EXPERT OPINION

Endothelial dysfunction markers as a therapeutic target for Sildenafil treatment and effects on metabolic control in type 2 diabetes

Elisabetta Mandosi, Elisa Giannetta, Tiziana Filardi, Manuela Lococo, Camilla Bertolini, Mara Fallarino, Daniele Gianfrilli, Mary Anna Venneri, Luisa Lenti, Andrea Lenzi & Susanna Morano[†]

[†]*Sapienza University of Rome, Policlinico Umberto I, Department of Experimental Medicine, Rome, Italy*

Objective: Endothelial dysfunction (ED) plays a role in diabetic cardiovascular complications. Hyperglycemia increases cytokines involved in vascular inflammation. Inhibition of phosphodiesterase type 5 (PDE5) exerts a relaxation on corpora cavernosa and has cardioprotective properties. The effect of chronic sildenafil treatment, on ED markers and metabolic parameters in a non-randomized study on men with type 2 diabetes (T2DM), was investigated.
Research design and methods: Twenty-eight T2DM patients (61.2 ± 7.8 years, hemoglobin A1c (HbA1c) 7.9 ± 1.3%, duration of diabetes 11.5 ± 7.8 years) were treated with sildenafil 100 mg/d for 3 months. Baseline and postprandial glycemia, insulin, HbA1c, HOMA index, lipids, glomerular filtration rate, homocysteine were assessed at each visit. P-selectin (CD62P), CD14/42b, CD14/41, ICAM (CD54), PECAM (CD31) and CD11b/CD18, were evaluated, after monocyte isolation with flow-cytometry, before and after treatment.
Results: After 3 months, sildenafil decreased P-selectin ($p < 0.05$), postprandial glycemia ($p < 0.01$), HbA1c ($p < 0.01$), low-density lipoprotein cholesterol ($p < 0.01$) and increased high-density lipoprotein ($p < 0.05$).
Conclusions: PDE5 inhibition, in T2DM patients, reduces the endothelial function marker P-selectin and exerts a beneficial effect on glycometabolic control.

Keywords: phosphodiesterase type-5 inhibitors, P-selectin, sildenafil, type 2 diabetes

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1. Introduction

Endothelial dysfunction (ED), defined as an abnormal response leading to a reduction in the bioavailability of nitric oxide (NO) and impaired vasodilation, is involved in the pathogenesis of vascular complications of type 2 diabetes mellitus (T2DM). It is accepted that standard cardiovascular disease (CVD) risk factors do not adequately explain the excess CVD in diabetes and heightened inflammation. An early finding of ED, induced by chronic hyperglycemia, is the release of chemokines such as Monocyte Chemoattractant Protein-1 and others molecules, from endothelium [1], involved in inflammatory pathways of vascular damage. These factors increase the expression of interstitial and vascular cellular adhesion molecules (such as selectins) that attract monocytes and immunocytes to the stressed site [2]. Moreover, according to experimental evidence, hyperglycemia increases reactive oxygen species (ROS) production that decreases the bioavailability of NO, leading to an impaired

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endothelial relaxation. Patients with diabetes are characterized by a reduced response to vasoactive agents [3,4].

Sildenafil is the first specific phosphodiesterase type-5 inhibitor (PDE5i) marketed for the treatment of erectile dysfunction. Inhibition of PDE5 exerts a relaxant effect on the smooth muscle cells of the trabecular structures in the corpora cavernosa by increasing NO availability [3,4]. PDE5 isozyme is widely expressed in the vasculature and recently, promising cardioprotective effects of long-term daily PDE5i administration have been demonstrated in a randomized placebo-controlled trial and in a meta-analysis matching > 1600 patients [5-7].

However, the efficacy of PDE5 inhibitor, used for the treatment of erectile dysfunction, seems to be lower in patients with diabetes compared with those without diabetes [3]. It has been suggested that a diffuse ED is responsible for this lack of response and recently it has been demonstrated in a systematic review of literature that chronic PDE5i administration exerts a beneficial effect on endothelial function, improving flow-mediated dilation and serum pro-inflammatory markers (IL6) in diabetic men [8]. The aim of the present study was to determine the effect of chronic therapy with a selective PDE5i, sildenafil, on ED markers and metabolic control in T2DM patients.

