PROFESSOR SERAP GUR (Orcid ID : 0000-0002-1730-7282)

DR WAYNE JOHN G. HELLSTROM (Orcid ID : 0000-0003-1284-959X)

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TESTOSTERONE POSITIVELY REGULATES FUNCTIONAL RESPONSES AND NITRIC OXIDE EXPRESSION IN THE ISOLATED HUMAN CORPUS CAVERNOSUM

Serap Gur[1,2], Laith Alzweri[1], Didem Yilmaz-Oral[3], Ecem Kaya-Sezginer[4], Asim Abdel-Mageed[1], Suresh C. Sikka[1], Cetin Volkan Oztekin[5] and Wayne J.G. Hellstrom[1]

[1]Tulane University Health Sciences Center, Department of Urology and Pharmacology, New Orleans, LA, USA, [2]Ankara University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey, [3]Cukurova University, Department of Pharmacology, Faculty of Pharmacy, Adana, Turkey, [4]Ankara University, Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey, [5]Department of Urology, Faculty of Medicine, University of Kyrenia, Girne, Turkish Republic of North Cyprus, Mersin 10, Turkey

Running head: Effects of testosterone on ED in the isolated HCC

Correspondence author: Wayne J.G. Hellstrom, MD, FACS

Professor of Urology, Chief, Section of Andrology, Tulane University,

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Health Sciences Center, Department of Urology,
1430 Tulane Avenue, 86-42, New Orleans, LA 70112
Phone: (504) 988-3361
Fax: (504) 988-5059; **e-mail:** whellst@tulane.edu

ABSTRACT

Background: Testosterone (T) deficiency is associated with erectile dysfunction (ED). The relaxant response of T on the corporal smooth muscle through a non-genomic pathway has been reported; however, the *in vitro* modulating effects of T on human corpus cavernosum (HCC) have not been studied.

Objectives: To compare the effects of various concentrations of T on nitric oxide (NO)-dependent and -independent relaxation in organ bath studies and elucidate its mode of action, specifically targeting the cavernous NO/cyclic guanosine monophosphate (cGMP) pathway.

Materials and Methods: HCC samples were obtained from men undergoing penile prosthesis implantation (n = 9). After phenylephrine (Phe) precontraction, the effects of various relaxant drugs of HCC strips were performed using organ bath at low (150ng/dL), eugonadal (400ng/dL), and hypergonadal (600ng/dL) T concentrations. The penile tissue measurements of endothelial nitric oxide synthase (eNOS), neuronal (n)NOS and phosphodiesterase type 5 (PDE5)A were evaluated via immunostaining, Western blot, cGMP and nitrite/nitrate (NOx) assays.

Results: Relaxation responses to ACh and EFS in isolated HCC strips were significantly increased at all T levels compared with untreated tissues. The sildenafil-induced relaxant response was significantly increased at both eugonadal and hypergonadal T levels. Normal and high levels of T are accompanied by increased eNOS, nNOS, cGMP and nitrate/nitrite (NOx) levels, along with reduced PDE5 protein expression.

Conclusion: This study reveals an important role of short-term and modulatory effects of different concentrations of T in HCC. T positively regulates functional activities, inhibition of PDE5 expression and formation of cGMP and NOx in HCC. These results demonstrate that T indirectly contributes to HCC relaxation via downstream effects on nNOS, eNOS, and cGMP and by inhibiting PDE5. This action provides a rationale for normalizing T levels in hypogonadal men with ED, especially when PDE5 inhibitors are ineffective. T replacement therapy may improve erectile function by modulating endothelial

function hypogonadal men.

Key words: Hypogonadism, testosterone, human corpus cavernosum, phosphodiesterase type 5 enzyme, relaxation

INTRODUCTION

Androgens are essential to the development and growth of the penis, and regulate erectile function via multiple mechanisms (Traish and Kim, 2005, Traish, et al., 2007). Testosterone (T) regulates central mechanisms of penile erection, and influences peripheral neural function. Studies have demonstrated that penile erection in rats is T-dependent (Suzuki, et al., 2007). The nitric oxide synthase/cyclic guanosine monophosphate (NOS/cGMP) pathway is critical for normal erectile function (Burnett, et al., 1992). Androgens regulate NOS levels, and modulate phosphodiesterase-5 (PDE5) activity (Traish, et al., 2003, Zhang, et al., 2005). Previous studies show that androgens regulate the expression and the activity of NOS

isoforms in the corpus cavernosum (CC) in animal models (Lugg, et al., 1995, Park, et al., 1999, Reilly, et al., 1997, Zvara, et al., 1995).

