Effect of Vasodilator And Immunosuppressive Therapy On The Endothelial Dysfunction In Patients With Systemic Sclerosis

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Abstract

Background: A comparative analysis of flow-mediated-vasodilation (FMD), vasoactive angiogenic, and fibrogenic mediators between treatment-naïve and treated Systemic sclerosis (SSc) patients is an unmet need.

Aim and Objective: 1) To assess the FMD and different pathogenic mediators in SSc patients [treatment naïve (Group-I, n=24) on vasodilator (Group-II, n=10), on vasodilator + immunosuppressive (Group-III, n=22)], and in healthy control (HC), n= 20]. To assess the proportion of circulating Endothelial cells in Group-I.

Materials and method: FMD was measured in all the participants. Serum levels of NO, ET1, NO/ET1, sVCAM, sICAM, TGF β , IL-6, VEGF were measured along with the gene expressions of eNOS, iNOS, ET-1, TGF β . CEC was measured in Group-I and HC.

Results: FMD was significantly decreased in all SSc patients though receiving treatment. Upregulation of serum NO and ET concentrations were noted post-treatment with an unaltered NO/ET1 ratio. NO was positively correlated with FMD ($r=0.6$), and negatively with TGF β ($r=-0.5$). ET-1 showed less correlation with TGF β ($r=-0.5$) but no significant correlation with FMD. Circulating endothelial cell (CEC) was significantly higher in Group-I (3.2%) than HC (0.8%) ($p=0.002$), and it showed a good correlation with NO ($r=-0.7$, $p=0.0001$) and NO/ET1 ($r=-0.6$, $p=0.007$).

Conclusion: Endothelial dysfunction was seen in SSc patients irrespective of treatment which might be due to the unaltered NO/ET1. NO might be the major driving molecule in the vascular pathophysiology of SSc.

Introduction

The pathological hallmarks of Systemic Sclerosis (SSc) constitute an inter-related triad of vasculopathy, autoimmunity, and tissue remodelling. Vasculopathy in SSc is associated with tissue ischemia which is responsible for various clinical manifestations, Raynaud's phenomenon being the most common [1, 2]. Vascular tonicity in healthy individuals is chiefly maintained by the vasoactive substances; vasodilator Nitric Oxide (NO) and vasoconstrictor Endothelin-1 (ET1), which are secreted by the endothelial layer. An imbalance in the production of these vasoactive substances leads to Endothelial Dysfunction (ED) [3]. Both NO and ET1 interact or have a concomitant regulative expression balance in maintaining vascular tone [4]. Systemic vasculopathy is the sine-qua-non feature of pathogenesis that is initiated by an imbalance between NO and ET-1, associated with some other vasoactive molecules. From the pathogenic viewpoint, endothelial injury is believed to be the initial event in SSc which subsequently leads to endothelial dysfunction (ED) [5, 6].

Flow-mediated dilatation (FMD) of the brachial artery by ultrasonography (USG) is a non-invasive method for the assessment of endothelial function. Impaired has been reported in various rheumatic

diseases and it is considered as a predecessor of cardiovascular diseases. It reflects the effects of several pathophysiological events such as vessel tone regulation, cell proliferation, and inflammatory responses. Impairment of FMD in SSc patients has also been documented in the literature [7–9].

Downregulation of the endothelial NO synthase (eNOS) enzyme and NO bioavailability has been linked with the ED. Three major isoforms of NOS catalyze NO production: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). eNOS is the major NOS isoform expressed in endothelial cells. It has been reported that with the advancement of the skin disease in SSc there is downregulation of eNOS expression whereas iNOS is upregulated [10]. In addition to the NO and ET1 imbalance, fibrogenic cytokines, angiogenic mediators, and cell adhesion molecules have a putative role in SSc vasculopathy. Transforming growth factor-beta (TGF β) and vascular endothelial growth factor (VEGF) can cause defective angiogenesis in SSc [11, 12]. Overexpression of the adhesion molecules like soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM) have been documented in SSc, and this is seen during the early part of microvascular damage of SSc [13, 14]. Currently, circulating endothelial cells (CECs) have emerged as a reliable marker of ED [15]. As a consequence of endothelial injury, there is a lack of endothelial integrity, and the detached endothelial cells in the circulation can be detected as CECs. A higher proportion of CEC is seen in patients with SSc [16].