2. Patients and methods

Eligible men were recruited from the outpatient clinics of Policlinico Umberto I, 'Sapienza' University Hospital of Rome between 2008 and 2009. The inclusion criteria were as follows: T2DM, age 35 – 75 years; diabetes duration > 1 year; hemoglobin A1c (HbA1c) < 10% (53 mmol/mol); normal blood pressure or treated hypertension with achievement of a target of < 130/80 mm Hg; and body mass index < 40 kg/m^2. The exclusion criteria were as follows: use of exogenous insulin, thiazolidinediones, or spironolactone; prior or current use of PDE5 inhibitors; substance abuse; history of CVD, proliferative retinopathy, or autonomic neuropathy; symptoms or signs of ischemic heart disease during cardiac evaluations at enrollment and contraindications to sildenafil use. Concomitant medications (e.g., antihypertensives, statins, oral antidiabetic medications) could not be changed between the 3 months before the study. All subjects gave their written informed consent. The protocol was approved by the hospital ethics committee.

2.1 Study design

This study was conducted in T2DM patients consecutively enrolled to receive 100 mg/day sildenafil in fractioned dose (25 mg at 8 AM plus 25 mg at 4 PM plus 50 mg at 10 PM). Patients were monitored monthly for the entire study duration. The study was designed, conducted, and monitored by the study team without industry support.

2.2 Laboratory investigations

The following parameters were assessed at each visit: baseline and postprandial glycemia, insulin, HbA1c, homeostasis model assessment index (HOMA index), lipid profile, glomerular filtration rate (GFR), omocistein and vital signs. Endothelial function markers, P-selectin (CD62P) and inflammatory indices, CD14/42b, CD14/41, ICAM (CD54), PECAM (CD31) and CD11b/CD18 were measured before starting sildenafil and after 3 months of treatment.

2.2.1 *Antibodies and other reagents*

Monoclonal antibodies directly conjugated to fluorochromes used in this study were purchased from the following sources: PE-conjugated CD14 (IgG2a) and CD11b (IgG2a) and FITC-conjugated CD41 (IgG1), CD54 (IgG2b), CD31 (IgG1) and CD18 (IgG1) were obtained from Sigma Chemical Company (Sigma, Aldrich; Milano, Italy); FITC-conjugated CD42b (IgG1) and CD62P (IgG1) were obtained from BioLegend (San Diego, California).

2.2.2 *Monocytes isolation and immunolabeling*

Blood samples were collected in heparizined tubes (10 IU/ml). Monocytes were isolated after centrifugation of the blood with a polysucrose-sodium diatrizoate solution, 1.077 g/ml density and 280 mOsm osmolarity (Lymphoprep; Nycomed, Oslo, Norway) at 800g at 20°C. The mononuclear cell layer was collected and the cells were thus washed two times in a solution of cold phosphate-buffered saline (PBS, pH 7.2), supplemented with 1% fetal calf serum and 2 mmol/l EDTA (Sigma, Aldrich, Milano, Italy). The cell suspension was then incubated with an anti-CD14 antibody (attached on microbead surface) (Miltenyi Biotec, Bergisch Gladbach, Germany) to separate the monocytes from other mononuclear cells [9]. The monocytes obtained (50 µl/1 $\times$ 10^6/ml) were washed and resuspended at a concentration of 1 $\times$ 10^6/ml in PBS and then incubated with a designed amount of specific paired FITC- and PE-conjugated antibodies for 30 min at room temperature. The different combinations were immunostained with different combinations of the following antibodies: PE-conjugated CD14 and FITC-conjugated CD42b, PE-conjugated CD14 and FITC-conjugated P-selectin (CD62P), PE-conjugated CD14 and FITC-conjugated CD41, PE-conjugated CD14 and FITC-conjugated ICAM (CD54), PE-conjugated CD14 and FITC-conjugated PECAM (CD31) and PE-conjugated CD11b and FITC-conjugated CD18. Then, 2 ml of PBS were added to all tubes. Cells were resuspended in 0.5 ml of 2% paraformaldehyde after 10 min centrifugation at 500 g and careful supernatant removal.

