Erectile Dysfunction

ORIGINAL ARTICLE

Tadalafil treatment had a modest effect on endothelial cell damage and repair ability markers in men with erectile dysfunction and vascular risk

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The number of the circulating angiogenic cells (CACs) and colony forming units (CFUs) derived from cultured circulating mononuclear cells (MNCs) represents a laboratory surrogate for endothelial cell repair ability. The serum of men with erectile dysfunction (ED) and vascular risk factors (VRFs) showed an increased level of endothelial cell damage/dysfunction markers and reduced the numbers of CACs and CFUs derived from the cells of healthy men. We analyzed whether treating men with ED and VRFs with the selective phosphodiesterase type 5 inhibitor tadalafil improved the endothelial cell repair ability and reduced the levels of the serum markers of endothelial cell damage/dysfunction. MNCs from healthy men were cultured with 20% serum from 36 ED patients to obtain CACs and CFUs. The ED patients were evaluated before and after 4 weeks of treatment with tadalafil (20 mg every other day) or with a placebo. The tadalafil treatment improved erectile function (P = 0.0028), but had no effect on the inhibitory effects of serum from ED patients on the CACs and CFUs derived from healthy men. The levels of endothelin-1 (P = 0.011) and tissue type plasminogen activator (P = 0.005) were reduced after treatment compared to baseline and those of the placebo group, whereas no changes were observed in the E-selectin levels. The tadalafil treatment in the ED patients with VRFs resulted in only a modest effect on the laboratory measures of the endothelial cell damage/dysfunction and repair ability. The proposed beneficial effect of phosphodiesterase type 5 inhibition on vascular homeostasis requires further analysis.
Asian Journal of Andrology (2014) **16**, 290–294; doi: 10.4103/1008-682X.122347; published online: 20 January 2014

INTRODUCTION

A current hypothesis for the initial lesions of atherosclerosis as well as for the pathogenesis of erectile dysfunction (ED) involves endothelial cell damage/dysfunction.[1] Elevated serum levels of cellular adhesion molecules (CAMs) released by a damaged endothelium are found under conditions associated with an increased risk of developing atherosclerosis and ED,[2,3] with or without vascular diseases or vascular risk factors (VRFs).[4–6] ED in men with VRFs or vascular diseases is associated with a dulled capacity for repairing endothelial damage, as suggested by a reduced number of bone marrow-derived circulating endothelial progenitor cells (PGCs) and by a reduced ability of *ex vivo* expanded blood mononuclear cells (MNCs) to form circulating angiogenic cells (CACs).[7–11] Biochemical and functional surrogates of vascular damage and of a reduced endothelial cell repair ability, as well as epidemiologic studies,[12–14] suggest that ED is an early sign of a systemic vascular disease.

Selective inhibitors (i) of phosphodiesterase type 5 (PDE5) in men with ED and VRFs are effective in treating ED, while improving the endothelial function at the brachial arteries and reducing the biochemical measures of endothelial cell damage/dysfunction.[11,15–19] These agents competitively inhibit cyclic guanosine 5'-monophosphate

hydrolysis by PDE5, thereby fostering NO-dependent cyclic guanosine 5'-monophosphate accumulation and the consequent relaxation of vascular smooth muscle cells.[20] It is suggested that the beneficial effect of PDE5i on endothelial cells is related to the stimulation of the synthesis and transcription of endothelial NO synthase mRNA and protein and to Akt-dependent eNOS phosphorylation.[21,22] eNOS-derived NO plays an essential role in the mobilization and function of bone marrow-derived PGCs;[23] whereas, the activation of the intracellular Akt pathway increases the number of *ex vivo* expanded CACs by modulating the survival and/or proliferation of freshly isolated cells.[23,24] PDE5i treatment of men with ED and VRFs is associated with an increased number of circulating PGCs and *ex vivo* expanded CACs.[11,16]