With this background, this study was conducted to measure the extent of endothelial dysfunction and the expressions of molecular markers between the treated and untreated SSc patients in comparison to the healthy individuals and to evaluate the association of ED with such molecular markers. Additionally, this study also evaluated the proportion of CECs in treatment naïve SSc patients and their association with ED.

Materials And Method

Recruitment of study participants and sample collection

This cross-sectional study was conducted at the Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata, India. Patients of SSc [fulfilling American College of Rheumatology (ACR) criteria for SSc, 2013] were recruited from the outpatient clinic of the Department of Clinical Immunology and Rheumatology, IPGME&R, between November 2019 to February 2020, along with age-sex matched healthy individuals (n= 20). All participants were ≥ 18 years and ≤ 55 years of age. All recruited patients had Interstitial Lung Disease (ILD). Co-existence of other rheumatological diseases, current or past history of tobacco usages, history of diabetes, hypertension, renal impairment, hypothyroidism and pregnancy were excluded from the study. SSc patients were subcategorized based on the ongoing treatment modalities into the following groups: treatment-naïve (Group-I, n=24), patients receiving vasodilator only (Group-II, n=10), and patients receiving a combination of vasodilator and immunosuppressive (Group-III, n=22). This study was approved by the institutional ethical committee of IPGME&R (No: IPGME&R/IEC/2018/533). Written, informed consent was taken from each participant. This study was done following the Declaration of Helsinki, 1964, and its later amendments.

Total 8ml of Peripheral blood were collected from all the study participants in ethylene diamine tetra-acetic acid (EDTA) vial as well as within clot vial to isolate serum and plasma to perform the following experimental proceedings.

Measurement of FMD of the Brachial Artery by Ultrasonography:

FMD of the brachial artery was measured by an experienced radiologist who was blinded of the participant's data. Before the FMD analysis, the subject rested for 30 minutes in a temperature-controlled room. Measurements were taken in the supine position. The right brachial artery above the antecubital fossa was imaged by a linear array transducer (7-12 MHz) using My Lab. 25 gold (eSaote, Italy) USG platform. The centre of the artery was identified and the clearest pictures of anterior and posterior intima layers were obtained. Intima to intima measurement was obtained at baseline (D1) and then a sphygmomanometer cuff was wrapped around the forearm distal to the scanned region and inflated to 200 mm Hg for 5 minutes. The luminal diameter was measured again 60 secs after cuff deflation (D2).

FMD was calculated based on the following formula ; $\frac{(D2 - D1) * 100}{D2}$. An FMD value lower than 4.5% was considered as a cut-off for ED [17–19].

Measurement of Nitrite by Griess assay:

Supernatant from deproteinized plasma samples was then used for the determination of NO. Reduction of nitrate to nitrite was done by adding 100 µL vanadium(III) chloride (8mg/ml) to each tube and the Griess reagents, [50 µL sulphanilamide (2%) and 50 µL 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD)] was added and incubated for 30 minutes at 37 °C. Absorbances were taken at 540 nm using a spectrophotometer (BIO-RAD, US). The concentration of NO in serum samples was determined from a linear standard curve established by 0-20 µmol/l sodium nitrite. [19]

Measurement of Serum Cytokines and Cell-adhesion Molecules by ELISA:

Blood samples were collected within a clot vial spun at 3000 Xg for 7 minutes to separate serum. The serum concentration of ET1, TGFβ, IL-6, sVCAM, sICAM, and VEGF were measured by using ELISA kits (KINESIS Dx, Los Angeles), according to the manufacturer's protocol.

Extraction of total RNA:

One milliliter of collected blood was incubated at room temperature with RBC lysis buffer at a 1:10 ratio. Cells were pelleted down and washed twice with 1x PBS. Cells were incubated with 1 ml of TRIzol reagent (Thermo Fisher Scientific, MA, USA) and chloroform (Himedia, Mumbai) (TRIzol: chloroform = 1: 0.25) in

ice. After centrifugal phase separation, total RNA was salted out with isopropanol, washed with 70% ethanol (Himedia, Mumbai), and air-dried. Then the air-dried RNA was dissolved in RNase-free water and stored at -400C for downstream experiments.

