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Oxidative Stress and Antioxidant Therapy: Their Impact in Diabetes-Associated Erectile Dysfunction

LING DE YOUNG,* DARRYL YU,* RYON M. BATEMAN,† AND GERALD B. BROCK*

*From the *Department of Urology, St Joseph's Health Care, Lawson Health Research Institute, and the †Department of Medical Biophysics, The University of Western Ontario, London, Canada.*

ABSTRACT: Oxidative stress is believed to affect the development of diabetic-associated vasculopathy, endothelial dysfunction, and neuropathy within erectile tissue. Our hypothesis is that, given adequate concentrations of the oxygen free radical scavenger vitamin E, enhanced levels of circulating nitric oxide (NO) should improve erectile function with the potential for a synergistic effect with a phosphodiesterase type 5 (PDE5) inhibitor. Twenty adult male Sprague-Dawley streptozotocin-induced (60 mg/kg intraperitoneally) diabetic rats were placed in 4 therapeutic groups (n = 5 per group) as follows: 1) peanut oil only (diabetic control), 2) 20 IU of vitamin E per day, 3) 5 mg/kg of sildenafil per day, and 4) vitamin E plus sildenafil using oral gavage for 3 weeks. In addition, 5 age-matched rats served as normal nondiabetic controls (normal). Erectile function was assessed by measuring the rise in intracavernous pressure (ICP) following cavernous nerve electrostimulation. Penile tissue was evaluated for neuronal NO synthase (nNOS), smooth muscle α-actin, nitrotyrosine, and endothelial cell integrity. Urine nitrite and nitrate (NO_x) concentration was quantified, and electrolytes were tested by a serum biochemistry panel. A significant decrease in ICP was recorded in the diabetic animals, with improvement measured in the animals receiving PDE5 inhibitors either with or without vitamin E; the controls had a pressure of 54.8 ± 5.3 cm H_2O, the vitamin E group had a pressure of 73.5 ± 6.6 cm H_2O, the sildenafil group had a pressure of 78.4 ± 10.77 cm H_2O, and the vitamin E plus sildenafil group had a pressure of 87.9 ± 5.5 cm H_2O ($P < .05$), compared with the normal cohorts at 103.0 ± 4.8 cm H_2O. Histoexaminations showed improved nNOS, endothelial cell, and smooth muscle cell staining in the vitamin E plus sildenafil group compared to the control animals. Urine NO_x increased significantly in all the diabetic groups but was blunted in the vitamin E and vitamin E plus sildenafil groups. A significant increase in positive staining for nitrotyrosine was observed in the vitamin E plus sildenafil group. Vitamin E enhanced the therapeutic effect of the PDE5 inhibitor in this study, supporting the potential use of oxygen free radical scavengers in salvaging erectile function in diabetic patients.

Key words: Impotence, vitamin E, sildenafil, nitric oxide, phosphodiesterase type 5.

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Erectile dysfunction (ED) is experienced by men with diabetes mellitus (DM) at a greater frequency than by the general population across all ages. Alterations in neural and impaired penile vascular systems are believed to be primarily responsible for ED rates approaching 75% in some reports (Kolodny et al, 1974; McCulloch et al, 1980; Maatman et al, 1987; Benet and Melman, 1995; Hakim and Goldstein, 1996). In contrast to the efficacy (70%–89%) of phosphodiesterase type 5 (PDE5) inhibition therapy in the general population, just slightly more than 50% of diabetic men respond favorably to treatment (Rendell et al, 1999). The possible underlying cause of this relative resistance to treatment has been reported previously by our group (De Young et al, 2003). Oxidative stress–mediated neurovascular alterations in diabetic patients likely play an integral role, inducing impaired endothelial function and neuropathy in the corpus cavernosum of diabetic men (Herman et al, 1978; Blanco et al, 1990).

Nitric oxide (NO), derived from vascular endothelial and neural sources, plays an essential role in the early steps of the normal cascade of relaxation of the penile vasculature and cavernous smooth muscle, and its action is mediated through the cyclic guanosine monophosphate system (Burnett et al, 1992; Andersson and Wagner, 1995; Moreland et al, 2001). The presence of oxygen free radicals inactivates NO and reduces its physiologic impact. NO is a free radical and can react with other radicals, such as superoxide anions, to produce peroxynitrite and contributes to numerous pathological conditions such as atherosclerosis, ischemia, and generalized reperfusion injury (Cooke and Tsao, 1993; Beckman and Koppenol, 1996). Impaired NO activity and endothelial dysfunction have been described in diabetic animal models as well as in human volunteers (Saenz et al, 1989; Escrig et al, 2002). Additionally, increased auto-oxidation of glucose and oxidation of low-density lipoproteins have been described in diabetes, which may result in the overproduction of free radical species, leading to smooth muscle dysfunction (Tesfamariam and Cohen, 1992; Baynes and

Correspondence to: Dr Gerald Brock, Department of Urology, St Joseph's Health Care, London, Canada N6A 4V2 (e-mail: gebrock@sympatico.ca).