Recently, we demonstrated that the serum from men with ED associated with VRFs may negatively modulate the number of the *ex vivo* expanded CACs and colony forming units (CFUs) derived from the MNCs of healthy men.[6] This finding was associated with increased levels of endothelin-1 (ET-1) and soluble (s) E-selectin compared to the controls. We analyzed the effects of treatment with the selective PDE5i tadalafil on the negative modulation of CACs and CFUs derived from healthy men by the serum of ED patients. As a

secondary outcome, we explored the effect of tadalafil treatment on the circulating markers of endothelial cell damage/dysfunction. The aim of the study was to explore the potential effects of PDE5 inhibition on the laboratory surrogates of endothelial cell damage and repair ability to lend support to the hypothesis of a beneficial effect of this inhibition on vascular homeostasis.[11,15–19]

MATERIALS AND METHODS

Study population
Men aged 30–70 years affected by ED for more than 6 months who were treated at the Andrology Clinic of the Department of Internal Medicine at the University Hospital of L'Aquila were invited to participate in the study, and the Sexual Health Inventory for Men[25] was used to evaluate erectile function in these patients. The exclusion criteria were a history of endocrine diseases other than type 2 or type 1 diabetes, hypogonadism (plasma testosterone level <250 ng dl^{-1}), pelvic surgery or trauma, prostatic disease, penile curvature, neurological or major psychiatric disorders, coronary artery disease, stroke and other overt vascular diseases. The exclusion criteria included active peptic ulcer disease; alcohol or substance abuse; major hematological, renal or hepatic abnormalities; and nitrate therapy. Thirty-six ED patients were enrolled in the study, along with a group of 10 healthy men (aged 38.2 ± 12.4) with no recognized VRFs and who were not suffering from ED. The participants were asked to sign an informed consent form, and the local ethics committee approved the study.

Protocol
The enrolled patients were first evaluated to assess the common carotid arteries to determine their intima-media thickness (IMT), and Doppler spectrum analysis was used to assess the cavernous arteries, as reported.[5] After a 4-week run-in period during which the patients were asked to avoid the consumption of PDE5i, acetyl salicylic acid and any antioxidant or vasoactive drug, the participants were randomized to treatment with tadalafil (20 mg tablets) (Cialis™, Lilly Icos, Indianapolis, IN, USA) or placebo, every other day for 4 weeks. A coded package of 14 capsules containing 20 mg of tadalafil or placebo was provided for each participant by an investigator who was not involved in the laboratory evaluations that followed. The blood sampling was performed in the morning after an overnight fast before and after 4 weeks of therapy.

Ex vivo expansion assay and characterization of circulating angiogenic cells
The MNCs were isolated from 20 ml of peripheral blood using Ficoll density-gradient centrifugation, washed three times in phosphate-buffered saline, and suspended in endothelial basal medium (EBM-2) (Euroclone SPA, Milan, Italy) supplemented with 20% fetal bovine serum (FBS) (Celbio, Milano, Italy); 10^6 MNCs per cm^2 were seeded on fibronectin-coated culture dishes (Beckton and Dickinson, Milan, Italy). After 4 days of culture at 37 °C with 5% CO_2, the nonadherent cells were discarded by washing with phosphate-buffered saline, and the adherent cells were maintained in culture for an additional 3 days before undergoing cytochemical analysis. The adherent cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine-labeled acetylated low-density lipoprotein (DiLDL) (Invitrogen, Milano, Italy) at a concentration of 2.4 µg ml^{-1} for 1 h at 37 °C. The cells were fixed in 1% paraformaldehyde for 10 min and incubated with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin 1 (UEA-1) (Sigma-Aldrich, Milano, Italy) at a concentration of 10 µg ml^{-1} for

1 h. The cells double positive for DiLDL and FITC-labeled UEA-1 were judged as functional CACs and were counted.[11] The CACs were counted manually in 10 randomly selected microscopic fields by two independent investigators with an inverted fluorescence microscope (× 20) (Zeiss, Oberkoken, Germany).