Quantitative Real-Time PCR (qRT-PCR) to analyze gene expression:

About 2.5 µg of total RNA was treated with 1 unit of DNase-I (Roche Diagnostics, Mannheim, Germany) and the first-strand cDNA was synthesized using RevertAid Reverse Transcriptase at 42°C following the manufacturer's protocol (Thermo Fisher Scientific, MA, USA). This cDNA was diluted (1:20) and used to quantify the expression of genes (eNOS, iNOS, ET1, TGFβ) using SyBr green (Roche Diagnostics, Mannheim, Germany) and respective primer sets in Quant studio 7 (Thermo Fisher Scientific). 18s ribosomal RNA was used as an internal control for relative quantification of the gene expression each reaction was run in triplicate for statistical accuracy.

Immunophenotyping of CECs by Flow cytometry Analysis:

Peripheral blood (100 µl) was incubated at room temperature with 1 ml. RBC lysis buffer (BD Biosciences, BD Biosciences, USA.). After lysis, the cells were pelleted down and washed twice with 1x PBS. Cells were incubated with specific antibodies : CD45 (cat no: 560777, V500; BD Pharmingen, USA), CD105 (cat no: 560819, BD Pharmingen, USA) CD31 (cat no: 560983, PE; BD Pharmingen, USA) to identify circulating endothelial cells (CECs). The percentage of circulating endothelial cell were measured by FACS. Whole blood cells were morphologically gated within characteristic linear forward scatter (FSC-A) and side-scatter (SSc-A). The gated population was plotted against SSc-A and CD45. CD45 negative populations were plotted against CD105 and CD31. Cells that were positive for CD105/CD31 were gated and % was calculated as CECs.

Statistical Analysis:

All statistical analyses were performed using GraphPad prism v 5.0, (GraphPad Software INC, CA, and USA) and MedcalC v 11.6, (Belgium). All normally distributed data were tested by applying the Shapiro-Wilk test. Normally distributed variables were presented as mean values with standard deviation (SD). The difference between groups was estimated by the independent *t* test and Man-Whitney *U* test for normally distributed and skewed data, respectively. Spearman (*r*) value was used for the correlation coefficient. All *p* values <0.05 were considered statistically significant.

Results

Baseline characteristics of the patients and HCs:

The mean ages of patients (n = 56): 35.4 ± 8.6 years (mean $\pm$ SD), [Group-I: (35.6 ± 10.5 , n=24), Group-II: (35.7 ± 7.7 , n=10), Group-III: (35.0 ± 8.1 , n=22)], and HC (35.3 ± 9.4 , n= 20) did not vary significantly. The male to female ratio in HC was 16:4 and in the patient, groups were 20:4, 8:2, and 18:4 respectively. The mean disease duration for the groups was: Group-I: 2.9 ± 2.3 , Group-II: 3.6 ± 1.9 , Group-III: 4.2 ± 2.2 . Among the total patient groups, 96.4% of patients were SCL70 positive. Positivity against the autoantibody production and the detailed demography have been tabulated in *Table 1*.

FMD among Patients and HCs:

Despite receiving the recommended standard of treatment, FMD proportion was significantly reduced in all groups of SSc patients compared to the HCs; [Group-I versus HC ($p < 0.0001$), Group-II versus HCs ($p = 0.001$), Group-III versus HCs ($p < 0.0001$). However, no significant differences in FMD were noted among the patient groups [$p = 0.2$ (Group-I vs Group-II), $p = 0.7$ (Group-I vs Group-III), $p = 0.4$ (Group-II vs Group-III)] (*Figure 1A*).