Clinical studies reveal that in men with ED and low to low-normal range of T, higher circulating androgen levels improve cavernosal arterial inflow, cavernosal vasodilatation and the overall erectile response to PDE5 inhibitors (Traish, Munarriz, O'Connell, Choi, Kim, Kim, Huang and Goldstein, 2003). Androgen supplementation has been demonstrated to regulate the activity of PDE5 in castrated animals (Armagan, et al., 2006, Morelli, et al., 2004, Traish, et al., 1999, Zhang, Morelli, Luconi, Vignozzi, Filippi, Marini, Vannelli, Mancina, Forti and Maggi, 2005). Upregulation of NOS by androgens leads to increased nitric oxide (NO) synthesis, which in turn upregulates PDE5 expression and activity (Traish, Goldstein and Kim, 2007). Androgens have important short- and long-term effects in the regulation of human umbilical artery (HUA) contractility. The short-term effects of T on HUA tone were investigated, and long-term effects of dihydrotestosterone (DHT) on the expression of various proteins involved in the contractile process are known (Saldanha, et al., 2013). Furthermore, the direct effects of T and its precursor/derivative dehydroepiandrosterone (DHEA) on isolated rat abdominal aortic rings were investigated (Oloyo, et al., 2011). T relaxed the abdominal aorta directly via a non-genomic pathway, which is independent of endothelial-derived vasoactive substances, but involves activation of inward rectifying potassium channel (K_{IR}) and blockade of L-type calcium channels (Oloyo, Sofola, Nair, Harikrishnan and Fernandez, 2011). T induced relaxation of HCC strips by activation of smooth muscle adenosine triphosphate-sensitive K(+) channels. In addition to its known endothelial action, T likely regulates erectile function locally by its action on HCC smooth muscle (Yildiz, et al., 2009).

Mechanistic approaches are needed to understand better the interplay among PDE5 expression, activation of the NO/cGMP pathway, and androgens. This study aimed to compare the enhancing effects of different concentrations of T on NO-dependent and -independent HCC relaxation in organ bath experimentation, and, specifically, targeted the NO/cGMP pathway.

MATERIALS AND METHODS

Drugs and solutions

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). T was prepared as a non-buffered aqueous solution (23.4 mg/L, at 25°C, pH ranging between 5.5 and 6.0)(Shoskes, et al., 2016).

Human corpus cavernosum (HCC) tissue strips

A total of eleven men with ED and/or Peyronie's disease were enrolled in this study, following Institutional Review Board guidelines. CC samples were obtained from men who underwent penile prosthesis surgery. The details of the patients' demographics especially the underlying disease or comorbidity were recorded (Table 1). Three to four strips were obtained from each sample, for a total number of 11 patients. HCC tissue strips were placed in cold Krebs isotonic solution [consisting in (mM): NaCl, 118; $NaHCO_3$, 25; glucose, 5.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$ 7(H_2O), 1.17; and $CaCl_2$ $2H_2O$, 2.5] and immediately transported (between 15–30 min) to the laboratory for *in vitro* experiments.

Measurement of isometric tension in HCC strips

HCC samples were placed in a petri dish containing Krebs-bicarbonate and then oxygenated with a mixture of 95% O_2 and 5% CO_2. On average, four strips of HCC tissue (1 × 1 × 6 mm) were prepared from each cavernosal sample. Strips were suspended in 20-mL organ bath chambers (Radnoti; Radnoti Glass Technology Inc, Monrovia, California) with one end fixed to a tissue holder and the other secured to a force transducer (FT03 Grass Instruments, Quincy, Massachusetts). The organ chamber temperature was maintained at 37°C via a circulating water bath. After the placement of tissue strips in the organ bath chamber, the preparations were allowed to equilibrate for approximately 60 minutes, and the bath solution was replaced every 15 minutes. Tissue segments were exposed to 10 μ M phenylephrine (Phe); after a stable contraction was reached, and several relaxing agents were added to the bath chambers. Electrical field stimulation (EFS) of the autonomic nerves (duration: 15 seconds; amplitude: 50-90 V; frequency: 1-20 Hz; pulse width: millisecond) was accomplished with the use of platinum electrodes, positioned on either side of the tissue strip (Grass Instruments, Quincy, MA). Preparations were preincubated for 30 min with guanethidine (5mM) and atropine (1mM) to block noradrenergic neurotransmission and muscarinic responses, respectively.