2.2.3 *Flow cytometry*

Two-color flow cytometry was used to identify the proportion of monocytes forming aggregates with platelets. Samples were run through a Beckman-Coulter XL2 flow cytometer,

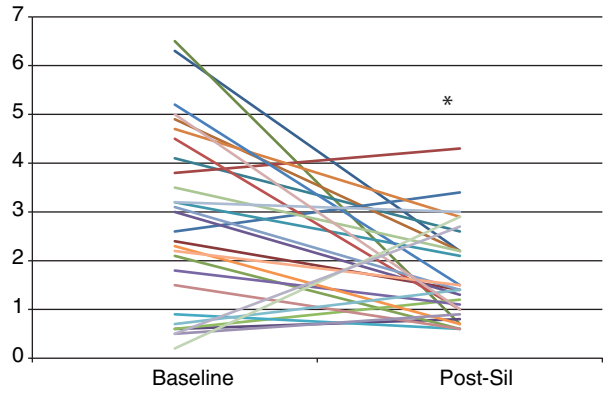

Figure 1. P-selectin reduction in Sildenafil-treated patients.
*p < 0.05.

and data analysis was performed using EXPO32 (Beckman-Coulter) software. For molecular mechanism studies of platelet–monocyte binding, samples were preincubated with saturating concentrations of inhibitory mAb for 15 min before labeling. Platelet–monocyte adhesion was determined using directly conjugated CD14-PE and CD42b-FITC mAb. Leukocyte/platelet fluorescence levels using these antibodies were unaltered by heparin and EDTA treatment.

2.3 Statistical analysis

Continuous variables are reported as mean ± 1 SD. Analysis were performed, as appropriate, by Student's *t*-test, two-way repeated-measures ANOVA, and post hoc comparison (Newman–Keuls and Bonferroni test). Statistical analyses were performed with the use of SPSS for Windows, version 18.0 (SPSS, Inc., Chicago, IL, USA). All statistical comparisons were performed with two-tailed significance tests, with $p < 0.05$ considered statistically significant.

3. Results

All the twenty-eight patients completed the study. They were assigned to receive sildenafil (n = 28, mean age = 61.2 ± 7.8 yrs, duration of diabetes = 11.5 ± 7.8 yrs) and monitored for 3 months. There were no drug-related adverse events.

After 3 months, P-selectin was reduced in the treated group (3.35 ± 3.51 vs 1.79 ± 1.1 %; $p < 0.05$) (**Figure 1**) and (**Table 1**). Moreover, in these patients a significant reduction in post prandial glycemia (10.1 ± 3.1 vs 8.8 ± 2.9 mmol/l; $p < 0.01$), HbA1c (7.9 ± 1.3 vs 7.3 ± 1.1 %; $p < 0.01$), low-density lipoprotein cholesterol (3.01 ± 0.98 vs 2.7 ± 0.9 mmol/l; $p < 0.01$), and a significant increase in high-density lipoprotein cholesterol (1.05 ± 0.2 vs 1.13 ± 0.2 mmol/l; $p < 0.05$) were observed (**Table 2**).

The CD14/42b, CD14/41, ICAM, PECAM and CD11b/CD18 levels were unchanged after sildenafil treatment (**Table 1**). At baseline, a significant correlation was observed only between P-selectin and CD14/41 ($p = 0.01$, data not shown), but after treatment this correlation was not found.

Table 1. Endothelial function and inflammation markers in sildenafil-treated patients.

	Sildenafil (n = 28)	
%	Baseline	3 months follow-up
P-selectin	3.35 ± 3.51	1.79 ± 1.1*
CD14/42b	6.78 ± 8.82	6.63 ± 12.37
CD14/41	12.39 ± 13.67	13.45 ± 18.36
ICAM	67.72 ± 27.6	62.0 ± 30.5
PECAM	98.66 ± 3.7	99.39 ± 0.69
CD11b/CD18	99.49 ± 0.49	99.14 ± 1.18

*p < 0.05.

Table 2. Metabolic parameters in Sildenafil-treated patients.

