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Endothelial Antioxidant Compound Prolonged the Endothelial Antiapoptotic Effects Registered After Tadalafil Treatment in Patients With Arterial Erectile Dysfunction

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ABSTRACT: This study evaluated the effects of a chronic treatment with tadalafil, a specific phosphodiesterase V inhibitor, on endothelial apoptosis through changes in the serum concentration of endothelial microparticles (EMP). EMPs were arbitrarily chosen as a marker of endothelial apoptosis, and the changes in their concentration were monitored before and after treatment. Additionally, administration of endothelial antioxidant compound (EAC) during the follow-up, after discontinuation of tadalafil, was evaluated to determine whether this treatment improved the potential effects of tadalafil on the endothelium. Seventy-five patients with arterial erectile dysfunction were evaluated at baseline and after administration of tadalafil (5 mg once daily for 90 days). The International Index of Erectile Function questionnaire was administered, and penile dynamic Doppler and flow-cytometric (serum concentrations of EMPs) analyses were performed before (T0) and after treatment. Time points after tadalafil discontinuation: T1, after 1 week; T2, after 3 months; and T3, after 6 months. Three different schemes of follow-up were evaluated: group A, follow-up with EAC administration, after tadalafil discontinuation, for 6 months; group B, follow-up without other treatment; and group C, follow-up with placebo during the follow-up, after tadalafil cessation. The events $CD45_{neg}/CD144_{pos}/annexinV_{pos}$ were defined EMPs. Patients treated with tadalafil showed a significant decrease in serum EMPs 1 week after discontinuing tadalafil (16.4% ± 3.6% vs 7.1% ± 3.3%). This effect was maintained for up to 3 months in the group without other treatment during follow-up and was maintained for up to 6 months in the group treated with EAC during follow-up. Chronic treatment with tadalafil reduces endothelial apoptosis in patients with arterial erectile dysfunction. Further, EAC treatment prolongs and stabilizes the duration of antiapoptotic effects on the endothelium that are initially promoted by tadalafil treatment.

Key words: Endothelial apoptosis, endothelial microparticles, endothelial antioxidant treatment, tadalafil cessation.

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Because endothelial dysfunction is a decisive component of erectile dysfunction (ED), additional endothelial diagnostic tools are necessary for a complete classification, mainly in poor-responder patients. Currently, the following are considered biomarkers of vascular endothelial dysfunction: insulin resistance, homocysteinemia, lipoprotein(a), endogenous nitric oxide (NO) synthesis inhibitors, adiponectin, inflammatory factors (eg, C-reactive protein, interleukin 1, interleukin-6, tumor necrosis factor-a, and monocyte chemotactic protein), endothelial progenitor cells, vasodilators (nitrites, nitrates, and 6-keto prostaglandin F1a), vasoconstrictors (endothelin, thromboxane A2, and reactive oxygen species [ROS]), adhesion molecules (vascular adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1], and P- and E-selectins), and thrombotic hemostatic factors (plasminogen activator inhibitor-1, tissue plasminogen activator, von Willebrand factor, and thrombomodulin; Costa and Virag, 2009; Hirata et al, 2010).

In particular, among the endothelial markers for dysfunctional apoptosis, the role of endothelial microparticles (EMP) is interesting in patients with ED. EMPs arise from the endothelial lining of blood vessels and are generally small (≤1.5 μm). In particular, externalization of phosphatidylserine (PS) is a key molecular event that indicates the initial loss of membrane integrity (Shet, 2008). Recent studies indicate that EMPs are able to decrease NO-dependent vasodilation, increase arterial stiffness, promote inflammation, and initiate thromboses at their PS-rich membrane, which highly coexpresses tissue factor. EMP levels are elevated in acute coronary syndromes, in severe hypertension with end organ damage, and in thrombotic thrombocytopenic purpura,

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conditions that are associated with endothelial injury and a prothrombotic state (Shet, 2008; Chironi et al, 2009). Moreover, Esposito et al (2008) reported that patients with ED and diabetes mellitus display a low EMP62/EMP31 ratio, which is considered an index of endothelial apoptosis.