Colony-forming assay
MNCs suspended in Medium199 (Sigma-Aldrich) supplemented with 20% FBS were seeded at 10^6 cm^{-2} on 24 well fibronectin-coated culture plates. After 48 h of culture at 37 °C with 5% CO_2, the nonadherent cells were collected and replated onto fibronectin-coated dishes with new medium for an additional 3 days. A colony unit consisted of multiple thin, flat cells emanating from a central cluster of rounded cells.[26] The colonies were counted manually in 10 randomly selected microscopic fields by two independent investigators with an inverted microscope (× 20) (Zeiss).[6]

Effect of human serum (HS) on the circulating angiogenic cells and colony forming units from healthy men
The CACs and CFUs obtained as described were compared to those obtained when replacing the FBS with 20% HS from the control subjects and from ED patients at baseline and at the end of treatment. In the assays, HS was added to fresh culture medium during the last 3 days of culture. The results were expressed as the percentage of variation compared to the number of CACs and CFUs obtained in the culture medium supplemented with 20% FBS.[6]

Blood determination of endothelial damage/dysfunction markers and the endothelial cell-dependent prothrombotic state
Enzyme-linked immunosorbent assays were used to assess the plasma concentrations of sE-selectin and ET-1 as markers for endothelial cell damage/dysfunction and of tPA as an endothelial prothrombotic mediator.[5,27] All the assays were performed in duplicate and according to the manufacturer's performance characteristics as follows: sE-selectin (DIACLONE, Besançon Cedex, France), minimum detectable level <0.5 ng ml^{-1}; the intra- and inter-assay coefficients of variation were 4.11% and 6.36%, respectively; ET-1 (BIOMEDICA, Wien, Austria), minimum detectable level 0.02 fmol ml^{-1}; the intra- and inter-assay coefficients of variation were 3% and 5%, respectively; and tPA (ASSAYPRO, St Charles, MO, USA), minimum detectable level 0.0625 ng ml^{-1}, the intra- and inter-assay coefficients of variation were 4.9% and 7.3%, respectively. The plasma fasting glucose, total cholesterol and low-density lipoprotein-cholesterol were obtained from all the participants following standard procedures. The total testosterone was determined through a chemiluminescent microparticle immunoassay (ARCHITECT System, Abbott, Longford, Ireland) according to the manufacturer's protocol, and the analytical sensitivity was < 0.08 ng ml^{-1}.

Statistical analysis
The data were presented as the means ± standard deviation (s.d.). The Kruskal-Wallis one-way analysis of variance by ranks followed by the Wilcoxon rank-sum test assessed the differences among groups. A downward adjustment of the alpha level on the Wilcoxon rank-sum test was applied to compensate for the multiple comparisons to maintain the overall probability at a level of 0.05. The differences in proportion were assessed by the Pearson c2 test. A two-factor analysis of variance for repeated measures on one factor (Proc GLM by SAS; Statistical Analysis System Institute, Inc. Cary, NC, USA) after the logarithmic transformation of data was applied to evaluate the pre- and posttreatment differences in the tadalafil and placebo groups. All the statistical analyses were performed using SAS Software.

RESULTS

Patient characteristics and the effect of tadalafil treatment on erectile function

There were no differences in the characteristics at baseline between the patients randomized to the tadalafil therapy or placebo group, whereas differences were observed between the patient groups and controls (**Table 1**). Erectile function assessed through the Sexual Health Inventory for Men score was similar in the tadalafil and placebo groups at baseline (9.11 ± 6.28 and 10.15 ± 6.88, respectively) and improved after tadalafil treatment (14.00 ± 8.13) compared to baseline and the placebo treatment (8.8 ± 7.5), as demonstrated by the treatment-by-time interaction in the two-factor analysis of variance for the repeated measures on one factor (F = 10.38; P = 0.0028) (**Figure 1**). No subjects on tadalafil had to discontinue the medication because of adverse events.

Effect of tadalafil on the inhibition of circulating angiogenic cells and colony forming units from healthy men by the serum from men with erectile dysfunction

An inhibitory effect on the CACs and CFUs derived from healthy men was observed when replacing FBS with 20% HS from the controls. The mean number of CACs was 83.0% ± 34.5% and the mean number of CFUs was 63.8% ± 18.3% when the MNCs of healthy men were cultured in the presence of 20% HS from the controls relative to the numbers for FBS (**Table 2**). The inhibitory effect in both assays was higher with serum from the ED patients than for serum from the controls ($P < 0.017$), and

no differences were observed between the baseline values of the patients treated with tadalafil and those treated with the placebo (**Table 2**). Treatment with tadalafil had no effect on the serum inhibition of the CACs and CFUs derived from healthy men (**Figure 1**).