Comparative Serum Concentration of Vasoactive Substances associated with Maintenance of vascular Tone:

The NO and ET1 are the major regulative factor in regulating the vascular tone. As there is no difference in FMD (%) among the different patient groups, we measured the concentration of NO and ET1. Serum NO concentration (pg/ml) was significantly lower in Group-I compared to the HC ($p < 0.0001$). Both the treated groups (II and III) have restored serum NO at a level comparable to the HC as no significant differences ($p = 0.6$, $p = 0.7$ respectively) are found between HC with Group-II and Group-III. Whereas, significant differences [Group-I vs Group-II: ($p = 0.0003$), Group-I vs Group-III ($p = 0.03$)] were present between the treated and untreated groups (*Figure 2A*).

Serum concentration ET1 (pg/ml) showed no significant difference between the HCs and Group-I ($p = 0.1$). Interestingly, patients of group-II and group-III showed significantly higher ET1 expression than HCs ($p = 0.03$, and $p < 0.0001$, respectively) as well as from Group-I ($p = 0.001$, and $p < 0.0001$, respectively). Inter-group analysis showed higher expression of ET1 in group-III compared to the Group-II ($p = 0.04$) patients (*Figure 2B*).

The ratio of serum concentration between NO and ET1 (NO/ET1) was also analyzed among the study groups, as this ratio is an important factor for maintaining vascular tone. NO/ET1 was significantly decreased in all patient groups compared to HCs (Group-I: $p < 0.0001$, Group-II: $p = 0.04$, Group-III: $p = 0.001$). Intergroup analysis showed no difference in NO/ET1 among the different patient subgroups (*Figure 2C*).

Comparative mRNA expression of the mediators associated with maintenance of vascular tone:

We also measured the expression of eNOS and iNOS. SSc patients showed statistically significant downregulation of eNOS compared to HCs [Group-I: ($p = 0.0005$), Group-II: ($p = 0.001$), Group-III: ($p = 0.001$)]. Group-I had a significantly lower expression of eNOS than group-III ($p = 0.03$), but no such

differences were noted between Group-I versus Group-II ($p=0.4$), and Group-II versus Group-III ($p=0.4$). (Figure 2D).

Compared to the HCs, significant downregulation of iNOS was noted in patient groups [Group-I ($p=0.0002$), Group-II ($p=0.0007$), Group-III ($p=0.0002$)]. Intergroup analysis among SSc patients did not show any significant differences in iNOS mRNA expression (Figure 2E).

No significant differences were observed in the ET1 mRNA expression among the study groups (Figure 2F).

Comparative Account on Molecular Parameters associated with Fibrosis, Angiogenesis, and Cell Adhesion:

The serum concentration of TGF β was significantly higher in all patient sub-groups compared to HCs ($p<0.0001$, $p=0.004$, and $p=0.002$ for Group-I, Group-II, and Group-III, respectively). However, treated SSc patients had a significant decrease in the serum concentration of TGF β . Group-I had a significantly higher concentration among the patient groups ($p=0.004$ and $p<0.0001$ for group-II and group-III, respectively). Group-III had a significantly lower concentration of TGF β compared to Group-II ($p=0.04$) (Figure 3A).

Concerning the serum concentration of TGF- β , its mRNA expression also showed a similar trend. All patient groups, except Group-III, showed significantly upregulated expression of TGF β compared to healthy control (Group-I: $p<0.0001$, Group-II: $p=0.05$, Group-III: $p=0.5$). Group-I had significantly higher TGF- β mRNA expression compared to the other patient groups (Group-II: $p=0.04$, Group-III: $p=0.0002$) (Figure 3B).

We also assessed the serum concentration of cell adhesion molecules (VCAM and ICAM) in response to the conventional therapeutic approaches. Significant upregulation of sVCAM was observed in all patient groups compared to HC (Group-I: $p=0.01$, Group-II: $p=0.03$, Group-III: $p=0.01$). No differences were present in the sVCAM concentration between the treated groups (Figure 3C). sICAM showed no statistically significant differences between the study groups (Figure 3D).

Regarding Serum VEGF expression, treated groups (Group-II and Group-III) had a significantly higher level than HCs ($p=0.03$, $p=0.006$ respectively), and Group-I ($p=0.04$, $p=0.007$ respectively). No difference was found regarding serum concentration of VEGF between Group-II and Group-III (Figure 3E).

