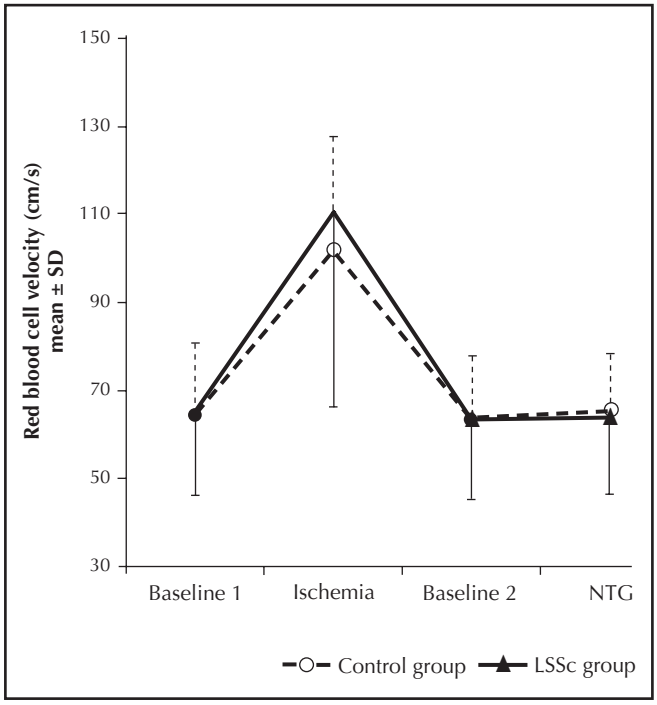

Figure 1
Red blood cell velocity during the experiment.

New measures of the BA diameter and RBCV (post-ischemia/reactive hyperemia phase) were taken 60 seconds after releasing ischemia. After a new ten-minute resting period (baseline 2), the individuals received a sublingual dose of 300 mcg of NTG, and the measures of the BA diameter and RBCV were taken after three minutes (post-NTG). The means of the three measures of BA diameter and RBCV obtained at each of the four phases were calculated (Figure 1).

Statistical analysis

For comparing baseline variables (numerical) between the LSSc and control groups, the Student *t* test or Mann-Whitney (non-parametric) test were used for independent samples. The homogeneity of variances between the groups was assessed by use of the Bartlett's test. Analysis of variance (ANOVA) for repeated measures was used to assess the behavior over the three phases (baseline, reactive hyperemia, and post-NTG) in each group. Bonferroni adjustment (adjusted for three phases) for multiple comparisons was used to identify which phases significantly differed between themselves. To assess whether the evolution throughout the experiment significantly differed between the groups, ANOVA for repeated measures for one factor (effect of the interaction group x time) was used. In ANOVA for repeated measures, logarithmic transformation

(natural log) was applied to the data. Non-parametric methods were used, because the variables showed no normal distribution (Gaussian) due to data dispersion and rejection of the Kolmogorov-Smirnov test in the groups studied. The significance level adopted was 5%. The statistical analysis was processed in the 6.11 SAS software (SAS Institute, Inc., Cary, NC). Data are shown as mean ± SD.

RESULTS

This study assessed 40 females, 20 comprising the LSSc group and 20 healthy females comprising the control group. Of those with LSSc, seven (35%) had disease duration > 10 years and 13 (65%) had disease duration < 10 years. Seven patients (35%) were on low oral prednisone doses (< 10 mg/day). Fourteen patients (70%) used vasodilators regularly and daily [calcium channel blockers (CCB), rheological modifiers of red blood cells, inhibitors of phosphodiesterases 1 and 5]. The groups did not significantly differ regarding the following characteristics: age (38.6 ± 9.2 and 43.4 ± 9.6 years in control and LSSc groups, respectively, P = 0.11); weight (62.1 ± 5.4 and 58.7 ± 9.3 kg in control and LSSc groups, respectively, P = 0.32); systolic blood pressure (SBP) (110.8 ± 16.9 and 116.5 ± 15.7 mmHg in control and LSSc groups, respectively, P = 0.27); diastolic blood pressure (DBP) (71.5 ± 12.8 and 75.0 ± 10.6 in control and LSSc groups, respectively, P = 0.35); and heart rate (73.2 ± 11.3 and 76.6 ± 11.9 in control and LSSc groups, respectively, P = 0.36).

Table 1 shows the baseline variants of weight, age, SBP and DBP in the LSSc and control groups. Data regarding the longitudinal BA diameters in the baseline 1, post-ischemia, baseline 2, and post-NTG phases in both groups are shown in Table 2. Data regarding RBCV in the baseline 1, post-ischemia, baseline 2, and post-NTG phases in both groups are shown in Table 3.

Baseline measures

The BA diameter was significantly lower in the LSSc group (P = 0.005), but no significant difference in RBCV was observed in the groups (P = 0.90).

Flow-mediated dilation (FMD)

No significant difference between the groups was observed regarding BA diameter (P = 0.07) and RBCV (P = 0.38).

