Original Article

The effects of combined free radical scavenger and sildenafil therapy on age-associated erectile dysfunction: An animal model

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Abstract

Introduction: Aging results in erectile dysfunction that is partially attributed to decreased nitric oxide (NO) and increased free radical generation. Vitamin E enhances endothelial cell function and acts as a free radical scavenger; however, its benefits on erectile function in the elderly are unknown.

Aims: The aim of the following study is to determine if Vitamin E alone, or in combination with the phosphodiesterase 5 inhibitor sildenafil, may improve erectile function and the NO signaling in a cohort of aged (13-15 month old) rats.

Materials and Methods: Male Sprague-Dawley rats (n = 28) were divided based upon age into young (4-5 months old, n = 7) and aged (13-15 months old, n = 21) cohorts. Aged rats were treated with Vitamin E, sildenafil or a combination of both. Penile cavernosal and dorsal nerve tissues were evaluated for neuronal nitric oxide synthase (nNOS) and caveolin-1 expression. Erectile function was assessed through intra-cavernous pressure (ICP) recordings.

Results: nNOS and cavoelin-1 were significantly decreased in aged rats compared with young controls. In aged rats, both Vitamin E and sildenafil partially recovered nNOS expression but when combined, a synergistic elevation in nNOS was observed. The significant decreases in ICP recorded in aged rats were improved with sildenafil; however, Vitamin E did not yield any additional improvements in ICP.

Conclusions: Diminished levels of nNOS and caveolin-1 are found in aged rats. When combined with sildenafil, Vitamin E synergistically increased nNOS expression. Since biochemical gains were not realized physiologically, other contributing factors likely exist.

Key Words: Aging, antioxidant, erectile dysfunction, impotence, nitric oxide, sildenafil, vitamin E

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INTRODUCTION

Older men experience erectile dysfunction (ED) at an increased frequency and severity compared to younger men.[1,2] While elderly men have an increased likelihood of contributing co-morbidities such as; hypertension, diabetes and peripheral vascular disease; increased age alone has been shown to play a critical role in the development of ED.[3-6] Indeed, age-related changes result in altered endothelial cell function that causes a reduction of cellular nitric oxide (NO) levels and subsequent impairment in penile smooth muscle relaxation. Furthermore, alterations in the levels of nitric oxide synthase (NOS) and accumulated reactive oxygen species (ROS) dampen the abilities of the penile vasculature in elderly males to evoke erection.[7-10] The previous studies have confirmed the beneficial effects

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of Vitamin E in enhancing the therapeutic effects of the phosphodiesterase (PDE) 5-inhibitor sildenafil in a diabetic rat model.[11] Given that time-dependent inhibition in NO signaling occurs with aging, we sought to determine whether Vitamin E could have similar beneficial effects on salvaging erectile function in an aged rat model.

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant and oxygen free radical scavenger.[12,13] It is an important antioxidant for countering oxidative stress-induced injury in the aged population.[14,15] Vitamin E has also been reported to improve NO-mediated arterial relaxation as well as maintain cellular membrane integrity and protein stability.[16-18] The true impact of Vitamin E supplementation on erectile function has yet to be proven; in spite of its widespread clinical use. Reports linking Vitamin E supplementation to an increased rate of cardiovascular events further supports the need for background studies into the biochemistry and physiology of its effects.[19] Vitamin E treatment appears to enhance relaxation in the corpus cavernosum of spontaneously hypertensive rats by improving neuronal or endothelial function related to NO.[20] A hallmark of aging is overproduction of ROS and Vitamin E has been shown to reduce oxidant damage produced by NO.[21,22] A precedent thus exists for potential improvements in NO signaling and erectile function with the use of Vitamin E, especially in the aged population.