Within the patient groups, group-I has the highest serum concentration of IL-6 compared to HCs ($p=0.001$). Whereas, no significant differences were found between the treated groups and HCs. Intergroup analysis of serum IL-6 concentration in SSc patients showed significant downregulation in Group-III compared to Group-I. No differences were found between group-I and Group-II, Group-II and Group-III (Figure 3F).

Correlation Analysis Between the Molecules associated with Endothelial Dysfunction and FMD:

Correlation statistics were done between NO, ET1, NO/ET1, and the mediators of fibrosis, angiogenesis, and FMD to study the comparative impact of NO and ET-1 on ED. A strong positive correlation of FMD was noted with NO ($r=0.6$, $p<0.0001$) (Figure 4A). Whereas no correlation was found between FMD and ET1 ($r=-0.04$, $p=0.8$) (Figure 4B). NO/ET1 had a positive correlation with FMD ($r=0.3$, $p=0.02$) (Figure 4C).

NO and ET1 showed negative correlations with TGF β ($r=-0.5$, $p=0.001$ and $r=-0.4$, $p=0.001$ respectively) (Figure 4D-4E). No correlation was found between NO/ET1 and TGF β (Figure 4F). VEGF showed a positive correlation with NO/ET1 ($r=0.4$, $p=0.01$) (Figure 4F-H) but not with these individual molecules. IL6, sVCAM, sICAM showed no significant correlation with NO, ET1, and NO/ET1.

The comparative proportion of CECs in HCs and Group-I patients and its correlation with FMD, NO, ET1, and NO/ET1:

Percentages of CECs were significantly higher in Group-I compared to HCs ($p=0.002$) (Figure 5B). FMD showed a strong negative correlation with the proportion of CEC ($r=-0.6$, $p=0.003$) (Figure 5D). A strong negative correlation was found between NO and CEC ($r=-0.7$, $p<0.0001$) and NO/ET1 with CEC ($r=-0.6$, $p=0.007$) (Figure 5E-G).

Discussion

SSc is a clinically heterogeneous autoimmune disorder typically characterized by vasculopathy, autoimmunity/inflammation, and fibrosis [20]. Vasculopathy is being considered as a leading event in the SSc pathogenesis which subsequently acts as a connection between autoimmunity and dysregulated fibrosis. Systemic autoimmunity and environmental triggers are the possible pioneers of vasculopathy, which is characterized by both abnormal activation and damaged endothelial cells [21]. Brachial arterial FMD measurement is a surrogate marker of endothelial function and a significant reduction in the FMD in SSc patients has been documented in the literature [9, 22–24]. There is a paucity of data regarding the effect of treatment on ED of SSc and the correlation among the markers of ED, vasoactive, angiogenic, and fibrogenic mediators. We stratified our SSc patients based on the therapeutic regimen and found a significant reduction in the FMD across all patient groups. It indicates that current therapeutic modalities may not be adequate to restore vasculopathy in SSc patients. This finding is also supported by the fact that currently there is no approved disease-modifying drug for SSc [25]. An imbalance between NO and ET1 is already established in SSc vasculopathy [26–27]. However, the role of ET-1 is yet to be elucidated properly. The current EULAR guidelines recommend using phosphodiesterase-5 inhibitor or endothelial receptor antagonist for the management of the different symptoms related to vascular insufficiency, aimed at counteracting the NO and ET-1 imbalance [28]. In reality, the symptoms of vascular insufficiency show a fluctuating course in the majority of SSc patients despite ongoing treatment strategies, and irreversible damage is seen with almost 1/5 of patients requiring digital amputation due to gangrene [29]. The impaired balance between NO and ET1 has an impact on the progression of vascular dysfunction. We found a significant downregulation of NO level in the treatment naïve SSc patients than

HCs. In the treated groups, increased NO levels were observed. This is probably because all patients recruited in this study were receiving phosphodiesterase-5 inhibitors as vasodilator therapy.