In the first series of experiments, acetylcholine (ACh, 10^{-8}-10^{-4} M), EFS (1-20 Hz), and sildenafil (10 μM)-induced relaxation responses were evoked with 30 min incubations of hypogonadal (150 ng/dL),

eugonadal (400 ng/dL), and hypergondadal (600 ng/dL) T concentrations after precontraction of the HCC strips with Phe (10 μM).

In the second series of experiments in those penile tissue strips, direct sympathetic stimulation-related contraction was induced with EFS (40 Hz).

Western Blotting

Human penile tissues were homogenized with liquid nitrogen in RIPA lysis buffer containing a protease inhibitor (Cell Signaling Technology, Beverly, MA, USA) and centrifuged at 12,000×g at 4°C for 10 min to obtain a supernatant. Total protein extracts were quantified by the bicinchoninic acid assay (BCA assay, Pierce Biotechnology, Rockford, IL, USA) and 20 μg of protein was loaded per well onto a 10 % SDS-PAGE gel, then fractionated and transferred to a polyvinylidene difluoride membrane by semi-dry electrotransfer for 1h at 100 V. After soaking in blocking buffer (1x phosphate-buffered saline (PBS) and 5% dry milk), membranes were incubated overnight at 4°C with primary antibodies, including endothelial (e)NOS and neuronal (n)NOS (1:1000; BD Transduction Laboratories), PDE5A (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, TX), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000, Cell Signaling Technology, Danvers, MA). Membranes were incubated with horseradish peroxidase-linked secondary antibody and visualized with the Odyssey Fc system (LI-COR Biosciences, Lincoln, NE, USA). Band intensity was quantified, and values were normalized to GAPDH, and these results were expressed as fold change.

Immunohistochemical Studies

For the immunohistochemical localization of eNOS, nNOS, and PDE5A, HCC from each group (n=4–5) were fixed in 10% formalin and stored until processing for paraffin embedding. Sections were cut at 8–10mm, adhered to glass slides, deparaffinized in xylene and hydrated through graded alcohol baths. Endogenous peroxidases were quenched with 3% H_2O_2, and nonspecific binding of immunoglobulin G was blocked using normal horse serum (1:50) in PBS containing 0.1% bovine serum albumin. Slides were treated with 0.1% Triton X-100 for 20 min, washed in PBS for 5 min, and then incubated with rabbit primary polyclonal antibodies (eNOS, nNOS, PDE5A 1:100) (BD Transduction Laboratories, San Diego, CA) for 1 hr at room temperature. Then, the sections were then washed and incubated for an additional 30

min with a biotinylated secondary antibody (Dako, Carpinteria, CA) followed by a further 30 min incubation with avidin-biotin-conjugated horseradish peroxidase (Dako), and then the substrate (diaminobenzidine, Vectastain, Vector Laboratories, Peterborough, UK) for 5 min. eNOS, nNOS and PDE5A protein-positive cells appeared brown against Harris hematoxylin counterstain. Negative control slides, stained with a secondary antibody, were carried out for each tissue specimen (data not shown). Images were visualized under light microscopy (DM4000B and DFC280 color digital camera system), and semiquantitative histomorphological assessment was performed on all of the stained specimens. The staining of the brown area was scored (0–1 no positive staining; 1–3 increasing degrees of intermediate staining; and 4 extensive staining).