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Thorpe, 1999). Direct inactivation of NO, largely by superoxide anions, which is believed to be present in higher-than-normal concentrations in diabetes, may also play a role in producing impaired cavernosal relaxation (Katusic, 1996).

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant and oxygen free radical scavenger (Burton, 1994). It has been shown to enhance endothelial cell function, trap oxygen free radicals (Willson, 1983), and inhibit monocyte-endothelial adhesion and cytokine release (Islam et al, 1998). Additionally, some reports describe the inhibition of platelet adhesion and aggregation by a protein kinase C–dependent mechanism (Mabile et al, 1999; Saldeen et al, 1999). In vivo as well as in vitro evidence demonstrates that vitamin E treatment can reverse protein kinase C activation, which is believed to be responsible for a component of glucose-induced vascular dysfunction in diabetes (Kinlay et al, 1999; Park et al, 1999). Vitamin E has also been reported to improve NO-mediated arterial relaxation and to maintain cell membrane integrity and protein stability (Keegan et al, 1995; Karasu et al, 1997).

The potential positive clinical impact of combining vitamin E and sildenafil to treat diabetes-induced ED (ED-DM) has been demonstrated by our group (De Young et al, 2003) and others previously. This animal study was designed to gain insight into the role of oxidative stress and the effect of antioxidant therapy on treating ED. We selected a group of animals shortly after the induction of diabetes through faulty control of sugar levels, believing they had the greatest likelihood of reversibility (Soriano et al, 2001).

Materials and Methods

Diabetic Model

Twenty young adult male Sprague-Dawley rats aged 20–24 weeks received streptozotocin (60 mg/kg intraperitoneally [IP]) in pH 4.5 citric acid buffer to induce diabetes. Rats that tested positive for glycosuria at 48 hours were placed in 4 therapeutic groups: 1) untreated diabetes (control given peanut oil), 2) 20 IU of vitamin E per day (vitamin E), 3) 5 mg/kg of sildenafil per day (provided by Pfizer Canada, Pointe-Clair, Canada), and 4) 20 IU of vitamin E per day plus 5 mg/kg of sildenafil per day. Five nondiabetic age-matched rats served as negative controls (normal). Peanut oil was chosen as the vehicle to deliver the treatments because of its low natural content of α-tocopherol. The dose of 20 IU per animal per day of vitamin E was selected following a literature review that demonstrated a therapeutic range of 2–120 IU. Treatments were administered by using daily oral gavage for 3 weeks. All supplements were stopped at least 20 hours before evaluation of erectile function. The final dose of sildenafil was administered 20 hours preceding the electrostimulation experiments. Given that, at this time point, greater than 5 half-lives of sildenafil expire in this animal model, changes in blood pressure attributable to the vasoactive effects of sildenafil are not expected; therefore, blood pressure was not measured.

Evaluation of Erectile Function

The lateral-prostatic space was dissected using a lower abdominal midline incision. The major pelvic ganglion and cavernous nerve were identified, isolated, and hooked with a stainless steel bipolar electrode. Through a sagittal perineal incision, the penile crus was exposed. A 23-gauge needle filled with heparin (250 IU/mL) and connected to Tygon tubing was inserted into the penile crus. The microsurgery procedure was facilitated by the use of a Zeiss SR operating stereomicroscope. Intracavernosal pressure (ICP) was evoked with 0.2-millisecond pulses of 2 mA at 20 Hz for a 40-second duration and recorded using LabVIEW 2 software (National Instruments, Austin, Tex). Three electrostimulations were replicated at intervals of 10 minutes. The animals were sacrificed using pentobarbital (200 mg/kg IP), after which penile tissue was harvested for analysis. The animal experimental protocol was approved by the University Council on Animal Care Animal Use Subcommittee, University of Western Ontario, London, Canada.