Effect of tadalafil treatment on markers of endothelial cell damage/dysfunction and the prothrombotic state

The circulating levels of all the studied markers were comparable at baseline in the two groups of patients (tadalafil and placebo) (**Table 2**). The serum levels of sE-selectin and ET-1 were higher in both groups

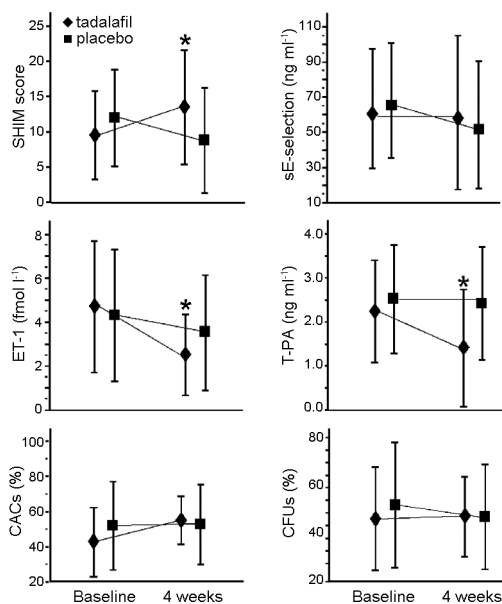**Table 1: Baseline clinical characteristics of the studied groups**

Variables	Control (n=10)	Tadalafil (n=18)	Placebo (n=18)	P value
Age (year)	38.2±12.4[a]	52.05±8.98	49.61±12.78	0.032
BMI (kg m⁻²)	24.3±0.9[a]	29.64±0.97	28.16±3.56	0.020
Waist (cm)	84.8±4.2[a]	102.67±13.92	98.5±8.1	0.0008
ATPIII score	2.6±4.09[a]	11.5±5.4	10.9±5.2	0.0005
Aspirin (%)	N	16.7	11.1	NS
β-blockers (%)	N	11.1	11.1	NS
ACE inhibitors (%)	N	27.7	38.8	NS
Calcium channel blockers (%)	N	22.2	16.6	NS
Diuretics (%)	N	27.7	22.2	NS
Insulin (%)	N	11.1	11.1	NS
Hypoglycemic agents (%)	N	27.7	22.2	NS
SBP (mmHg)	115±20[a]	135.17±19.0	134.44±17.48	0.029
DBP (mmHg)	80±10[a]	88.3±7.86	86.39±9.52	0.016
Fasting glucose (mg dl⁻¹)	85.8±4.4[a]	124.0±44.93	120.93±36.67	0.0003
Total cholesterol (mg dl⁻¹)	137.5±8.9[a]	213.63±43.06	201.06±38.2	<0.0001
LDL cholesterol (mg dl⁻¹)	80.8±10[a]	129.33±41.16	125.03±29.58	0.0002
Testosterone (ng ml⁻¹)	5.3±1.1	6.0±3.0	5.9±2.37	0.29
Cavernous arteries PSV (cm s⁻¹)	-	44.60±8.74	46.58±8.46	0.37
Carotid artery IMT (cm)	0.06±0.01[a]	0.09±0.03	0.10±0.03	0.0003
SHIM score	25±00[a]	9.11±6.28	10.15±6.88	<0.0001

ACE: angiotensin converting enzyme; BMI: body mass index; DBP: diastolic blood pressure; IMT: intima-media thickness; LDL: low density lipoprotein; N: none; NS: not significant; PSV: peak systolic velocity; SBP: systolic blood pressure; SHIM: sexual health inventory for men. [a]$P<0.017$ compared to ED groups treated with tadalafil or placebo with the Wilcoxon rank-sum test. Differences evaluated by Kruskal-Wallis one-way analysis of variance by ranks or by the X^2 test, as appropriate. The values are expressed as the mean±standard deviation (s.d.).