Measurement of cGMP levels

An ELISA kit with 96 wells (Cat. 900-014, Assay Designs, Ann Arbor, MI, USA) was used to quantify the cGMP levels. Selected plate wells contained known concentrations of cGMP (50, 10, 2, 0.4, 0.08 pmol/mL) prepared by serial dilutions to make the standard curve. The frozen tissues were ground into very fine pieces and dissolved in 0.1M HCl. An aliquot of the supernatant containing cGMP from the penile tissues was placed in the wells after centrifugation, while another aliquot was used for measurement of protein levels. Both the standard and test wells were treated with a yellow antibody, and after incubation and washing, the plate was read by a microplate reader at 405 nm; cGMP levels were calculated using the standard curve and expressed as pmol/mg protein.

Nitrate and nitrite (NOx) analysis

NOx concentrations were calculated with a NOx Colorimetric Assay kit (Cayman Chemical Company, USA) and measured with the Griess method. HCC tissue samples were homogenized in PBS, pH 7.4, and centrifuged at 10,000×g for 20 min. The supernatant solution was then ultracentrifuged at 100,000×g for 30 min. After being passed through 30kD ultrafilters (Millipore, Bedford, MA), 40mL of the tissue lysates was diluted with 240mL assay buffer and mixed with 10mL cofactor and 10mL nitrate reductase (colorimetric assay kit, Cayman Chemical Co). After the samples had been kept at room temperature for 3 h to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthalene–ethylene diamine dihydrochloride) in a microplate reader to obtain nitrite

plus nitrate concentrations. Amounts of nitrite in the samples were estimated by a standard curve obtained from the enzymatic conversion of sodium nitrate ($NaNO_3$) to nitrite.

Data Analysis

All results are expressed as the mean (± SEM). Statistical differences were determined by ANOVA followed by Bonferroni's complementary analysis, with $P<0.05$ considered to indicate statistical significance.

RESULTS

***In Vitro* Relaxant and Contractile Responses**

Endothelium-dependent relaxation response to ACh in isolated HCC strips was significantly increased in the presence of all T concentrations as compared to untreated tissues (Fig.1A). EFS-induced relaxation responses were increased in hypogonadal, eugonadal and hypergonadal T concentrations at 20 Hz (Fig. 1B). EFS-induced nitrergic relaxation did not change at lower frequencies (1-5 Hz). However, EFS-induced relaxation at 10 Hz frequency increased at hypogonadal and eugonadal concentrations. Sildenafil-induced relaxant responses of HCC strips were significantly increased at eugonadal and hypergonadal concentrations ($p< 0.001$; Fig. 2A). EFS-induced contractile responses were not altered between the groups at 40 Hz (Fig. 2B).

Western blot Analysis

Western blot analysis using penile tissue demonstrated that the protein levels of eNOS and nNOS were significantly increased in eugonadal and hypergonadal conditions compared to the control group ($p< 0.01$ and $p< 0.001$; Fig. 3A and B). PDE5A protein levels in HCC were decreased by normal and high T level incubations (Fig. 3A and B).

Immunohistochemical Analysis

Figure 4 indicates a significant increase in the abundance of nNOS (upper panel) and eNOS (middle panel) in eugonadal and hypergonadal conditions compared to the control group. At eugonadal and hypergonadal T concentrations, PDE5 protein staining was decreased compared to untreated and

hypogonadal states, as observed in Figure 4 (lower panel).

cGMP Levels

The cGMP generation induced by eugonadal and hypergonadal conditions was 4.2 and 5 times greater than untreated tissues ($p< 0.01$; Fig. 5A).

NOx Levels

The isolated HCC tissue levels of NO_x in eugonadal and hypergonadal T levels were significantly higher than the untreated tissues ($p< 0.05$; Fig.5B).

DISCUSSION

This study demonstrates that T indirectly and specifically contributes to relaxation of HCC via nNOS, eNOS, and cGMP, and by inhibiting PDE5 which results in an augmented erectile response. The relaxation responses of HCC to ACh and EFS were significantly increased at all levels of T compared to untreated tissues. Furthermore, the relaxant response to sildenafil was significantly increased in normal (400 ng/dL) and higher T (600 ng/dL) levels. No change in EFS-induced neurogenic contractions was observed at all levels of T. Normal and high levels of T were accompanied by increased eNOS, nNOS and cGMP, and diminished PDE5 protein expression. Tissue NOx concentration (NO production marker) was enhanced by 400 and 600 ng/dL concentrations of T.