Immunohistologic Analysis

Penile tissue was fixed in cold fresh 2% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours; cryoprotected in 15% sucrose for 20 hours at 4°C; and then embedded in optical cutting compound (OCT) (Tissue-Tek, Sakura, Torrance, Calif) and stored at −70°C. The OCT-embedded tissues were cut into 5-μM sections and adhered to superfrost plus slides (Fisher Scientific, Nepean, Canada). Immunostaining for nerves positive for neuronal NO synthase (nNOS), endothelial cell marker, smooth muscle α-actin, and nitrotyrosine was performed. The sections were air dried for 10 minutes, hydrated in phosphate-buffered saline (PBS) buffer, and then treated with 0.3% hydrogen peroxide in methanol. After 2 rinses with water and PBS buffer, the sections were blocked with 3% goat serum in PBS buffer for 3 hours at room temperature. The sections were then incubated in blocking buffer at 4°C overnight with primary mouse anti-nNOS, anti-CD31 (Transduction Lab BD PharMingen, Mississauga, Canada), mouse anti–α-actin (Roche Diagnostics, Quebec, Canada), and rabbit anti-nitrotyrosine (Upstate New York, NY) with dilutions of 1:100, 1:500, 1:300, and 1:50, respectively. The sections were washed 3 times with PBS buffer and incubated with the secondary antibody biotin conjugated goat anti-mouse immunoglobulin G (IgG; Sigma Chemical Co, St Louis, Mo) at a dilution of 1:250 in PBS with 1% bovine serum albumin for 2 hours at room temperature; for anti-nitrotyrosine, the sections were incubated in a 1:100 dilution of a biotin conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif). After 3 washings in PBS buffer, the sections were incubated with an anti-biotin clone BN-34 peroxidase conjugate IgG fraction (Sigma) for 2 hours. The antigen localization was visualized using a diaminobenzidine peroxidase substrate (Sigma). The sections were counterstained (except for nitrotyrosine-staining slides) with hematoxylin, dehydrated through graded alcohols to xylene, and coverslipped.

Table 1. *Profile of serum biochemistry*

	Normal	Control	Vitamin E	Sildenafil	Vitamin E + Sildenafil
Glucose (mM/L)	5.3 ± 1.3	32.5 ± 2.5	30.4 ± 1.2	33.8 ± 1.8	32.4 ± 2.1
Triglycerides (mM/L)	0.35 ± 0.1	13.6 ± 5.7	8.77 ± 0.67	11.2 ± 4.6	7.7 ± 1.2
Cholesterol (mM/L)	0.83 ± 0.1	2.8 ± 0.6	3.7 ± 1.1	3.02 ± 0.6	3.4 ± 0.2
Urea (mM/L)	7.74 ± 0.6	13.5 ± 1.6	11.5 ± 0.9	12.4 ± 1.2	12.2 ± 0.6
Creatinine (mM/L)	40 ± 7	50.4 ± 4.2	70.4 ± 5.6	62.8 ± 2.4	61.2 ± 2.2

Urine Nitrite and Nitrate Measurement

Urine samples were collected from rats before surgery and stored at −20°C for nitrite and nitrate (NO_2^-/NO_3^-), or NO_x^-, analysis. NO_x^- was analyzed using an optimized NO chemiluminescence system (Bateman et al, 2002), which reduced NO_x^- to NO and detected released NO by a chemiluminescent reaction with ozone using an NO analyzer (NOA; Sievers, Boulder, Colo). In brief, thawed 10-μL urine samples were injected into a purge vessel containing 0.05 M vanadium chloride in 1 M HCl (Sigma) at 90°C. Liberated NO was transported to the NOA by helium carrier gas. The system was calibrated against known concentrations of NO_3.

Serum Biochemistry Panel

The blood glucose level was measured by tail vein sampling at the time of the erectile function assessment. Blood samples were collected after surgery, and serum was separated and kept at −70°C until analyzed. All the tests were performed by standard procedures used in our clinical biochemistry laboratory.

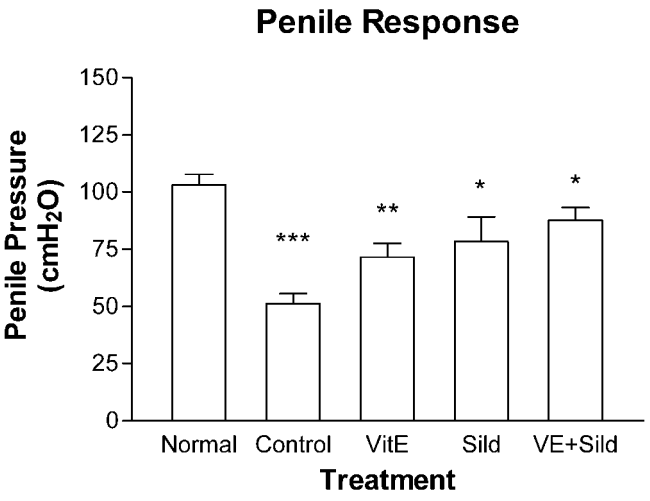

Figure 1. Penile pressure measured in response to electrostimulation of the cavernous nerve in normal and diabetic rats. The mean maximum intracavernous pressures (ICPs) ± the standard error of 5 experiments are as follows: normal, 103.0 ± 4.8 cm H_2O; control (untreated diabetes), 54.82 ± 5.3 cm H_2O; vitamin E, 73.51 ± 6.58 cm H_2O; sildenafil, 78.43 ± 10.77 cm H_2O; and vitamin E plus sildenafil, 87.86 ± 5.46 cm H_2O. All in the diabetic group showed decreased ICPs compared with the normal control ($P < .05$). Significantly increased ICPs were observed in the sildenafil and vitamin E plus sildenafil groups compared with the control group ($P < .05$).