Interestingly, a similar increasing trend was found in serum ET-1 concentration in treated SSc patients. Vasodilator treatment to counteract the downregulated mediator of vasodilation might have some impact on the balancing mechanism within the biological system to increase the ET1 level. We did not find any significant alteration of NO/ET1 ratio between treatment naïve and treated groups. This result indicates that the ratio between NO and ET1 could be more important in SSc vasculopathy, rather than the absolute value of either one. This finding also explains the persistence of FMD abnormality across all SSc patients irrespective of the treatment. From the clinical viewpoint, it indicates that the current treatment modalities may not be sufficient enough to achieve the optimum benefit in SSc vasculopathy. We also showed a fair correlation of FMD with the NO and NO/ET1 ratio. In concurrence with this finding one recent study documented the superior efficacy of combined phosphodiesterase-5 inhibitors with ET1 receptor antagonists in the management of pulmonary arterial hypertension of connective tissue disease than monotherapy [30].

We also noted a significant downregulation of the eNOS gene expression in the group-I and II patients compared to those receiving immunosuppressive treatment (group-III). Reduced eNOS expression in SSc has been reported in the literature [31]. Reduced eNOS expression in SSc has been linked with inflammation, and our study showed that immunosuppressives in addition to the vasodilators could be more effective in the restoration of downregulated eNOS expression in SSc.

As documented in the literature, TGF β is considered as the master regulator of fibrosis and it may have a role in the angiogenic defect seen in SSc [5, 32]. TGF β was significantly upregulated in all SSc patients. SSc patients treated with both the vasodilator and immunosuppressive agents had a lower TGF β expression compared to the other two groups but it was still significantly higher than HCs. Simultaneously, TGF β gene expression was significantly downregulated in treated groups than in non-treated patients. However, these favourable findings were not translated into the significant increment of the FMD in the treated patients, though FMD was numerically higher in the treated groups than naïve patients. Treatment modalities showed effectiveness in reducing the serum IL-6 concentration which was most prominent in patients receiving immunosuppressive therapy compared to treatment-naïve patients. We also noted significant upregulation of sVCAM level but not of sICAM level in our SSc patients, and one recent study also reported a similar result [33]. According to the previous studies, TGF β induced endothelial injury is mediated by the upregulation of sICAM whereas has no relationship with sVCAM [34, 35]. In this regard, our data indicates the efficacy of the treatment modalities in controlling TGF β and in controlling the expression of sICAM and sVCAM.

CEC has gained much attention in recent years and it is considered a marker of endothelial damage [15, 19]. One recently published study showed that CEC can be a direct indicator of the systemic vascular damage of SSc [36]. Considering both FMD and CEC as indicators of ED, we tried to find the CEC level in treatment naïve SSc patients, as this group had the lowest FMD. We found a significantly higher

proportion of CEC in the treatment naïve group, and the CEC proportion showed a strong negative correlation with both FMD and NO but no correlation with ET1. These findings indicate that NO downregulation may be the major cause of ED in SSc patients rather than ET1 upregulation.

The relatively small size of group-II and cross-sectional design are the major limitations of our study. A prospective study with SSc patients aiming at FMD evaluation before initiation of any treatment and follow-up at different time frames after initiation of vasodilator and/or immunosuppressive treatment would be a more pragmatic approach. It will also provide valuable information regarding the additional benefit of immunosuppressive agents in addition to the vasodilators in counteracting SSc vasculopathy if any. Despite these limitations, our study has some unique aspects. A comparative assessment of the FMD in treatment naïve SSc and different treatment groups has been done in our study to get information regarding treatment effects on ED. Simultaneously, we also assessed different vasoactive and inflammatory mediators and their gene expressions in SSc patients. Most importantly, we evaluated the CEC proportion in treatment naïve SSc patients and showed its correlation with FMD. To the best of our knowledge, this would be the first instance when such findings are being reported.

Declarations

1. Conflict of interest: Nothing to declare.

2. Author Contributions:

All authors contributed to the study conception and design and helped in the perusal of the study. The first draft of the manuscript was written by, Dipanjan Bhattacharjee and all authors commented on the subsequent versions of the manuscript. All authors read and approved the final manuscript.

3. Funding statement: Nil.

4. Ethical approval:

This study was approved by the Institutional ethical committee of IPGME&R (No: IPGME&R/IEC/2018/533).

5. Consent to participate :

Written, informed consent was taken from each participant. This study was done following the Declaration of Helsinki, 1964, and its later amendments.