To the best of our knowledge, this study is the first to report enhanced relaxant responses of HCC to ACh in the presence of various T concentrations. T contributes to the regulation of cavernosal endothelial reactivity. An increase in endothelium-dependent relaxation and protein expression of eNOS highlights the importance of androgens in maintaining the integrity of the structure and endothelial function of HCC, which was consistent with previous studies (Armagan, Kim, Goldstein and Traish, 2006, Baba, et al., 2000, Baba, et al., 2000, Wang, et al., 2015). A recent study demonstrated that T deficiency caused endothelial dysfunction via elevation of asymmetric dimethylarginine and oxidative stress in castrated rats (Kataoka, et al., 2017). In addition, hypogonadal men show impaired vascular reactivity, including endothelial-dependent vasodilation because of reduced NO availability (Bernini, et al., 2006). Furthermore, normal and high levels of T were accompanied by increased eNOS protein levels in the HCC. The activity and protein

level of eNOS were enhanced by exposure to physiological concentrations of T, but not to high concentrations of T in steroid-deprived human umbilical vein endothelial cells (Goglia, et al., 2010). Compelling evidence suggests that T is involved in the regulation of corporeal expression/activity of NOS isoforms (eNOS and nNOS), thus maintaining an adequate NO supply (Abidu-Figueiredo, et al., 2011). However, the exact molecular basis underlying the regulation of NOS by T still remains to be fully elucidated. A recent study by Seo et al. showed that exercise training has beneficial effects on erectile function in aged rats by increased T production from the testis and strengthening of the cavernous endothelium with the activation of eNOS (Seo, et al., 2018). In a previous study, T was shown to induce NO production in human amniotic epithelial cells via androgen receptor-dependent activation of eNOS(Yu, et al., 2010). Another study showed that in human umbilical vein endothelial cells, both T and DHT induced NO synthesis via eNOS activation, along with increased levels of eNOS(Goglia, Tosi, Sanchez, Flamini, Fu, Zullino, Genazzani and Simoncini, 2010).

Consistent with our results, previous studies demonstrated that *in vitro* administration of eugonadal and higher T upregulated penile nNOS expression (Baba, Yajima, Carrier, Morgan, Nunes, Lue and Iwamoto, 2000, Park, Kim, Kim and Paick, 1999). Park et al. showed that eNOS mRNA expression was independent of androgen and androgens enhance nNOS gene expression in the CC of rats, suggesting that they play an important role in maintaining NOS activity (Park, Kim, Kim and Paick, 1999). Any decrease in T levels influences strongly the number of NOS‐positive nerve fibres in the penis by down-regulating them (Baba, Yajima, Carrier, Morgan, Nunes, Lue and Iwamoto, 2000). A study by Reilly et al. demonstrated the role of T in stimulating nNOS expression (Reilly, Zamorano, Stopper and Mills, 1997). In addition, early findings suggest that three isoforms of NOS are expressed in the testis and epididymis of horse and these enzymes play important roles in the biology of interstitial cells that produce T (Ha, et al., 2004). Parenteral T administration enhances vasodilatation in men and women in part via endothelial-dependent actions that are likely to involve the release of NO(Rosano, et al., 1999, Worboys, et al., 2001).

eNOS is an established target of signalling actions of T through both genomic and non-genomic mechanisms (Fu and Simoncini, 2008). Several of the genes known to be regulated by T could directly modulate the activity of pathways involved in erectile function, such as eNOS, nNOS and PDE5 activity (Podlasek, et al., 2016). However, the exact molecular basis underlying the regulation of NOS by T remains to be fully elucidated (Abidu-Figueiredo, Ribeiro, Chagas, Cardoso, Costa and Sampaio, 2011).

In addition, T replacement therapy in hypogonadal men improved erectile function by modulating endothelial progenitor cells (Hwang, et al., 2016). Omar et al. indicated a significant increase in endothelial progenitor cells and endothelial microparticles levels in patients with both vasculogenic ED and late onset hypogonadism (Omar, et al., 2017). Increased NO bioavailability in erectile tissue due to varying T levels may lead to both parasympathetic hyperactivity and up-regulation of NO signaling in HCC.