Figure 1: Erectile function (Sexual Health Inventory for Men (SHIM) score), circulating levels of soluble endothelial (sE)-selectin, endothelin (ET)-1, tissue type plasminogen activator (t-PA), percentages of circulating angiogenic cells (CACs) and colonies (CFUs) derived from circulating mononuclear cells of healthy men in the presence of human serum compared to standard culture medium. The symbols indicate the mean value, and the bars indicate the standard deviations in the patients with ED and VRFs before and after treatment with tadalafil or placebo. All the parameters were comparable at baseline between the two groups of patients with ED. The effect of tadalafil treatment compared to placebo and to the baseline values, as assessed by two-factor analysis of variance for repeated measures on one factor, resulted in a significant increase (*) in the SHIM score (F = 10.38; P = 0.0028) and in a reduced level (*) of ET-1 (F = 7.26; P = 0.011) and of t-PA (F = 8.99; P = 0.05).

Table 2: Baseline laboratory characteristics of the studied groups

Parameters	Control (n=10)	Tadalafil (n=18)	Placebo (n=18)	P value
sE-selectin (ng ml⁻¹)	33.5±8.6[a]	63.1±33.8	68.1±32.0	0.0018
ET-1 (fmol ml⁻¹)	1.6±0.43[a]	4.7±2.9	4.3±3.0	0.0057
t-PA (ng ml⁻¹)	2.7±0.65	2.2±1.1	2.5±1.2	0.2067
CACs %	83.0±34.5[a]	45.7±19.5	50.0±25.0	0.0103
CFUs %	63.8±18.3[a]	43.7±20.4	46.6±25.2	0.0353

CACs % and CFUs %, percentages of circulating angiogenic cells (CACs) and colony forming units (CFUs), respectively, from the circulating mononuclear cells of healthy men after replacing fetal bovine serum with 20% human serum compared to standard culture medium; ET-1: endothelin-1; t-PA: tissue type plasminogen activator. [a]$P<0.017$ compared to ED groups treated with tadalafil or placebo with the Wilcoxon rank-sum test. Differences evaluated by Kruskal-Wallis one-way analysis of variance by ranks. The values are expressed as the mean±standard deviation (s.d.).

of patients than in the controls ($P < 0.017$). No differences were observed in the circulating tissue type plasminogen activator (t-PA) levels among the three groups (**Table 2**). Tadalafil treatment resulted in a reduction in the ET-1 levels compared to baseline and to placebo treatment (F = 7.26; $P = 0.011$), and the results were comparable to the baseline values obtained for the controls (**Figure 1** and **Table 2**). Tadalafil treatment was not associated with changes in the circulating levels of sE-selectin, whereas the t-PA levels were reduced (F = 8.99; $P = 0.005$) (**Figure 1**).

DISCUSSION

This study revealed that continuous treatment with tadalafil, a selective PDE5i with a long half-life,[28] did not affect the inhibitory effect of the serum of men with ED and VRFs on the *ex vivo* expanded CACs and CFUs from healthy men.[6] The tadalafil treatment was associated with a reduction in the ET-1 levels, but not the sE-selectin levels, both of which were higher at baseline than in the healthy men. A reduced level of t-PA, a marker for the endothelium-dependent prothrombotic state,[27] was observed after treatment, although this factor was not different at baseline in the ED patients and in the healthy men, thus suggesting that its variation after treatment deserves confirmation in a larger study. Tadalafil treatment of ED patients with VRFs improved erectile function but had only a modest effect on the laboratory measures of endothelial cell damage/dysfunction and repair ability.