6. Consent to publish

No individual patient information has been disclosed in the manuscript.

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Tables

Table 1. Positivity against the autoantibody production and the detailed demography have been tabulated in table.

Variables	Healthy (n=20)	SSc Patients (n= 56)			P value
		Group-I (n=24)	Group-II (n=10)	Group-III (n=22)	
Age (Years, mean $\pm$ SD)	35.3 $\pm$ 9.4	35.6 $\pm$ 10.5	35.7 $\pm$ 7.7	35.0 $\pm$ 8.1	NS
Sex (female/male)	16: 4	20: 4	8 : 2	18 : 4	NS
Disease duration (Years, mean $\pm$ SD)	-	2.9 $\pm$ 2.3	3.6 $\pm$ 1.9	4.2 $\pm$ 2.2	NS
mRDss (mean $\pm$ SD)	-	23.4 $\pm$ 9.0	18.3 $\pm$ 10.2	18.9 $\pm$ 12.1	NS
Drugs received	-	N.A	Tadalafil (n=8, mg/day) Nifedipin (n=2, mg/day)	Tadalafil (n=22) Prednisolone (n= 20, 6.92 mg/day) Rituximab, 5 th dose (n=1) Cyclophosphamide (n=2) HCQs (n=2) Methotrexate (n=2)	
SCL 70	-	n= 24	n = 8	n= 22	
NOR 90	-	n = 5	-		
Centromere A	-	n= 2	n = 1		
Centromere B	-	n = 4	-		
Th/TO	-	n= 10	n = 1		
PM-SCL 75	-	n= 3	-		
PM-SCL 100	-	n= 1	-		
RNA POL-III (RP-11)	-	n = 5	n = 1		
RNA POL-III (RP-155)	-	n = 4	n = 1		
RO 52	-	n = 5	n = 2		
Ku	-	n = 4	n = 1		
Ribosomal -P- protein (RIB)	-	n= 1	-		
Fibrillarin	-	n= 1	-		
PDGFR	-	-	-	n= 1	

Figures

Figure 1

See image above for figure legend.

Figure 2

The comparative serum concentration of vasoactive substances associated with maintenance of vascular tone.(A-B) Comparative Serum concentration of NO and ET1 in different study populations. (C) Intergroup comparison of NO/ET1 ratio in different study groups. (D-E) Comparative intergroup analysis of eNOS and iNOS within the study populations. (F) A comparative account of mRNA expression of ET1 in different study groups.

Figure 3

The comparative account on molecular parameters associated with fibrosis, angiogenesis, and EC adhesion in response to the conventional therapeutic approaches: (A-B) Comparative intergroup analysis of translational and transcriptional expression of TGF β . ***(C-D)*** Relative intergroup analysis of cell adhesion molecules: sVCAM and sICAM respectively. ***(E-F)*** Comparative statement on the serum concentration of VEGF and IL6 in between the study groups.

Figure 4

Relative correlation analysis of NO, ET1, and NO/ET1 with molecules associated with endothelial dysfunction: (A-C) Correlation analysis of FMD (%) with NO, ET1, and NO/ET1 respectively. ***(D-F)*** Correlation of NO, ET1, and NO/ET1 with master regulator TGF β . ***(G-I)*** Correlation analysis of VEGF with NO, ET1, and NO/ET1.

Figure 5

Comparative % of CEC and its correlation with vasoactive molecules (NO, ET1) and NO/ET1: (A-B) Representative quadrant plot of immunophenotyping of CECs (%) from whole blood of healthy controls and SSc patients by flow-cytometry. Whole blood cells were morphologically gated within characteristic linear forward scatter (FSC-A) and side-scatter (SSc -A). The gated population was plotted against SSc -A and CD45. CD45⁻ populations were plotted against CD105 and CD31. Cells that were positive for CD105/CD31 were gated and % was calculated as CECs. ***(C)*** Box and whisker plot showing the comparative frequency(%) of CECs in naïve SSc patients (Group-I) and healthy control. ***(D-G)*** A comparative account of the correlation between CECs with FMD, NO, ET1, and NO/ET1.