In the present study, it was observed that EFS induced a greater relaxation response to all concentrations of T. A combined treatment, consisting of a brief period of electrical nerve stimulation and systemic supraphysiologic T, induced functional recovery following a crush of the recurrent laryngeal nerve (Monaco, et al., 2015). It is well known that type 2 diabetes is associated with androgen deficiency (Kataoka, et al., 2014). The erectile response in the Otsuka Long-Evans Tokushima Fatty (OLETF) rats (animal model of type II diabetes with obesity) was significantly decreased, and androgen replacement produced a significant improvement their erectile function (Kataoka, Hotta, Maeda and Kimura, 2014). nNOS protein levels in HCC were increased after incubation with normal and high levels of T (Schirar, et al., 1997). Similarly, androgens stimulated the expression of nNOS in the rat penis, thus maintaining an adequate quantity of NO (Park, Kim, Kim and Paick, 1999, Reilly, Zamorano, Stopper and Mills, 1997). Consistent with our results, previous studies demonstrated that *in vitro* administration of eugonadal and higher T levels upregulated penile nNOS expression (Baba, Yajima, Carrier, Morgan, Nunes, Lue and Iwamoto, 2000, Park, Kim, Kim and Paick, 1999). Penile erection in the rat is totally dependent on the presence of androgens (Penson, et al., 1996). This facilitatory action by androgens on penile erection involves an up-regulation of constitutive NOS isoenzymes in the CC (Marin, et al., 1999).

In our data, incubation of HCC strips with eugonadal and hypergonadal concentrations of T increased the levels of NOx compared to untreated tissues. A previous study revealed that T increased NO production linked to the phosphorylation/activation of eNOS in human aortic endothelial cells (Yu, Akishita, Eto, Ogawa, Son, Kato, Ouchi and Okabe, 2010). A study by Goglia et al. demonstrated that the synthesis of NO was related to the concentration of T, with maximal effects being observed in the presence of physiological concentrations of T and lesser benefits with higher doses in steroid-deprived human umbilical vein endothelial cells (Goglia, Tosi, Sanchez, Flamini, Fu, Zullino, Genazzani and Simoncini, 2010). Previous findings showed increased release of NO in the penile circulation during T replacement in castrated rats(Marin, Escrig, Abreu and Mas, 1999), as well as of the identified role of NO in mediating the

actions of androgens secreted from the Leydig cells (Pomerantz and Pitelka, 1998). There was increased NOS activity and NO synthesis in a dose-dependent manner.

Androgens maintain the erectile response by alternate pathways, including one that is independent of NO but involves the synthesis of cGMP (Reilly, et al., 1997). We found increased levels of cGMP in the presence of 400 and 600 ng/dL T levels. The previous study demonstrated that modulation of tissue levels of the intracellular second messenger cGMP is involved in the downstream signaling of steroid hormones(Traish and Kim, 2005). In porcine coronary artery smooth musculature, the non-genomic relaxant effect of T is mediated by an increase in cGMP tissue levels(Deenadayalu, et al., 2001). T-stimulated cGMP production in microvessels ultimately induced vasodilation (Puttabyatappa, et al., 2013). Deenadayalu et al. showed that T-induced relaxation of endothelium-denuded coronary arteries is mediated, at least in part, by enhanced NO production, leading to cGMP synthesis and PKG activation (Deenadayalu, et al., 2012).

We observed that the major cGMP hydrolyzing enzyme, PDE5 protein levels were lower in eugonadal and hypergonadal levels compared to control group. A sequence for the androgen receptor was described in the PDE5 promoter (Lin, et al., 2001). At the molecular level, the existence of a single and imperfect human PDE5A gene promoter indicates that it is non-functional(Scheller, et al., 1998). This is further supported by genome-wide studies that failed to identify PDE5A as an androgen-regulated gene in human prostate epithelial cells(Bolton, et al., 2007, Massie, et al., 2007). Recent studies failed to find androgen response elements in the rat PDE5A gene(Lin, et al., 2013, Yang, et al., 2009). Thus, androgen regulation of PDE5A gene is likely an interpretation of experimental data that lack the critical smooth muscle control. However, previous data reported conflicting results about the effect of T on PDE5 expression. Previous studies in experimental animal models, as well as clinical investigations, suggested that androgens positively affected PDE5 expression and activity. PDE5 function decreases in the penis after castration or under conditions of hypogonadism and is restored with T replacement (Morelli, Filippi, Mancina, Luconi, Vignozzi, Marini, Orlando, Vannelli, Aversa, Natali, Forti, Giorgi, Jannini, Ledda and Maggi, 2004, Traish, Park, Dhir, Kim, Moreland and Goldstein, 1999, Zhang, Morelli, Luconi, Vignozzi, Filippi, Marini, Vannelli, Mancina, Forti and Maggi, 2005). Frequent low-dose use of sildenafil and/or tadalafil supplemented with T has a marked effect on ameliorating cavernous oxidative stress in aged diabetic rats (Mostafa, et al., 2012). Previous data show that T positively regulates PDE5 expression and