Additional experiments have evaluated endothelial apoptosis in men with ED after treatment with tadalafil or phosphodiesterase inhibitor type 5 (PDEi-5), suggesting a decrease in apoptosis with treatment (Lysiak et al, 2008; Park et al, 2008). Other data suggest that daily use of PDEi-5 improves endothelial and erectile functions, but these benefits are lost upon drug withdrawal (Aversa et al, 2008).

Recently, we demonstrated that treatment with endothelial antioxidant compounds (EAC) in patients with arterial ED improves the success rate of sildenafil, another phosphodiesterase V inhibitor, suggesting that a combined treatment may be considered in such patients to increase bioavailable NO and to neutralize radical oxygen species, which in turn inactive NO (Vicari et al, 2010). Moreover, there is experimental evidence that treatment with antioxidants may improve erectile function in an acute fashion. In fact, Angulo et al (2009) recently evaluated the effects of the antioxidant AC3056 on diabetic ED by measuring erectile responses to cavernosal nerve electrical stimulation in streptozotocin-induced diabetic rats. The influence of AC3056 on erectile responses, lipid peroxidation, nitrite plus nitrate serum content, and nuclear factor-kappaB expression in penile tissue was determined. Complete reversion of ED was achieved after 3 days of treatment with 0.3% AC3056 (Angulo et al, 2009).

On this basis, this study evaluated the effects of chronic treatment with tadalafil on endothelial apoptosis via changes of the serum concentration of endothelial microparticles (EMP), which were arbitrarily chosen as a marker of endothelial apoptosis, before and after treatment. In addition, we evaluated whether administration of EACs during the follow-up, after tadalafil discontinuation, improves the potential effects of tadalafil on the endothelium.

Materials and Methods

We evaluated 75 patients (average age, 56 ± 6 years; range, 50–62 years) with arterial ED who fulfilled the following characteristics: International Index of Erectile Function (IIEF-5) <21 (Rosen et al, 1999), peak systolic velocity <30 cm/s (Benson et al, 1993), and acceleration time >110 ms (Speel et al, 2003) in both cavernous arteries.

Exclusion criteria included smokers (>5 cigarettes per day), hypogonadism, carotid or femoral atherosclerosis, and/or chronic ischemic heart disease.

Clinical (IIEF-5 administration), ultrasound (penile dynamic Doppler), and flow-cytometric evaluations were repeated at baseline (T0) and at 1 week (T1), 3 months (T2), and 6 months (T3) after cessation of tadalafil administration (5 mg once daily for 90 days).

Another group of 20 healthy participants (average age, 58 ± 4 years; range, 51–62 years) served as a control group for the assessment of EMPs in the pretreatment condition. They had no ED (IIEF-5 > 21), systemic diseases, and/or cardiovascular risk factors.

Groups of Study and Design of Treatment

Three age-matched groups with ED were randomly assigned to three different follow-up schemes after tadalafil treatment. Scheme A was follow-up with EAC compound administration for 24 weeks (group A; n = 25). Scheme B was follow-up without other therapy for 24 weeks (group B; n = 25). Scheme C was follow-up with placebo administration for 24 weeks (inert capsule of sugar; group C; n = 25).

The patients in group A were prescribed an EAC containing L-arginine (2500 mg), propionyl-L-carnitine (250 mg), and nicotinic acid (20 mg; ie, Ezerex, Sigma Tau Pharmaceuticals, Rome, Italy; 1 small envelope daily for 24 weeks).

Flow Cytometric Analysis

Evaluation of EMPs with externalization of PS was conducted using an EPICS XL flow cytometer (Coulter Electronics Instrumentation Laboratory, Milan, Italy) equipped with a single argon laser (488 nm) as the source of emission and 3 detectors: FL1, green color (wavelength 525 nm); FL2, orange color (wavelength 575 nm); and FL3, red (wavelength 620 nm). The flow cytometer was operated at the medium flow-rate setting with a log gain on light scatter and fluorescence.