Nitroglycerin-mediated dilation (NMD)

No significant difference between the groups was observed regarding BA diameter (P = 0.24) and RBCV (P = 0.97).

Table 1
Data regarding the baseline variants of weight, age, SBP, and DBP in the LSSc and control groups

Baseline variable	Group	Mean	SD	Median	Minimum	Maximum	P[a]
Age (years)	P	43.4	9.6	44	28	60	0.11
	C	38.6	9.2	39.5	27	54	
SBP (mmHg)	P	116.5	15.7	115	95	160	0.27
	C	110.8	16.9	110	85	150	
DBP (mmHg)	P	75.0	10.6	77.5	60	100	0.35
	C	71.5	12.8	70	55	100	
HR (beat/min)	P	76.6	11.9	72.5	60	104	0.36
	C	73.2	11.3	71	58	99	
BA diameter – baseline 1	P	3.57	0.52	3.45	2.93	5.07	0.005
	C	3.93	0.39	3.92	3.17	4.73	
RBCV – baseline 1	P	65.3	19.0	59.3	38.1	113	0.97
	C	64.6	16.1	63.8	35.7	99.2	

SD: standard deviation; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; BA: brachial artery; RBCV: red blood cell velocity.
[a] Student *t* test or Mann-Whitney test for independent samples.

Table 2
Data regarding the BA diameter in the baseline 1, post-ischemia, baseline 2, and post-NTG phases in both groups

	Time	Mean ± SD	Median	P[a]	Significant differences[b]	P[c]
LSSc group	Baseline 1	3.57 ± 0.52	3.45	0.0001	Baseline 1 ≠ ischemia Baseline 1 ≠ NTG Ischemia ≠ NTG Baseline 2 ≠ NTG	0,31
	Ischemia	3.77 ± 0.59	3.74			
	Baseline 2	3.66 ± 0.62	3.58			
	NTG	4.44 ± 0.64	4.33			
Control group	Baseline 1	3.93 ± 0.39	3.92	0.0001	Baseline 1 ≠ ischemia Baseline 1 ≠ NTG Ischemia ≠ baseline 2. NTG Baseline 2 ≠ NTG	0,31
	Ischemia	4.14 ± 0.49	4.12			
	Baseline 2	3.98 ± 0.56	3.90			
	NTG	4.70 ± 0.58	4.69			

BA: brachial artery; SD: standard deviation.
[a]ANOVA for repeated measures in each group (effect of time); [b] Bonferroni's multiple comparisons at 5%; [c]ANOVA for repeated measures between the two groups (effect of the interaction group*time).

Table 3
Data regarding the RBCV in the baseline 1, post-ischemia (reactive hyperemia), baseline 2, and post-NTG phases in both groups

	Time	Mean ± SD	Median	P[a]	Significant differences[b]	P[c]
LSSc group	Baseline 1	65.3 ± 18.96	59.3	0.0001	Baseline 1 ≠ ischemia Ischemia ≠ baseline 2 Ischemia ≠ NTG	0.77
	Ischemia	110.2 ± 43.86	103.1			
	Baseline 2	63.3 ± 17.78	61.0			
	NTG	63.8 ± 17.69	62.9			
Control group	Baseline 1	64.6 ± 16.10	63.8	0.0001	Baseline 1 ≠ ischemia Ischemia ≠ baseline 2 Ischemia ≠ NTG	0.77
	Ischemia	102.0 ± 25.89	97.5			
	Baseline 2	63.6 ± 14.57	59.8			
	NTG	65.4 ± 12.90	63.9			

RBCV: red blood cell velocity; SD: standard deviation.
[a]ANOVA for repeated measures in each group (effect of time); [b] Bonferroni's multiple comparisons at 5%; [c]ANOVA for repeated measures between the two groups (effect of the interaction group*time).

Dilating response of the BA after induced ischemia

No statistically significant difference in the BA vasodilating response after induced ischemia (endothelium-dependent) was observed between the control (8.9%) and LSSc groups (8.6%).

DISCUSSION

Analysis of endothelial dysfunction by measuring both FMD, which is an endothelium-dependent dilation, and NMD, which is an endothelium-independent dilation, shows controversial results in SSc. Lekakis et al.[17] and Cypiene et al.,[19] studying patients with the diffuse form of SSc (DSSc), have reported a reduction in both FMD and NMD as compared with those of healthy controls. Rossi et al.[20] have also reported a significant reduction in both FMD and NMD when studying patients with DSSc or LSSc, and comparing them with healthy controls. Szucs et al.[18] have found a reduction in FMD, but no change in NMD, when studying individuals with DSSc or LSSc as compared with healthy women. Andersen et al.[9] have found no alterations in FMD and NMD when comparing healthy individuals with those having DSSc or LSSc. Rajagopalan et al.,[21] comparing individuals with primary RP and secondary RP

with SSc and other connective tissue diseases, have confirmed changes in the microcirculation by using laser Doppler fluxmetry after brief digital arterial occlusion; however, they have found no difference in FMD in the groups analyzed. Roustit et al.,[22] comparing healthy individuals with primary RP and SSc, have not found any significant change in FMD in the three groups. D'Andrea et al.[25] have reported a discreet reduction in FMD in patients with SSc as compared with healthy individuals. Bartoli et al.[26] have also reported smaller FMD when comparing individuals with SSc with a control group.