PDE5 inhibitors are effective agents in the treatment of ED across a broad range of etiologies.[23,24] Sildenafil acts by selectively inhibiting the cyclic guanosine monophosphate (cGMP)-specific type 5 PDE, the major isozyme metabolizing cGMP in the corpus cavernosum.[25] Inhibition of cGMP metabolism acts synergistically with the release of NO from endothelial and neuronal sources to elevate cGMP levels. This enhances and maintains penile smooth muscle relaxation and facilitates increased cavernosal blood flow during sexual stimulation and erection.[26,27]

The presence of NO, derived from vascular endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthases (nNOS), represents an essential component that functionally relaxes penile vasculature and cavernous smooth muscles. NOS function is regulated by plasma membrane organelles known as caveolae, which also act to store NO thus playing an important role in cell signaling.[28,29] Caveolins are a family of integral membrane proteins necessary for caveolae formation and have been implicated in a variety of disease processes including ED (Bakircioglu *et al.* 2001). While their role is incompletely understood, decreased levels of NOS and caveolin-1 has been reported in aged rats – a finding associated with increased oxidative stress-induced injury.[8,30-32] Subsequently, the impaired veno-occlusive mechanism limits cavernous smooth muscle relaxation yielding age-associated ED.[8,33]

Vitamin E combined with sildenafil has been previously shown to evoke a synergistic and therapeutic effect by increasing nNOS as well as penile pressures in an animal model of diabetes.[34] Since aging alters endothelial cell function and decreases NOS activity,[7] we sought to investigate the effects of Vitamin E and sildenafil on erectile function on aged rats.

MATERIALS AND METHODS

Young and aged animal model

After approval from the Institutional Review Board at the University of Western Ontario (London, Ontario, Canada), twenty-eight aged male Sprague-Dawley rats were divided based upon age into young (4-5 months old, $n = 7$) and aged (13-15 months old, $n = 21$) cohorts. The Aged rats (13-15 months old) were then further sorted into control and treatment arms. The treatment arm of the aged subgroup received Vitamin E (30 IU/day), Sildenafil (provided by Pfizer Canada, Pointe-Clair, Quebec; 5 mg/kg/day) or combined Vitamin E and sildenafil (30 IU/day + 5 mg/kg/day). Each treatment was administered by oral gavage. The results of the aged rats were then compared with both aged control rats and young control rats (4-5 months old). This was done in order to help determine the effects of age, as well as the changes seen as a result of vitamin E, sildenafil or the combination of these agents.

The dose of Vitamin E was selected from our previous studies that demonstrated a therapeutic range between 2 and 120 IU units of vitamin E.[34] The dose of sildenafil used was based on previous dose response studies conducted in our laboratory that identified 5 mg/kg to be the optimal erectogenic dose in this animal model.[34] We have also chosen to give a dose of Vitamin E 3 h and sildenafil 10 min before surgery to optimize drug concentration in the plasma.[35,36] The "University Council on Animal Care-Animal Use Subcommittee" approved all experimental protocols.

Immunohistological analysis

Rat penile tissue was fixed in cold, fresh 2% formaldehyde 0.1 M phosphate buffer (pH 7.4) for 4 h, cryoprotected in 15% sucrose for 20 h at 4°C, embedded in an optical cutting compound (OCT; Tissue-Tek, Sakura, USA) and stored at-70°C. The OCT embedded tissues were cut into 5 μM sections and adhered to superfrost plus slides (Fisher Scientific, Nepean, ON Canada). Immunostaining for nNOS, endothelial cell marker (CD31) and smooth muscle α-actin was then performed as described previously.[11,34]

Individual sections were air dried (10 min), hydrated in a phosphate-buffered saline (PBS) buffer and treated with

0.3% hydrogen peroxide in methanol. Multiple rinses with water and PBS were performed followed by blocking with 3% goat serum for 3-5 h at room temperature. The sections were incubated in blocking buffer at 4°C overnight with primary antibody mouse anti nNOS (1:100 dilution), anti CD31 (1:500 dilution; Transduction Lab BD PharMingen, Mississauga Canada) and mouse anti-α-actin (1:300 dilution, Roche Diagnostics, Quebec, Canada). Sections were then washed five times with PBS buffer and incubated with the secondary antibody biotin conjugated goat anti-mouse immunoglobulin G (IgG) in PBS (1:250 dilution; Sigma, St. Louis, MO USA) with 1% bovine serum albumin for 2-5 h at room temperature. After several PBS washings, the sections were incubated with an anti-biotin clone BN-34 peroxidase conjugate IgG fraction (Sigma) for 2 h. Antigen localization was visualized by using diaminobenzidine peroxidase substrate (Sigma). Sections were counterstained with hematoxylin, dehydrated with graded alcohols to xylene and placed under a coverslip.