Endothelium-Derived Microparticles With PS Externalization

The measurement of blood EMPs requires careful attention to the collection and processing of blood samples. While separating the cellular elements of blood from the plasma, which contains EMPs, the centrifugation speed must be closely monitored. In our experience, a 2-step centrifugation using 1500 × *g* for 10 minutes and then 13 000 × *g* for 10 minutes results in platelet-free plasma (as assessed by flow cytometry and light microscopy). The second centrifugation step is particularly efficient at rendering plasma relatively "platelet free." Flow cytometric analysis of blood MPs appears to be the most favored method to characterize these particles. Typically, EMPs are identified as particles with a forward angle light scatter smaller than an internal standard consisting of 1- to 1.5-μm latex particles.

ECD anti-human CD45 (Instrumentation Laboratory), phycoerythrin-conjugated anti-human CD144 (Instrumentation Laboratory), and fluorescein isothiocyanate–conjugated annexin V (Instrumentation Laboratory) were used for EMP flow detection by flow cytometry. To exclude microparticles originating from leukocytes, we considered only events within the $CD45_{neg}$ gate. $CD144_{pos}$ events expressing PS in the outer membrane leaflet following annexin V staining were defined as

Table 1. *Body mass index and main metabolic parameters in erectile dysfunction (ED) patients and controls during enrollment*

Parameters	ED Patients (n = 75)	Controls (n = 20)
Age	56 ± 6	58 ± 4
Body mass index, kg/m^2	25.2 ± 3.0	26.0 ± 3.5
Waist circumference, cm	88 ± 3.0	89.0 ± 7.0
Triglycerides, mg/dL	165.0 ± 15.0	167.5 ± 8.5
Cholesterol HDL, mg/dL	54.0 ± 6.0	50.0 ± 5.0
Systolic blood pressure, mm Hg	142.0 ± 8.0	144.5 ± 8.0
Diastolic blood pressure, mm Hg	90.0 ± 5.0	93.0 ± 3.0
Fasting glycemia, mg/dL	91.0 ± 10.0	93.5 ± 8.5

Abbreviation: HDL, high-density lipoprotein.

EMPs. EMP concentrations are reported as the percentage of total events.

Appropriate isotype controls were used for each staining procedure as negative controls to define the appropriate regions. Flow cytometric analysis was conducted for 600 seconds or 100 000 events, whichever occurred first (Jy et al, 2004; Khan et al, 2005; Mariucci et al, 2010; Masouleh et al, 2010; van Ierssel et al, 2010). The same operator, blinded with respect to the sample origin (controls or patients), performed all of the tests in this study.

All patients provided informed consent, and the study was approved by the institutional review board.

Statistical Analysis

Results are reported as means ± SEM thoughout the study. The data were analyzed by 1-way analysis of variance followed by the Duncan multiple range test. SPSS 9.0 for Windows (SPSS Inc, Chicago, Illinois) was used for statistical evaluation. A statistically significant difference was defined as $P < .05$.

Results

At Baseline (T0)

There were no statistically significant differences relative to body mass index or main metabolic parameters in ED patients and controls during enrollment (Table 1). Likewise, there were no statistically significant differences relative to these parameters among the ED groups (A–C) (Table 2).

ED patients had significantly lower IIEF-5 scores and peak systolic velocities, whereas their acceleration times and intima-media thicknesses were significantly higher compared with controls ($P < .05$; Table 3). There were no statistically significant differences in these variables among the patients with ED.

Patients with arterial ED had concentrations of circulating EMPs ($CD45_{neg}/CD144_{pos}$/annexin V_{pos}) significantly higher than the control group ($P < .05$; Table 3). However, among patients with ED, there was no statistically significant difference relative to the concentrations of circulating EMPs (Table 3).