Statistical Analysis

Values are expressed as the mean ± standard error of 5 experiments for each group. Data were compared by 2-tailed *t* tests, $\alpha = .05$.

Results

The animals in all 4 diabetic groups showed persistent hyperphagia, polydipsia, and polyuria consistent with faulty control of diabetes. No differences in body weight loss or hyperglycemia were measured between the active treatment groups. All experimental groups had significantly greater body weight loss than the normal animals. The diabetic groups showed significant elevations in serum triglycerides and cholesterol levels compared to the normal animals (Table 1). In spite of similar elevations in measured glucose levels, the animals receiving vitamin E and vitamin E plus sildenafil demonstrated lower serum triglycerides than the animals receiving peanut oil and/or sildenafil alone. However, given the small numbers of animals and the wide variation in values within each group, no statistically significant difference between treatment arms was produced. Elevated serum urea values were measured in all the treatment groups, $P < .05$.

The peak ICP (PICP) during electrostimulation of the cavernous nerves is shown in Figure 1. It rose significantly in all treatment groups compared with the diabetic controls but did not reach the level measured among the normal animals. The group receiving vitamin E combined with sildenafil showed the greatest increase in PICP with the smallest intragroup variation among the 3 active treatment arms. The sildenafil-treated group showed a wide intragroup variation in PICP that is consistent with clinical experience among diabetic men with ED, in whom the efficacy of sildenafil is 50%–60%. The responder animals did not display improved sugar control or other evident biochemical parameters distinct from the nonresponders.

The histologic examination was performed using a Zeiss microscope with a computerized imaging system (Northern Eclipse, Empix, Canada). The reviewer was blinded to the groups, and the counts were repeated for consistency. As shown in Table 2, the sum of NOS nerve

Table 2. *Summary of immunohistological results†‡*

	Normal	Control	Vitamin E	Sildenafil	Vitamin E + Sildenafil
nNOS	616 ± 27.4	264 ± 18*	360 ± 16*	380 ± 15*	555 ± 22
% endothelial cell	62.6 ± 3.2	16 ± 1.9*	24.0 ± 2.5*	20.0 ± 4.5*	33 ± 1.2*
% SM α-actin	14.42 ± 0.3	9.35 ± 0.3*	9.5 ± 0.3*	10.4 ± 0.3*	12.9 ± 0.6
% nitrotyrosine	0.94 ± 0.1	2.54 ± 0.01*	1.89 ± 0.03*	3.22 ± 0.1*	1.57 ± 0.2

† nNOS indicates neuron nitric oxide synthase; SM, smooth muscle.

‡ The sum of positive staining of nNOS within the dorsal nerve and 3 other sites in the penile corpus cavernosum (power, 400×) is reported. The proportion of positive staining of endothelial cells within the major penile sinusoids (power, 400×) relative to normal animals demonstrated a significant decrease in all of the diabetic groups, with the vitamin E plus sildenafil group reaching only 50% of normal controls. The proportion of positive staining of SM α-actin to the cross-sectional areas of midshaft penile sections (power, 25×) in the vitamin E plus sildenafil group approached the level in normal controls, whereas it was significantly lower in the other treatment groups. The proportion of positive staining of nitrotyrosine (power, 400×) was significantly increased in 3 treatment groups but not in the vitamin E plus sildenafil group.

* $P < .05$.

fibers that stained positive was calculated as the total from the dorsal nerve, an area adjacent to the dorsal vein, and 2 regions from the right and left posterior corpus cavernosum in the field of view, at 400× magnification. All treatment groups showed a significantly higher degree of positive staining for nNOS than did the untreated diabetic animals (control). The greatest increase relative to the diabetic control was observed among the animals receiving a combination of vitamin E and sildenafil. The vitamin E, sildenafil, and vitamin E plus sildenafil groups showed a degree of staining that was 36%, 44%, and 110% higher than that of the diabetic controls. Penile cavernosum sinusoidal endothelial cell staining was altered in all groups to varying degrees compared with normal animals. The proportion of endothelial cell marker CD31 staining was greatest in the normal group, and the treatment groups were ordered as follows: vitamin E plus sildenafil > vitamin E > sildenafil > diabetic control, measured as 53%, 38%, 32%, and 25%, respectively, of normal levels, indicating impaired endothelial function (Table 2). A significant recovery in the positive staining for penile smooth muscle α-actin was measured in the vitamin E plus sildenafil group compared with the controls and the vitamin E group (Table 2). A significant increase in positive staining for nitrotyrosine was measured in the controls ($P < .001$), the vitamin E group ($P < .05$), and the sildenafil group ($P < .001$) when compared to that of the normal animals (Table 2).