Histological examination was conducted by a blinded reviewer using a Zeiss microscope and a computerized imaging system (Northern Eclipse, Empix, Canada). Positive staining of nNOS within the dorsal nerve and three other sites in penile cavernosum was tabulated. Positive staining of CD31 within the major penile sinusoidal lining was measured under ×400 power. The area of positive staining of smooth muscle α-actin was calculated as the ratio of total sectional area under ×25 power in 4 duplicate sections of each sample.

Western blot analysis

Proteins were prepared from frozen penile tissues as described previously.[34] A sample containing 20 ug of protein was separated on 10% polyacrylamide gel electrophoresis and transferred to Hyboud-enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, Piscataway, NJ, USA). Mouse anti-smooth muscle alpha-actin, nNOS and caveolin-1 were used as primary antibodies. Goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz, California) was the secondary antibody. Antibody specific bands were detected using ECL reagents (Amersham-Pharmacia Biotech, England) by exposing blots to autoradiographic film for 20-50 s (Raw data not shown). The autoradiographic film was scanned using Alp ImagerTM 2200 Documentation and Analysis System (Alpha Innotech Corporation Canada).

Evaluation of erectile function

For each rat, the lateral-prostatic space was dissected utilizing 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 transverse perineal incision the penile crus was exposed. A 23-Gauge needle filled with heparin (250 IU/mL) was connected to Tygon tubing and inserted into the penile crus. The microsurgical procedure was facilitated by the use of a Zeiss SR operating stereomicroscope. Intra-cavernosal pressure was evoked with 0.2 ms pulses of 2 mA at 20 Hz for 40 s duration and recorded using LabVIEW 2 software (National Instruments, Austin, Texas, USA). Three electro-stimulations were replicated at intervals of 10 min. The animals were sacrificed using pentobarbital (200 mg/kg i.p.) after which the penile tissue was harvested for analysis.

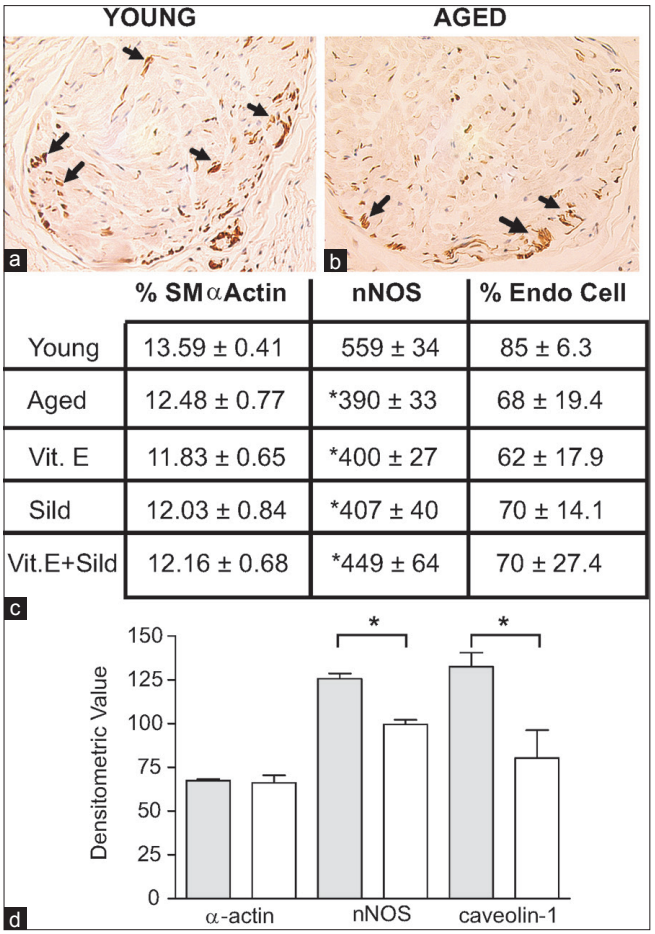
Statistical analysis

Values are expressed as mean ± standard error of seven experiments for each group. Data were compared by two-tailed *t*-tests with $\alpha = 0.05$.