One Week After Cessation (T1)

ED patients displayed significantly higher mean IIEF-5 scores compared with baseline, and additionally had significantly higher peak systolic velocities. In contrast, acceleration time was significantly lower ($P < .05$) compared with baseline (Table 3). In addition, there were no statistically significant differences relative to the following values compared with baseline: end diastolic velocity, resistance index, and intima media thickness.

Finally, serum concentrations of circulating EMPs were significantly lower compared with baseline ($P < .05$; Table 3).

Among patients with ED (groups A, B, and C), there were no statistically significant differences in the concentrations of circulating EMPs, IIEF-5 scores, or instrumental Doppler parameters (Table 3).

Table 2. *Body mass index and main metabolic parameters of erectile dysfunction (ED) patients (groups A–C) during enrollment*

Parameters	ED Patients, Group A (n = 25)	ED Patients, Group B (n = 25)	ED Patients, Group C (n = 25)
Age	56 ± 4	55 ± 5	57 ± 4
Body mass index, kg/m^2	24.0 ± 2.0	25.0 ± 3.0	25.0 ± 1.0
Waist circumference, cm	87 ± 6.0	86.0 ± 4.0	88.0 ± 10.0
Triglycerides, mg/dL	163.0 ± 8.0	161.0 ± 4.0	170.0 ± 5.0
Cholesterol HDL, mg/dL	52.0 ± 3.0	56.0 ± 5.0	58.0 ± 8.0
Systolic blood pressure, mm Hg	141.0 ± 10.0	143.0 ± 6.0	140.0 ± 2.0
Diastolic blood pressure, mm Hg	92.0 ± 5.0	95.0 ± 3.0	93.0 ± 6.0
Fasting glycemia, mg/dL	90.0 ± 15.0	93.0 ± 8.0	92.0 ± 5.0

Abbreviation: HDL, high-density lipoprotein.

Table 3. *Penile dynamic echo-color Doppler parameters, endothelial microparticles (EMP) serum concentrations, and International Index of Erectile Function (IIEF-5) scores (mean ± SEM) of the patients with erectile dysfunction (ED) and controls before and after cessation of tadalafil treatment*

			ED Group A			ED Group B			ED Group C		
Parameter	Controls	ED T0	T1	T2	T3	T1	T2	T3	T1	T2	T3
IIEF-5	23.0 ± 1.0	6.0 ± 4.0[a]	18.0 ± 2.0[b]	16.0 ± 2.0[b]	16.0 ± 2.0[b]	18.0 ± 3.0[b]	14.0 ± 2.0[b]	13.0 ± 2.0[b]	17.0 ± 2.0[b]	14.0 ± 5.0[b]	14.0 ± 3.0[b]
PSV	76.3 ± 0.5	21.0 ± 3.0[a]	36.0 ± 4.0[b]	35.0 ± 5.0[b]	35.0 ± 4.0[b]	37.0 ± 5.0[b]	33.0 ± 7.0[b]	32.0 ± 4.0[b]	35.0 ± 4.0[b]	34.0 ± 2.0[b]	31.0 ± 3.0[b]
EDV	1.5 ± 0.10	1.2 ± 2.0	1.5 ± 1.0	1.3 ± 1.0	1.3 ± 1.0	1.6 ± 2.0	1.4 ± 1.0	1.1 ± 1.0	1.2 ± 1.0	1.2 ± 1.0	1.2 ± 2.0
AT	55.3 ± 3.0	140.0 ± 10.0[a]	104.0 ± 8.0[b]	104.0 ± 5.0[b]	109.0 ± 8.0[b]	106.0 ± 8.0[b]	108.0 ± 5.0[b]	111.0 ± 8.0[b]	104.0 ± 6.0[b]	100.0 ± 8.0[b]	112.0 ± 6.0[b]
RI	0.97 ± 0.1	0.90 ± 0.6	0.92 ± 0.5	0.95 ± 0.3	0.93 ± 0.3	0.94 ± 0.5	0.95 ± 0.3	0.94 ± 0.3	0.93 ± 0.5	0.92 ± 0.5	0.90 ± 0.5
IMT	0.12 ± 0.4	0.45 ± 0.5[a]	0.44 ± 0.2	0.44 ± 0.5	0.44 ± 0.5	0.47 ± 0.3	0.47 ± 0.5	0.47 ± 0.5	0.43 ± 0.2	0.46 ± 0.4	0.40 ± 0.4
EMPa	1.8 ± 0.4	16.4 ± 3.6[a]	7.0 ± 3.0[b]	8.0 ± 1.2[b]	8.6 ± 1.5	7.2 ± 4.0[b]	8.2 ± 1.3[b]	16.1 ± 1.5[c]	7.1 ± 3.0[b]	8.4 ± 2.3[b]	16.2 ± 3.0[c]