	Sildenafil (n = 28)	
Metabolic parameters	Baseline	3 months
Basal Glycemia (mmol/l)	8.4 ± 2.4	8.0 ± 2.4
Post-prandial Glycemia (mmol/l)	10.1 ± 3.1	8.8 ± 2.9 *
HOMAi	8.1 ± 6.1	7.9 ± 7.9
HbA1c (%)	7.9 ± 1.3	7.3 ± 1.1*
Total Cholesterol (mmol/l)	4.78 ± 1.0	4.5 ± 0.87
HDL Cholesterol (mmol/l)	1.05 ± 0.2	1.13 ± 0.2*
LDL Cholesterol (mmol/l)	3.01 ± 0.98	2.7 ± 0.9*
Triglycerides (mmol/l)	1.51 ± 0.7	1.54 ± 0.7
eGFR (ml/min)	87.8 ± 16.5	86.0 ± 16.4
CrCl (ml/min)	141.7 ± 42.4	132.4 ± 34.4
Omocistein (μmol/L)	11.5 ± 3.8	12.9 ± 3.5

*p < 0.05.
CrCl: Creatinine clearance; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c; HDL: High-density lipoprotein; HOMAi: Homeostasis model assessment index; LDL: Low-density lipoprotein.

4. Discussion

This study shows that a 3-month treatment with sildenafil at a dose of 100 mg/day improves endothelial function marker (P-selectin) and metabolic control in men with T2DM. Poor glycemic control and associated hyperglycemia are related to the development of vascular complications in diabetes through promotion of ED and atherosclerosis, a chronic and progressive disease characterized by an inflammatory response of the arterial wall [10]. The development of atherosclerotic plaques is influenced by oxidative stress, an imbalance between the accumulation of ROS and limited antioxidant defences; this imbalance compromises NO availability and leads to ED [11,12]. The presence of ED represents a major promoter for atherosclerosis and thrombosis and it is an independent prognostic predictor for the risk of future cardiovascular events in several groups of patients, supporting the

need for drugs to improve endothelial function and to reduce the progression of atherosclerosis [13,14].

Hyperglycemia may be linked to ED and atherogenesis through the production of inflammatory factors that play an important role in recruiting monocytes, neutrophils, and lymphocytes in the site of endothelium damage [15]. This study demonstrated that sildenafil decreases P-selectin in men with T2DM. Other authors did not observe an effect of sildenafil treatment on oxidative stress biomarkers or cytokines, probably because of the short duration of the treatment [16]. P-selectin is involved in the pathogenesis of ED and atherosclerosis calling monocytic cells in the site of vascular injury. The selectins mediate adhesion of hematopoietic cells to vascular surfaces and to each other [17]. P-selectin acts binding to a specific receptor, the pSGL-1, expressed by all leukocytes: neutrophils, NK cells, monocytes, T-lymphocytes. It is involved in the interaction between leukocytes themselves and the endothelium and between platelets and endothelium.

Treatment with sildenafil, reducing the levels of P-selectin, could therefore play a role in improving the ED associated with diabetes mellitus [3-6]. This mechanism might underline the effects of PDE5i in improving parameters of endothelial function as recently shown in a recent meta-analysis in T2DM patients [8]. During the last years, sildenafil has evolved from possible anti-angina drug to the treatment of erectile dysfunction and more recently it has found a new place in the treatment of pulmonary hypertension.

In addition, potential cardioprotective effects of long-term daily PDE5i administration have been demonstrated in a large (>1600 patients) meta-analysis of placebo-controlled trials on heart architecture and performance [7].

The results of the present study could indicate that the effect of sildenafil is exerted not only at a hemodynamic level, inducing vasodilation, but also on a marker involved in vascular damage in diabetes. Chronic administration of this drug in patients with diabetes mellitus might act on some of the mechanisms involved in the pathogenesis and progression of diabetic vascular complications.