RESULTS

Initially, we examined cross-sections of the mid-shaft of the corpus cavernosum and dorsal penile nerve to determine nNOS-positive staining in young and aged rats. Histological examinations were conducted by a blinded reviewer using a Zeiss microscope from a subtotal of five sections in each condition [aged control vs. young control; Representative image shown in Figure 1]. The results for nNOS are reported as the sum of positive staining NO synthase nerve fibers from the dorsal nerve, an area adjacent to the dorsal vein and two regions from the right and left posterior corpus cavernosum (at 400 power) similar to previously described.[34] Young control rats showed a significantly higher positive staining for nNOS (559 ± 34) compared with the aged controls (390 ± 33) [Figure 1a-c]. Following exposure to Vitamin E and sildenafil, the expression of nNOS increased to 400 ± 27 and 407 ± 40, respectively [Figure 1c] in treated aged rats. A synergistic increase in nNOS was observed (449 ± 64) upon treatment in aged rats with both Vitamin E and sildenafil, suggesting that simultaneous application of both agents resulted in a biochemical stimulation of nNOS exposure above levels that would be normally expected. Over the same time-points, smooth muscle α-actin and the endothelial cell marker CD31 were statistically unchanged proving the effects were localized to the NO signaling cascade [Figure 1c].

Protein expression was examined through Western Blot and a significant decrease in nNOS was seen in aged control animals [Shaded bars, Figure 1d] compared with the young controls [Hollow bars, Figure 1d]. Given that recent studies in caveolin-1 knockout mice have found abnormal arterial function with aging[29] and that caveolin-1 acts to store NO,[28,29] we wanted to examine caveolin-1 protein expression in aged animals. We identified a decrease in caveolin-1 in aged control animals compared with young controls [Figure 1d] suggesting a possible contribution of this protein in the ED observed in aged rats.



	% SMαActin	nNOS	% Endo Cell
Young	13.59 ± 0.41	559 ± 34	85 ± 6.3
Aged	12.48 ± 0.77	*390 ± 33	68 ± 19.4
Vit. E	11.83 ± 0.65	*400 ± 27	62 ± 17.9
Sild	12.03 ± 0.84	*407 ± 40	70 ± 14.1
Vit.E+Sild	12.16 ± 0.68	*449 ± 64	70 ± 27.4

Figure 1: Synergistic, biochemical enhancement of neural nitric oxide synthase (nNOS) following treatment with Vitamin E and sildenafil in aged rats. Mid-shaft penile sections immunostained with nNOS in the tissues of young (Panel A) and aged (Panel B) control rats. Representative micrographs (magnification ×400) demonstrated positive staining for nNOS (dark color + arrows) in young animals (Panel A) associated with decreased staining in aged animals (Panel B). This was confirmed via analysis of the sum of positively stained nNOS within the dorsal nerve and at three other random sites within the corpus cavernosum (power = ×400; *, $P < 0.01$). No differences between the proportions of positively stained smooth muscle α-actin or endothelial cells within the major penile sinusoids were observed (Panel C). Western blot analysis on penile tissue extracts assessed nNOS, smooth muscle α-actin and caveolin-1 protein content using monoclonal antibodies (Panel D). Densitometric analysis ($n = 5$ penile extracts for each group) showed that while smooth muscle α-actin remained constant, young rats (solid bars) had significantly increased levels of nNOS and caveolin-1 expression levels compared with aged rats (Open bars; *, $P < 0.05$)