Abbreviations: AT, acceleration time; ED group A, follow-up with EAC; ED group B, follow-up without EAC; ED group C, follow-up with placebo; EDV, end diastolic velocity; IMT, intima-media thickness; EMPa, apoptotic endothelial microparticles; PSV, peak systolic velocity; RI, resistance index; T0, baseline; T1, after 1 week from cessation; T2, after 3 months from cessation; T3, after 6 months from cessation.

[a] $P < .05$ vs controls.

[b] $P < .05$ vs ED T0.

[c] $P < .05$ vs T3 group A.

Three Months After Cessation (T2)

ED patients displayed significantly higher mean IIEF-5 scores and peak systolic velocities compared with baseline, whereas acceleration time was significantly lower ($P < .05$) compared with baseline (Table 3).

In addition, there were no statistically significant differences in the following values compared to baseline: end diastolic velocity, resistance index, and intima media thickness.

The serum concentration of circulating EMPs was significantly lower compared with baseline ($P < .05$; Table 3).

There was no statistically significant difference in EMP serum concentration compared with T1, but the values recorded at T2 did demonstrate an increasing trend.

Among patients with ED, there were no statistically significant differences in the concentrations of circulating EMPs, IIEF-5 scores, or instrumental Doppler parameters (Table 3).

Six Months After Cessation (T3)

ED patients displayed significantly higher mean IIEF-5 scores and peak systolic velocities compared with baseline, whereas acceleration time was significantly lower ($P < .05$; Table 3).

No statistically significant difference compared with baseline was found for the other parameters investigated (ie, end diastolic velocity, resistance index, and intima-media thickness).

There was no statistically significant difference compared with T2 found relative to IIEF-5 score and other penile dynamic Doppler parameters investigated.

Finally, no statistically significant difference was found in the EMP serum concentration compared with baseline, although the mean values of EMPs detected at T3 were significantly higher than at T2 ($P < .05$; Table 3).

Among patients with ED, group B and group C displayed significantly higher mean EMP values than group A, but there was no statistically significant difference between groups B and C. Finally, the IIEF-5 score and instrumental Doppler parameters investigated were slightly (but not significantly) worse in groups B and C than in group A (Table 3).

Discussion

Our study, designed to evaluate the effects of chronic therapy with tadalafil (5 mg/d) on endothelial apoptosis, revealed a temporal response characterized by an initial antiapoptotic effect observed via a significant decrease

in serum EMP concentration relative to the pretreatment group. This beneficial proendothelial effect was transient and maintained until 12 weeks after tadalafil withdrawal; at 24 weeks, the EMP levels increased, reaching mean concentrations similar to those found before treatment. The parallel therapeutic arm, including the group of patients with arterial ED treated with EAC after tadalafil withdrawal, helped us partially explain this phenomenon. Indeed, after EAC treatment the mean EMP concentration significantly decreased again. The EMP values of patients in this group overlapped with those measured during the first 12 weeks of observation after tadalafil cessation and were lower than those found in the group of patients (without EAC) observed 24 weeks after tadalafil withdrawal. Thus, our data indicate that treatment with an EAC is able to prolong and stabilize the duration of the antiapoptotic effect on endothelium initially promoted by PDEi-5 treatment.