Moreover, this study suggests that chronic PDE5 inhibition improves glycometabolic control in patients with type 2 diabetes. It has been demonstrated that chronic treatment with sildenafil improves energy balance and enhances insulin action *in vivo*, in a mouse model [18]. Phosphodiesterase-5 is the predominant phosphodiesterase in vascular smooth muscle and the inhibition of PDE5 results in smooth muscle relaxation and vasodilation. In addition to modulating vascular tone, cGMP signaling can also regulate muscle glucose uptake [18]. A potential mechanism by which phosphodiesterase-5 inhibition may improve insulin action could be through the prevention of ED, characterized by decreased levels of NO, reduced production of cGMP and altered muscle glucose uptake. There is evidence supporting that ED may be causative of insulin resistance and T2DM [19,20].

Moreover, recent data demonstrated that the inhibition of PDE5 plays a role in the regulation of energy homeostasis in mouse skeletal muscle cells, increasing fatty acid oxidation [21].

Furthermore, sildenafil has been shown to cross the blood-brain barrier, and phosphodiesterase-5 expression has been detected in the brain. Thus, the enhanced insulin action may also result from an effect on the central nervous system [22]. This study has some limitations, such as the lack of placebo-controlled group and the treatment duration, 3 months, which is not sufficient to evaluate the effects of a longer sildenafil therapy in a real-life clinical set.

5. Conclusion

The results of this study indicate that the chronic inhibition of PDE5 reduces a biomarker of vascular damage and improves the glycometabolic control in patients with T2DM.

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Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT00692237.

Declaration of interest

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Ballantyne CM, Nambi V. Markers of inflammation and their clinical significance. Atheroscler Suppl 2005;6:21-9
• **In this work biomarkers implicated in the inflammatory process leading to atherothrombosis, and the effect of statins on these markers and their potential relationship to cardiovascular events, were investigated.**
2. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74
• **Pathways promoting thrombosis, complication of atherosclerosis are discussed. Moreover the clinical benefits of lipid-lowering therapies are evidenced.**
3. Phé V, Rouprêt M. Erectile dysfunction and diabetes: a review of the current evidence-based medicine and a synthesis of the main available therapies. Diabetes Metab 2012;38(1):1-13
• **Endothelial dysfunction is the link between diabetes-induced ED and coronary artery disease.**
4. Vardi M, Nini A. Phosphodiesterase inhibitors for erectile dysfunction in patients with diabetes mellitus. Cochrane Database Syst Rev 2007(1):CD002187
• **A review on the effects of PDE-5 inhibitors in diabetic men.**
5. Kanwar M, Agarwal R, Barnes M, et al. Role of phosphodiesterase-5 inhibitors in heart failure: emerging data and concepts. Curr Heart Fail Rep 2013;10(1):26-35
• **The favorable myocardial effect of PDE5 inhibitors.**
6. Giannetta E, Isidori AM, Galea N, et al. Chronic Inhibition of cGMP phosphodiesterase 5A improves diabetic cardiomyopathy: a randomized, controlled clinical trial using magnetic resonance imaging with myocardial tagging. Circulation 2012;125(19):2323-33
•• **The cardiac antiremodeling effect of Sildenafil resulting in improved cardiac kinetics and circulating markers. This effect is independent of any other vasodilatory or endothelial effects and is apparently exerted through a direct intramyocardial action.**
7. Giannetta E, Feola T, Gianfrilli D, et al. Is chronic inhibition of phosphodiesterase type 5 cardioprotective and safe? A meta-analysis of randomized controlled trials. BMC Med 2014;12(1):185
•• **PDE5i astherapy for men with cardiac hypertrophy and early stage heart failure.**
8. Santi D, Giannetta E, Isidori AM, et al. Therapy of endocrine disease: effects of chronic use of phosphodiesterase inhibitors on endothelial markers in type 2 diabetes mellitus: a meta-analysis. Eur J Endocrinol 2014
•• **Chronic Sildenafil administration seems to improve hemodynamic (FMD) and serum pro-inflammatory makers (IL6) in diabetic men.**
9. Morano S, Mandosi E, Fallarino M, et al. Antioxidant treatment associated with sildenafil reduces monocyte activation and markers of endothelial damage in patients with diabetic erectile dysfunction: a double-blind, placebo-controlled study. Eur Urol 2007;52(6):1768-74
•• **The synergic effect of propionyl L-carnitine (PLC) plus Sildenafil in reducing monocyte oxidative activity and endothelialdysfunction markers in diabetic patients with erectile dysfunction (ED).**
10. Ross R. Atherosclerosis - an inflammatory disease. N Engl J Med 1999;340:115-26
• **A review on atherosclerosis as inflammatory disease.**
11. De Meyer GRY, Herman AG. Vascular endothelial dysfunction. Prog Cardiovasc Dis 1997;39:325-42
• **A short overview of therapeutic approaches of the dysfunctional endothelium.**
12. Meyrelles SS, Peotta VA, Pereira TM, Vasquez EC. Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. Lipids Health Dis 2011;10:211
• **The evidence relative to the influence of gender on the development of atherosclerosis in a murine model and the main mechanisms underlying the endothelial are discussed.**
13. Anderson TJ, Uehata A, Gerhard MD, et al. Close relation of endothelial function in the human coronary and peripheral circulations. Endothelial dysfunction as a target for prevention of cardiovascular disease. J Am Coll Cardiol 1995;26:1235-41
• **Flow-mediated vasodilation in the brachial artery as a useful surrogate method in assessing the predisposition to atherosclerosis.**
14. Versari D1, Daghini E, Virdis A, et al. Endothelial dysfunction as a target for prevention of cardiovascular disease. Diabetes Care 2009;32(Suppl 2):S314-21
• **The endothelium as a surrogate end point of the therapeutic approach to cardiovascular risk.**
15. Iribarren C. Glycemic control and heart failure among adult patients with diabetes. Circulation 2001;103:2668-73
• **Poor glycemic control increases the risk of heart failure among patients with diabetes.**
16. Burnett LA. Serum biomarker measurements of endothelial function and oxidative stress after daily dosing of sildenafil in Type 2 diabetic men with erectile dysfunction. J Urol 2009;181(1):245-51
• **Short-term, continuous sildenafil treatment and systemic endothelial function.**
17. Vestweber D, Blanks JE. Mechanism that regulate the function of selectins and their ligands. Physiol Rev 1999;79(1):181-213
• **Selectins and glycoprotein ligands of the selectins participate in the activation of leukocyte integrins.**
18. Ayala JE, Bracy DP, Julien BM, et al. Chronic treatment with sildenafil improves energy balance and insulin action in high fat-fed conscious mice. Diabetes 2007;56(4):1025-33
• **Phosphodiesterase-5 inhibitors prevent diet-induced energy imbalance and insulin resistance.**
19. Tooke JE, Goh KL. Endotheliopathy precedes type 2 diabetes. Diabetes Care 1998;21:2047-9
• **Endothelial damage in diabetes.**
20. Pinkney JH, Stehouwer CD, Coppack SW, Yudkin JS. Endothelial dysfunction: cause of the insulin