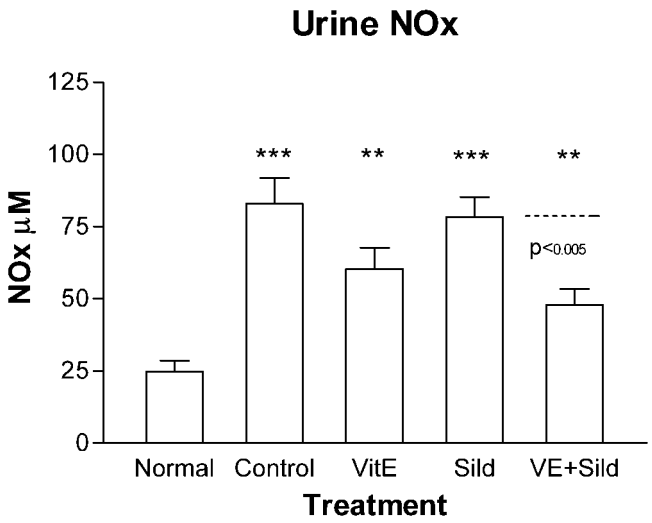

Figure 2. Immunostaining for the endothelial cell marker CD31 (upper panels) and nitrotyrosine (lower panels) in the normal control and all the diabetic groups. The antigen localization was visualized by using a diaminobenzidine peroxidase substrate. The positive staining (dark color and arrows) for CD31 decreased in diabetic groups and partially recovered in the vitamin E and vitamin E plus sildenafil groups. Stronger positive stainings (dark color and arrows) for nitrotyrosine are shown in the diabetic control, vitamin E, and sildenafil groups compared to normal animals. Significant improvement was seen in the vitamin E plus sildenafil group (400× magnification).

Figure 2 presents representative cross sections of penile tissue showing immunologic staining with the endothelial cell marker CD31 (upper panel) and nitrotyrosine (lower panel). The sinusoidal endothelial cell staining of the vitamin E plus sildenafil group demonstrated an increase in the number and intensity of endothelial cells staining for CD31 and a decrease in the intensity of staining for nitrotyrosine relative to diabetic-positive controls.

The measured concentrations of urine nitrite and nitrate (NO_x) (Figure 3) are 24.8 ± 3.8 μM in the normal group and show a 3.3-, 2.4-, 3.1-, and 2-fold increase in the control, vitamin E, sildenafil, and vitamin E plus sildenafil groups, respectively. This represented a significant increase in all the diabetic groups. The animals receiving vitamin E and vitamin E plus sildenafil demonstrated smaller rises.

Discussion

The underlying cause of the reduced efficacy of PDE5 inhibitors in clinical practice among diabetic men remains to be fully elucidated. Recent reports that describe large clinical trials with vardenafil and tadalafil among diabetic populations support earlier reports from sildenafil studies that demonstrate impaired efficacy as assessed by all commonly used end points (Rendell et al, 1999; Brock et al,