Increased ET-1 levels were reported in the ED patients with or without VRFs and in the patients with overt vascular disease.[4,5,29–31] The blockade of ETa receptors improved the endothelial function in the patients with early atherosclerosis, suggesting a pathogenetic role of increased ET-1 levels in endothelial dysfunction.[32] PDE5 inhibition downregulates the ET-1 system,[33] which explains the reduced levels of ET-1 in the men with ED and VRFs treated with PDE5i.[15,17,34] Our data confirmed this finding and demonstrated that the ET-1 levels were restored to those observed in healthy men, although its clinical relevance has yet to be defined. The men with ED treated with an ETa receptor antagonist (BMS.193884) showed no improvement in erectile function,[35] suggesting that ET-1 might not have a relevant role in the pathogenesis of ED. Whereas available studies, including this one, documented a reduced level of ET-1 in ED patients treated with PDE5i,[15,17,34] conflicting results were obtained when biochemical measures of endothelial activation/damage other than ET-1 were considered. The levels of sE-selectin,[17,18] sPlatelet (P)-selectin and s-Intercellular adhesion molecule-1 (ICAM-1) were not altered in the ED men with VRFs treated with PDE5i;[18,36] whereas, the same treatment was associated with reduced levels of sICAM,[19] sP-selectin and sVascular cell adhesion molecule-1 (VCAM-1).[18,37] A limitation in these trials is the lack of a healthy group to determine whether the levels of CAMs released by the endothelial cells were elevated in ED men. As reported for ET-1, the sE-selectin levels were increased at baseline in the ED patients compared to the healthy men, but no changes were observed after the tadalafil treatment. The major reason for the conflicting results between the different studies could be because of differences in patient selection and the treatment protocol. The variability after treatment between the different biochemical measures might suggest a poor beneficial effect of PDE5i on systemic endothelial cell activation/damage, at least with the proposed treatment protocol.

Tadalafil treatment in men with ED and VRF is associated with an improved capacity for repairing endothelial damage, as suggested by the increased number of *ex vivo* expanded CACs.[11] This effect is expected after considering the PDE5i-dependent stimulation of eNOS[21–22] and the essential role of eNOS-derived NO in stimulating

the number of *ex vivo* expanded CACs.[23,24] This apparently conflicts with the finding that tadalafil treatment did not remove the inhibitory effect of the serum of men with ED and VRFs on the CACs and CFUs derived from healthy men (**Figure 1**). In a previous paper, we showed that the vascular risk score, but not the levels of biochemical measures of endothelial cell damage/dysfunction, was related to the inhibition of the CACs and CFUs derived from the MNCs of healthy men and cultured in the presence of serum from ED men.[6] This finding suggested that unidentified soluble factors in the serum of men with ED induced dysfunction of the cells involved in vascular homeostasis, and this dysfunction was dependent on the load of VRF exposure.[6] PDE5i improved erectile function and exerted some positive effects on the functions that benefit from increased eNOS-dependent NO availability such as selected measures of endothelial cell activation/ damage. VRFs, the main determinants of endothelial dysfunction and progression to vascular damage,[38] are not modified by PDE5i treatment. The beneficial effect on endothelial cell by PDE5i is balanced by the detrimental effects on vascular homeostasis exerted by the VRFs. The finding that tadalafil treatment did not remove the VRF-dependent inhibition induced by the serum of ED patients on the CACs derived from healthy men corroborates this view.

Tadalafil treatment of ED patients with VRFs improved erectile function, but had only a modest effect on the laboratory measures of the damage/dysfunction and repair ability of endothelial cells. This finding suggests reconsidering the hypothesis that PDEi has a beneficial effect on vascular homeostasis relying on surrogate measures such as biochemical and/or functional parameters of endothelium health.[11,15–19] Whether PDE5i treatment has a clinically relevant positive effect on vascular health should be investigated by longitudinal studies that have the clinical measures of vascular health as primary outcomes.

The main limitations of this study are the short duration of the treatment protocol and the small number of patients. A larger population and/or longer treatment might demonstrate sharper differences between treatment with PDE5i and the placebo.

AUTHOR CONTRIBUTIONS

FP and SF conceived, designed, coordinated the study, conducted the interpretation of the data and drafted the manuscript. AD and SF performed the cell culture measurements and carried out the biochemical measurements; SD and AP conducted the acquisition and participated in the interpretation of the data; SN and AB performed the statistical analysis and participated in the analysis and interpretation of the data; FA and FF participated in the analysis and interpretation of the data and revised the manuscript. All the authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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