Similarly to those by Andersen et al.,[9] Rajagopalan et al.[21] and Roustit et al.,[22] our study found no evidence of vascular dysregulation confirmed by FMD in patients with SSc as compared with a control group comprised by healthy individuals. In accordance with Szucs et al.[18] and Andersen et al.,[9] our study showed that NMD is not altered in individuals with SSc as compared with that same control group.

Unlike our study, most of those authors have studied patients with predominance of the DSSc. We chose to study individuals with the limited form of disease because we knew that the incidence of vasculopathy is greater in such cases (severe RP, telangiectasia, and a primary type of pulmonary hypertension in late disease). Anticentromere antibody is a known risk factor for digital ischemia and appears, mainly, in the long-standing limited form.[27,28]

Cheng et al.[29] have conducted a study on the biomechanical properties and intima-media thickness of the carotid and femoral arteries in 19 patients with DSSc, 33 with LSSc, and 21 healthy controls. A progressive and significant reduction in the carotid artery elastic properties of patients with LSSc was observed as compared with those of the control group. In addition to the microvascular changes characteristic of SSc, macrovascular involvement can occur, exacerbating existing distal hemodynamic disorders, responsible for the digital changes.

Based on our findings, we believe that, although chronically damaged, the endothelium remains responsive in the population studied. The macrovascular abnormalities in individuals with SSc are attributed to structural and anatomical factors of the vessel wall rather than to endothelium functional changes.[11] Significant difference in the baseline BA diameter measure was identified between the LSSc and control groups. That fact *per se* can increase the risk of cardiovascular diseases in that population. An association has been shown between smaller BA diameter and the presence of subclinical atherosclerosis, estimated by measuring the intima-media thickness of the internal carotid arteries, indicating that arterial remodeling is a systemic process in SSc. Whether BA dilation in those cases is consequent to a structural change in vessel wall components or whether it is directly caused by the effect of cardiovascular risk factors on the vascular sympathetic tonus remains uncertain. A significant reduction in the arterial compliance of patients with SSc relates to vascular elasticity, and can be consequent to connective tissue changes and increase cardiovascular risk.[30] The BA diameter, a simple and reproducible index, can be a valuable indicator of cardiovascular risk, its variation being inversely proportional to that risk.[31]

Some of our data conflict with those from the literature and can be explained due to bias present in our study. Our sample size was small due to the low prevalence of the disease in our population and the difficulty in selecting patients with LSSc who met all the inclusion criteria. Another confounding factor was that many of the individuals studied (70%) were on regular vasoactive drugs at the time of the FMD and NMD assessment with BA Doppler ultrasound. Similarly to most published studies,[9,19–22,25] we chose to not suspend those drugs due to the severity of the peripheral vasculopathy of those patients.

Andersen et al.[9] have allowed the use of angiotensin-converting-enzyme inhibitors (ACEI) and corticosteroids during their study, and observed no significant difference between the treated and untreated groups, although plasma nitrate levels tended towards being lower in the group on corticosteroid. Bartoli et al.[26] and Szucs et al.[18] have chosen to suspend the vasoactive and antioxidant drugs for 24 hours before the exam. Rajagopalan et al.[21] have allowed patients with SSc to maintain their regular use of CCB or ACEI as long as it had been initiated more than four months before beginning assessing endothelial function, but they have excluded patients on lipid-lowering therapy or more than two anti-hypertensive drugs combined. Of the 42 patients with SSc assessed by Roustit et al.,[22] 16 were on regular use of CCB, three on ACEI, one on angiotensin II receptor blockers, two on hydroxychloroquine, two on cyclophosphamide, one on corticosteroid, two on azathioprine, and one on methotrexate. No patient used prostacyclin analogues during this study. Of the 36 patients with primary RP, only two were on CCB.

Disease duration also varied between the patients with LSSc, and only 30% of the sample had a disease duration since diagnosis over ten years. Regarding laboratory findings, the exposure to risk factors and their influence on cardiovascular diseases in both groups were not studied. In addition, anthropometric differences that might influence cardiovascular risk, such as the abdominal circumference measure, were not assessed in the groups studied. It is worth noting that the primary mechanism responsible for determining the

BA dilating response varies with the position of the sphygmomanometer on the upper limb of the individual assessed. Most of the response is NO-dependent when the sphygmomanometer is placed on the forearm; however, when placed on the arm, the response is partially NO-dependent. We chose to place it on the arm because cutaneous sclerosis is more common on the forearm, which makes the area very painful when compressed.[32]

In conclusion, our findings show that the BA diameter is reduced in patients with LSSc. However, both endothelium-dependent and endothelium-independent dilating responses remained preserved, encouraging the treatment of those patients in an attempt to reduce morbidity and mortality during the course of disease.

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