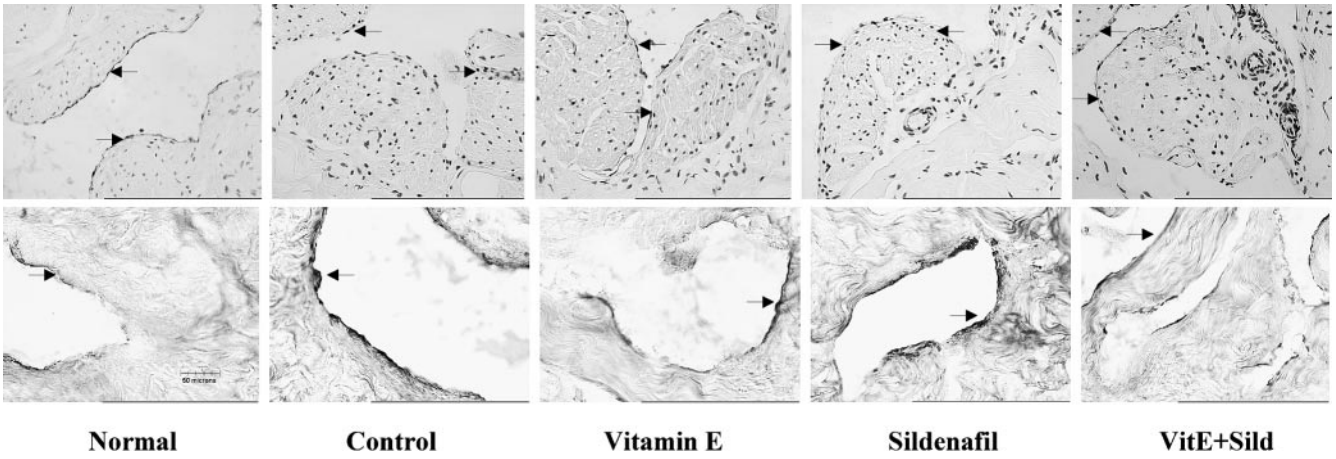

Figure 3. Urine nitrite and nitrate (NO_x) concentration was assessed by a chemiluminescent NO_x analyzer. The results are presented as the mean ± SE of 5 experiments, measured as 24.8 ± 3.8 μM in normal animals, 82.94 ± 8.9 μM in untreated diabetic animals (control), 60.25 ± 7.4 μM in the vitamin E group, 78.31 ± 7.0 μM in the sildenafil group, and 47.86 ± 5.5 μM in the vitamin E plus sildenafil group.

2002; Goldstein et al, 2003). The data presented in this report support our hypothesis of improved penile function in response to a combined therapy of vitamin E and the PDE5 inhibitor, sildenafil. We measured a consistent rise in the ICP and positive changes in penile tissue as measured by immunohistologic staining for nNOS, endothelial cell marker, smooth muscle α-actin, and nitrotyrosine among animals receiving the combined regime.

The protocol used in this study, in which the treatment was started just 2 days after diabetes was induced and then continued for 3 weeks, is clearly artificial and may not lend itself to direct human comparison, where diabetes is often detected and treated months or years after onset. We chose this early time point and faulty level of diabetic control to evaluate penile function without extensive end organ damage, believing these animals would be the most likely to show a reversible effect of therapy (Soriano et al, 2001). There is supporting evidence from the literature concerning the beneficial role of vitamin E in the aorta, lung, and other organs (Pathania et al, 1998; Lang et al, 2000). Vitamin E appears to be the first line of defense against the peroxidation of polyunsaturated fatty acids that are contained in cellular and subcellular membrane phospholipids, because it binds to peroxyl free radicals and forms stable molecules (Mayes, 2000; van Haaften et al, 2003). Also, vitamin E binds to a variety of active oxidant species such as singlet oxygen and superoxide free radicals (Mayes, 2000). NO can react with superoxide anions ($O_2^{\bullet -}$), forming the toxic oxidizing agent peroxynitrite ($ONOO^-$). Peroxynitrite induces nitration of tyrosine residues, leading to changes in protein structure and function and alterations in signaling pathways (Ceriello et al, 2001). In this study, we measured an increase in the urine levels of NO_x metabolites (nitrite and nitrate), which indicate the production of NO (Tanaka et al, 1997). An increased peroxynitrite formation and an elevated presence of nitrotyrosine in protein have previously been reported in diabetic patients (Ceriello et al, 2001; Hoeldtke et al, 2003). Positive tissue staining of nitrotyrosine has been used as an indirect indicator of oxidant stress (Ceriello et al, 2002; Fries et al, 2003). In this study, the diabetic animals demonstrated increased levels of urine NO_x concentration as well as increased amounts of nitrotyrosine staining in the penile tissue when compared with the normal animals. This supports the belief that oxidative stress is an important factor contributing to the pathological changes seen in diabetic-associated ED. Furthermore, it provides supporting experimental evidence for our findings, indicating that antioxidant treatment in association with PDE5 inhibition is a useful salvage treatment approach.

This report supports the theory that ED-DM is a multifaceted condition affecting neural, muscular, vascular, and metabolic functions. The vitamin E–treated group showed improved endothelial cell staining, with the vitamin E plus sildenafil group most closely resembling the normal animals.

In summary, vitamin E was shown to protect penile tissue from injury and to preserve nerve and endothelial function, as demonstrated by nNOS and endothelial cell staining patterns and a measured rise in intracorporal pressure. There is support in the literature for the potential neurotrophic role of vitamin E in ischemic animal models (Gonzalez-Perez et al, 2002). Additionally, vitamin E has been shown to reduce oxidative stress in congestive heart failure (Shite et al, 2001); the mechanism is not yet known but may have contributed, in part, to the improved intracorporal pressure measured in this report. Finally, recent evidence strongly supports the potential benefit of vitamin E within the diabetic population. As described by Evans et al (2002), hyperglycemia within the diabetic population may lead to the activation of kinases and

stress-activated protein kinases responsible for many of the pathological changes observed in this population.

As such, the utility of antioxidants should be considered an alternative new strategy for treatment. The addition of a PDE5 inhibitor to the vitamin E cocktail may further enhance endothelial function. Recent evidence demonstrating a protective role for PDE inhibitors in patients with endothelial dysfunction has been published (Halcox et al, 2002).

To our knowledge, this report is the first to record the potential interaction of oxygen free radical scavenger therapy with daily PDE5 inhibitors in an animal model of diabetes. Future work defining the dose, timing, and optimal parameter of these agents may provide an enhanced erectile response for this challenging-to-treat population.