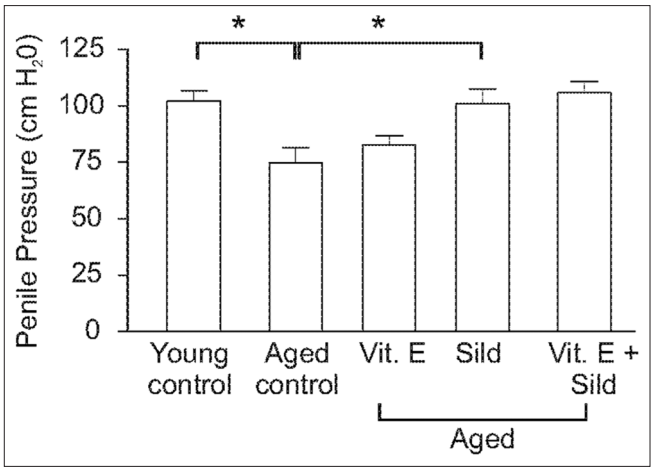


Figure 2: Penile pressure measurements in response to cavernous nerve electro-stimulation in young and aged rats. The mean maximum intra-cavernous pressure ± standard error of the mean was recorded ($n = 7$ for each). Young control rats were found to have significantly increased penile pressures compared to aged control rats (102.1 ± 4.6 vs. 74.9 ± 4.6 cm H_2O). While Vitamin E alone failed to significantly increase the penile pressures (82.2 ± 4.2 cm H_2O) with respect to aged rats, Sildenafil was able to recapture most of the losses seen in the aged population (101.3 ± 6.4 cm H_2O). The addition of short-term Vitamin E therapy increased penile pressures slightly (105.9 ± 5.0 cm H_2O) but not enough to achieve significance (*, $P < 0.05$)

As a means to determine whether the biochemical findings showing synergistic up regulation of nNOS with combination therapy (vitamin E and sildenafil) had physiological significance, we measured cavernous nerve electro-stimulation generated penile pressure [Figure 2]. As anticipated, a clear age-induced impact on erectile function amongst the animals studied existed with young control animals exhibiting improved responses [Figure 2]. Examination of the peak intra-cavernous pressure during electro-stimulation of the cavernous nerves identified a significant decrease in penile pressure from young control compared to aged control rats [102.1 ± 4.6 vs. 74.9 ± 4.6 cm H_2O; $P < 0.05$; Figure 2]. Administration of Vitamin E improved responses slightly compared to aged controls (82.2 ± 4.2 cm H_2O) but these results did not reach statistical significance. Sildenafil improved penile pressures in aged rats significantly compared to aged controls (101.3 ± 6.4 cm H_2O) and reached similar levels a young control rats [Figure 2]. When Vitamin E was combined with sildenafil, the improvement was significant compared to aged controls but not significant with respect to sildenafil alone (105.9 ± 5.0 cm H_2O) [Figure 2]. As such, the synergy observed in the biochemical analysis did not translate to the physiological findings given the context of our experimental protocol.

DISCUSSION

ED increases in prevalence proportional to age in a manner independent of genetics, diet, race, culture or co-morbid condition. Aging introduces a multitude of confounding variables that contribute to ED such as higher rates of diabetes, hypertension and hypogonadism. However, across all age strata, age remains an independent predictor of ED prevalence. Older populations are particularly at higher risk for ED given that they have increased comorbidities. Furthermore, these illnesses may occur at more severe levels of intensity, particularly testosterone deficiency,[37] in which higher levels of oxygen free radicals have been reported.[31,32] Vasculogenic compromise

resulting in end organ (penile) failure[8,9] has also been shown to be age-dependent. Characterized by reduced smooth muscle content, increased collagen fibers and reduced neural function, vascular ED is primarily treated with PDE5 inhibitors at the outset.[8,9] Unfortunately, owing to disease progression and other factors, the efficacy of PDE5 inhibitors frequently attenuates with time among significant proportions of men. The remaining ED treatment options are more invasive; such as intracavernous injection and insertion of a penile prosthesis. As such, adjuncts capable of increasing the potency of PDE5 inhibitors would be desirable.

Previous studies have shown that Vitamin E can increase PDE5 inhibitor effectiveness in diabetic rats.[11,34] Given that increasing age results in an elevation of free radicals and that free radicals inactivate NO signaling, we postulated that concurrent treatment of ED with sildenafil and the antioxidant Vitamin E, may decrease free radical production, enhance NO signaling and potentiate erectogenic responses evoked following PDE5 inhibition.

Initially, we evaluated the effects of aging on the expression of both nNOS and the scaffolding protein caveolin-1. We demonstrated that aged rats exhibit biochemically-decreased expression of caveolin-1 and nNOS that could be recovered via treatment with Vitamin E and sildenafil. Unfortunately, these biochemical gains were not echoed in improvements of erectile function. It is thus tempting to speculate that a longer duration of treatment could improve physiological outcomes.