resistance syndrome. Diabetes 1997;46(Suppl 2):S9-S13

- **An hypothesis for both the endothelial and metabolic abnormalities associated with atherogenesis.**

21. Sabatini S, Sgrò P, Duranti G, et al. Tadalafil alters energy metabolism in C2C12 skeletal muscle cells. Acta Biochim Pol 2011;58(2):237-41

- **Tadalafil modulates energy homeostasis in mouse skeletal muscle cells.**

22. Phillips BG, Kato M, Pesek CA, et al. Sympathetic activation by sildenafil. Circulation 2000;102:3068-73

- **Sympathetic activation by sildenafil may have implications for understanding cardiovascular events associated with its use.**

Affiliation

Elisabetta Mandosi[1], Elisa Giannetta[1], Tiziana Filardi[1], Manuela Lococo[1], Camilla Bertolini[1], Mara Fallarino[1], Daniele Gianfrilli[1], Mary Anna Venneri[2], Luisa Lenti[1], Andrea Lenzi[1] & Susanna Morano[†1]
[†]Author for correspondence
[1]Sapienza University of Rome, Policlinico Umberto I, Department of Experimental Medicine, Viale del Policlinico 155, 00161, Rome, Italy
Tel: +390 649 978 381;
Fax: +390 649 978 381;
E-mail: susanna.morano@uniroma1.it
[2]Sapienza University, Departement of Histology and Embryology, Rome, Italy