Conclusion

Diabetic men with ED represent a difficult population to treat, as they are often refractory to standard oral PDE5 inhibitor therapy. In this report, we evaluated the potential synergistic role of upstream optimization of NO levels via oxygen free radical scavenger therapy (vitamin E) coupled with downstream facilitation using a PDE5 inhibitor (sildenafil). This potential salvage approach enhanced erectile response among a small cohort of diabetic animals and may provide a clinically meaningful approach to the hard-to-treat diabetic male with ED.

References

Abderrahmane A, Salvail D, Dumoulin M, Garon J, Cadieux A, Rousseau E. Direct activation of K(Ca) channel in airway smooth muscle by nitric oxide: involvement of a nitrothiosylation mechanism? *Am J Respir Cell Mol Biol.* 1998;19:485–497.

Andersson KE, Wagner G. Physiology of penile erection. *Physiol Rev.* 1995;75:191–236.

Anggard EE. Endogenous and exogenous nitrates. *Acta Anaesthesiol Scand Suppl.* 1992;97:7–10.

Bateman RM, Ellis CG, Freeman DJ. Optimization of nitric oxide chemiluminescence operating conditions for measurement of plasma nitrite and nitrate. *Clin Chem.* 2002;48:570–573.

Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes.* 1999;48:1–9.

Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol.* 1996;271:C1424–C1437.

Benet AE, Melman A. The epidemiology of erectile dysfunction. *Urol Clin North Am.* 1995;22:699–709.

Blanco R, Saenz de Tejada I, Goldstein I, Krane RJ, Wotiz HH, Cohen RA. Dysfunctional penile cholinergic nerves in diabetic impotent men. *J Urol.* 1990;144:278–280.

Brock GB, McMahon CG, Chen KK, Costigan T, Shen W, Watkins V, Anglin G, Whitaker S. Efficacy and safety of tadalafil for the treatment of erectile dysfunction: results of integrated analyses. *J Urol.* 2002;168:1332–1336.

Burnett AL, Lowenstein CJ, Bredt DS, Chang TS, Snyder SH. Nitric oxide: a physiologic mediator of penile erection. *Science.* 1992;257: 401–403.

Burton GW. Vitamin E: molecular and biological function. *Proc Nutr Soc.* 1994;53:251–262.

Ceriello A, Mercuri F, Quagliaro L, Assaloni R, Motz E, Tonutti L, Taboga C. Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia.* 2001;44:834–838.

Ceriello A, Quagliaro L, D'Amico M, et al. Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. *Diabetes.* 2002;51:1076–1082.

Cooke JP, Tsao PS. Cytoprotective effects of nitric oxide. *Circulation.* 1993;88:2451–2454.

De Young L, Yu D, Freeman D, Brock GB. Effect of PDE5 inhibition combined with free oxygen radical scavenger therapy on erectile function in a diabetic animal model. *Int J Impot Res.* 2003;15:347–354.

Escrig A, Marin R, Abreu P, Gonzalez-Mora JL, Mas M. Changes in mating behavior, erectile function, and nitric oxide levels in penile corpora cavernosa in streptozotocin-diabetic rats. *Biol Reprod.* 2002; 66:185–189.

Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev.* 2002;23:599–622.

Fries DM, Paxinou E, Themistocleous M, et al. Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species. *J Biol Chem.* 2003;278:22901–22907.

Goldstein I, Young JM, Fischer J, Bangerter K, Segerson T, Taylor T. Vardenafil, a new phosphodiesterase type 5 inhibitor, in the treatment of erectile dysfunction in men with diabetes: a multicenter double-blind placebo-controlled fixed-dose study. *Diabetes Care.* 2003;26: 777–783.

Gonzalez-Perez O, Gonzalez-Castaneda R, Huerta M, Luquin S, Gomez-Pinedo U, Sanchez-Almaraz E, Navarro-Ruiz A, Garcia-Estrada J. Beneficial effects of alpha-lipoic acid plus vitamin E on neurological deficit, reactive gliosis and neuronal remodeling in the penumbra of the ischemic rat brain. *Neurosci Lett.* 2002;321:100–104.

Hakim LS, Goldstein I. Diabetic sexual dysfunction. *Endocrinol Metab Clin North Am.* 1996;25:379–400.

Halcox JP, Nour KR, Zalos G, et al. The effect of sildenafil on human vascular function, platelet activation, and myocardial ischemia. *J Am Coll Cardiol.* 2002;40:1232–1240.

Herman A, Adar R, Rubinstein Z. Vascular lesions associated with impotence in diabetic and nondiabetic arterial occlusive disease. *Diabetes.* 1978;27:975–981.

Hoeldtke RD, Bryner KD, McNeill DR, Hobbs GR, Baylis C. Peroxynitrite versus nitric oxide in early diabetes. *Am J Hypertens.* 2003;16: 761–766.