Aging alters erectile functioning as well as endothelial signaling through a reduction in NOS expression.[38] Moreover, recent studies on caveolin-1 knockout mice have found an association between this scaffolding protein and premature vascular aging in mesenteric vessels coupled and altered NOS expression.[29] Our results found a significantly lower nNOS expression among the aged rats, consistent with previous studies in other animals.[30,31,39]

Caveolin-1, an integral membrane protein associated with caveolae and trans-Golgi network derived vesicles, is important in smooth muscle contraction. It is an important factor in endothelial cell growth and has several postulated roles including cholesterol transport, caveolae formation, G-protein subunit regulation, oncogenic transformation, insulin signaling and eNOS regulation.[40,41] Here, we report significantly decreased levels of caveolin-1 in 13-15 month old rats concurrent with a decrease in NOS. The reduction of caveolin-1 shown in this study is an important finding in that it can partially explain ED in the aged population. Indeed, research using caveolin-1 deficient mice identified impaired relaxation of the corpus cavernosum in response to both nerve stimulation and sodium nitroprusside, supporting its role in erectile function.[42] Others have demonstrated reduced expression of caveolin-1 following pelvic nerve injury and have suggested the possibility of using caveolin-1 as a biomarker for cavernosal health.[43,44]

Studies in the rat have previously shown decreased expression of caveolin-1 in very old (24 month) rats coupled with concurrently increased NOS expression.[8] Combining the results of the Bakircioglu *et al.* study with our own, it appears that caveolin-1 exhibits a concurrent age-dependent decrease. Furthermore, NOS decreases in 13-15 month olds (this study) and then rebounds with an increase at 24 months.[8] Perhaps this is indicative of a compensatory response to some, as yet unknown, penile signal transduction pathway? Therefore, this relationship between caveolin-1 and NOS is more complicated than previously anticipated and needs to be further explored.

Oxidative stress plays an important factor in the pathological changes seen within the penis during aging.[32] Vitamin E supplementation, through its scavenging effect, may reduce this oxidative stress resulting in decreased levels of tissue damage. Recently, a study by Helmy and Senbel[38] explored this theory using treatment arms consisting of a 21-day course of either Vitamin E or Sildenafil. Vitamin E was shown to ameliorate many of the changes associated with age-related ED including improvement in intracavernosal pressures and restoration of NO content.[38] In addition, sildenafil also appeared to exert some antioxidant properties.[38]

In the present study, Vitamin E antioxidant therapy and PDE5 inhibition were examined as it was postulated that by protecting NO against ROS degradation, a synergistically increase in physiological erections could be attained. While this effect was previously demonstrated in diabetic mice,[34] it was hoped that a similar effect could be seem in order rats. We speculated that if Vitamin E provided benefit to erectile function in those men on PDE5 inhibitors, improved erectile function should be seen in the elderly population. Initially, we were optimistic when we noted that biochemically, nNOS was synergistically elevated following Vitamin E and sildenafil treatment in aged rats; however, the physiological benefits did not follow.

It is possible that a longer-term treatment with Vitamin E may have yielded improved benefits. Furthermore, it may be that the benefits of combination therapy may be more pronounced in older animals (19-24 months) as reported by other researchers.[33,45] Further work examining the effects of Vitamin E with ED in 24 month-old aged animals may resolve these questions.

Age-associated ED represents an important clinical challenge, affecting large numbers of men who are often refractory to oral

PDE5i therapy. Reduced nNOS and caveolin-1 expression, elevated oxygen free radicals and decreased bio-available NO appear to be significant pathological factors in the early phase of this condition. Herein, we detail a decrease in nNOS expression that is rescued by short-term treatment with Vitamin E and sildenafil in a synergistic manner. Intra-cavernosal pressures were lowered in aged controls but were recovered following treatment with Vitamin E and sildenafil. It can be hypothesized that declines in nNOS and caveolin-1 may mirror early physiologic changes that result in ED. Additional long-term clinical studies evaluating the combination of Vitamin E and PDE5 inhibition should be undertaken in order to prolong the length of time in which PDE5 inhibition would be effective.

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