Several studies have established the effects of tadalafil and/or other PDEi-5 drugs on endothelial apoptosis.

Lysiak et al (2008) demonstrated that treatment with tadalafil prevents apoptosis and apoptotic-related mechanisms, resulting in a decreased number of apoptotic cells and an increase in the phosphorylation of the 2 survival-associated kinases, Akt and extracellular signal-regulated kinase 1/2. Park et al (2008) demonstrated that chronic treatment with a PDEi-5 suppresses corporal apoptosis via potentiation of Akt signaling in diabetic rats with ED. This treatment also significantly inhibited the activities of cavernosal caspase 3 and caspase 9, the main effectors of apoptosis (Park et al, 2008). Aversa et al (2007) demonstrated that chronic (20 mg on alternate days for 4 weeks) but not on-demand tadalafil improves some markers of endothelial function (eg, VCAM, ICAM, endothelin-1, insulin, and C-reactive protein) with sustained effects after its discontinuation. Finally, in another recent study of diabetic patients, after once-daily treatment with 2.5 mg or 5 mg of tadalafil (for 12 weeks), endothelial dysfunction biomarkers (ie, C-reactive protein, nitrotyrosine, ICAM-1, and VCAM-1) were unchanged (Hatzichristou et al, 2008). The authors suggest that underlying vascular pathology could limit the effects of tadalafil on the endothelium.

ROS, especially superoxide anion and hydrogen peroxide, are important signaling molecules in cardiovascular cells (Griendling et al, 2000; Cathcart, 2004). Enhanced superoxide production increases NO inactivation and leads to an accumulation of peroxynitrites and hydrogen peroxide (Kojda and Harrison, 1999; Vicari et al, 2010). Potential sources of vascular superoxide production include NADPH-dependent oxidases, xanthine oxidases, lipoxygenases, mitochondrial oxidases, and NO synthases (Lassegue and Clempus, 2003; Mueller et al, 2005). Studies performed during the last decade demonstrate that NADPH oxidase is the most important source of superoxide anion in phagocytic and vascular cells (Helal et al, 2011). Interestingly, the oxidative stress markers, plasma glutathione peroxidase and urinary 8-iso-prostaglandin F(2) alpha, independently influence the levels of annexin V–positive EMPs and microparticles derived from platelets, whereas the levels of inflammatory markers did not change, regardless of circulating microparticle type (Helal et al, 2011). ROS participate in growth, apoptosis, and the migration of vascular smooth muscle cells, in the modulation of endothelial function (including endothelium-dependent relaxation and expression of a proinflammatory phenotype), and in the modification of the extracellular matrix (Li et al, 1997). All of these events play important roles in endothelial dysfunction, suggesting that the sources of ROS and the signaling pathways that they modify may represent important therapeutic targets.

Finally, evidence that oxidative stress affects endothelial function is provided by the effect of antioxidant drugs. For example, tempol reduces oxidative stress markers and exerts a marked antihypertensive effect in a number of experimental models of hypertension. In addition, in humans, the use of vitamins C and E, allopurinol, flavonoid, and folic acid reduces the concentrations of homocysteine. Vitamin C strongly inhibits the oxidation of lipids, particularly low-density lipoprotein (Hirata et al, 2010), and relative to this aspect, our results confirm the beneficial effects of antioxidants.

In conclusion, our results suggest that further evaluation of these markers with different molecules belonging to phosphodiesterase V inhibitors or different schemes of treatment (eg, on demand compared with chronic therapy) should be performed. Finally, only large studies based on correlation between practical routine parameters (IIEF and penile Doppler evaluation) and the serum concentrations of EMPs will clarify the clinical significance of the changes in EMP levels after pharmacologic treatment.

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