Islam KN, Devaraj S, Jialal I. alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells. *Circulation.* 1998;98:2255–2261.

Karasu C, Ozansoy G, Bozkurt O, Erdogan D, Omeroglu S. Antioxidant and triglyceride-lowering effects of vitamin E associated with the prevention of abnormalities in the reactivity and morphology of aorta from streptozotocin-diabetic rats. Antioxidants in Diabetes-Induced Complications (ADIC) Study Group. *Metabolism.* 1997;46:872–879.

Katusic ZS. Superoxide anion and endothelial regulation of arterial tone. *Free Radical Biol Med.* 1996;20:443–448.

Keegan A, Walbank H, Cotter MA, Cameron NE. Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta. *Diabetologia.* 1995;38:1475–1478.

Kinlay S, Fang JC, Hikita H, et al. Plasma alpha-tocopherol and coronary endothelium-dependent vasodilator function. *Circulation.* 1999;100: 219–221.

Kolodny RC, Kahn CB, Goldstein HH, Barnett DM. Sexual dysfunction in diabetic men. *Diabetes.* 1974;23:306–309.

Lang D, Kredan MB, Moat SJ, Hussain SA, Powell CA, Bellamy MF, Powers HJ, Lewis MJ. Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions. *Arterioscl Thromb Vasc Biol.* 2000;20:422–427.

Maatman TJ, Montague DK, Martin LM. Erectile dysfunction in men with diabetes mellitus. *Urology.* 1987;29:589–592.

Mabile L, Bruckdorfer KR, Rice-Evans C. Moderate supplementation with natural alpha-tocopherol decreases platelet aggregation and low-density lipoprotein oxidation. *Atherosclerosis.* 1999;147:177–185.

Mayes PA. Structure and function of the lipid-soluble vitamins. In: Murray RK, Granner DK, Mayes PA, Rodwell VW, eds. *Harper's Biochemistry.* 25th ed. Stamford, Conn: Appleton & Lange; 2000:642–652.

McCulloch DK, Campbell IW, Wu FC, Prescott RJ, Clarke BF. The prevalence of diabetic impotence. *Diabetologia.* 1980;18:279–283.

Mercuri F, Quagliaro L, Ceriello A. Oxidative stress evaluation in diabetes. *Diabetes Technol Ther.* 2000;2:589–600.

Moreland RB, Hsieh G, Nakane M, Brioni JD. The biochemical and neurologic basis for the treatment of male erectile dysfunction. *J Pharmacol Exp Ther.* 2001;296:225–234.

Park JY, Ha SW, King GL. The role of protein kinase C activation in the pathogenesis of diabetic vascular complications. *Perit Dial Int.* 1999; 19(suppl 2):S222–S227.

Pathania V, Syal N, Pathak CM, Khanduja KL. Changes in rat alveolar macrophageal antioxidant defense and reactive oxygen species release by high dietary vitamin E. *J Nutr Sci Vitaminol (Tokyo).* 1998;44: 491–502.

Rendell MS, Rajfer J, Wicker PA, Smith MD. Sildenafil for treatment of erectile dysfunction in men with diabetes: a randomized controlled trial. Sildenafil Diabetes Study Group. *JAMA.* 1999;281:421–426.

Saenz de Tejada I, Goldstein I, Azadzoi K, Krane RJ, Cohen RA. Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N Engl J Med.* 1989;320:1025–1030.

Saldeen T, Li D, Mehta JL. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. *J Am Coll Cardiol.* 1999;34:1208–1215.

Shite J, Qin F, Mao W, Kawai H, Stevens SY, Liang C. Antioxidant vitamins attenuate oxidative stress and cardiac dysfunction in tachycardia-induced cardiomyopathy. *J Am Coll Cardiol.* 2001;38:1734–1740.

Soriano FG, Pacher P, Mabley J, Liaudet L, Szabo C. Rapid reversal of the diabetic endothelial dysfunction by pharmacological inhibition of poly(ADP-ribose) polymerase. *Circ Res.* 2001;89:684–691.

Tanaka S, Yashiro A, Nakashima Y, Nanri H, Ikeda M, Kuroiwa A. Plasma nitrite/nitrate level is inversely correlated with plasma low-density lipoprotein cholesterol level. *Clin Cardiol.* 1997;20:361–365.

Tesfamariam B, Cohen RA. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol.* 1992;263:H321–H326.

van Haaften RI, Haenen GR, Evelo CT, Bast A. Effect of vitamin E on glutathione-dependent enzymes. *Drug Metab Rev.* 2003;35:215–253.

Willson RL. Free radical protection: why vitamin E, not vitamin C, beta-carotene or glutathione? *Ciba Found Symp.* 1983;101:19–44.