functional activities in the prostate, and T ablation not only suppresses prostate size but also reduces prostatic smooth muscle contractility, with several potential smooth muscle contraction/relaxation pathways implicated (Zhang, et al., 2012). While this effect may be paradoxical, in that androgens upregulate both NOS and PDE5, this is a homeostatic mechanism that maintains a relatively constant ratio of critical enzymes for this pathway(Traish, Goldstein and Kim, 2007). The upregulatory effect of T on PDE5 expression is secondary to T-induced increases in the expressions and function of constitutive NOS enzymes(Podlasek, Mulhall, Davies, Wingard, Hannan, Bivalacqua, Musicki, Khera, Gonzalez-Cadavid and Burnett, 2016, Traish, Goldstein and Kim, 2007). Upregulated NOS, through increased accumulation of intracellular cGMP, may increase PDE5 protein expression through cGMP responsive sequences in the PDE5 promoter(Lin, et al., 2002, Lin, et al., 2000). Furthermore, PDE5 inhibitor responsiveness was positively regulated by T (Mancina, et al., 2005, Morelli, Filippi, Mancina, Luconi, Vignozzi, Marini, Orlando, Vannelli, Aversa, Natali, Forti, Giorgi, Jannini, Ledda and Maggi, 2004, Vignozzi, et al., 2009), which was confirmed in humans (Shabsigh, 2005). In this study, we showed that the relaxant response to sildenafil was enhanced after incubation with normal and high levels of T. Similarly, previous data in humans suggested that when improvement in the erection induced by T plus sildenafil might be greater than the sum of the improvement caused by each treatment(Rochira, et al., 2006). Combination therapy was shown to be beneficial in hypogonadal men with comorbidities (Chatterjee, et al., 2004, Foresta, et al., 2004, Greenstein, et al., 2005). Furthermore, a systematic review by Alhathal et al. (Alhathal, et al., 2012) indicated that T treatment in men with low-normal T levels (300 to 400 ng/dL) appeared helpful for patients with ED who did not respond to initial treatment with PDE5 inhibitors alone. In addition, in a previous study, T administration increased sildenafil-induced relaxant response of CC in high fat diet-induced rabbit model of metabolic syndrome (Filippi, et al., 2009). Furthermore, Vignozzi et al. showed that T supplementation, in combination with tadalafil, restored smooth muscle content, eNOS expression, as well as the relaxant response of penile strips to ACh in a rat model of bilateral cavernous neurotomy(Vignozzi, Filippi, Morelli, Marini, Chavalmane, Fibbi, Silvestrini, Mancina, Carini, Vannelli, Forti and Maggi, 2009). Furthermore, the reason for the differences in the expression of PDE5 between previous studies and our results might be due to acute or chronic effects of T. In addition, T treatment was found to upregulate PDE5 expression in animals but not in cultured cells; and the upregulation in animals is possibly mediated by cGMP, not by a direct action of androgens (Andric, et al., 2010). Furthermore,

another previous study found no effect of androgen on PDE5 expression in cultured cavernous smooth muscle cells(Kim, et al., 2014). This study hopes to have solved the androgen-PDE5 paradox.

The concentrations of T required to elicit relaxant effects *in vitro* were within 0.15–0.6 μ M in this study; these findings are consistent with previous studies, which also showed that micromolar concentrations of T were required to obtain a maximum relaxation of isolated arteries *in vitro*(Chou, et al., 1996, Tep-areenan, et al., 2002, Yue, et al., 1995). These concentrations are significantly higher than the circulating levels in adult human male plasma (10–50 nM). However, it is well recognized that in *in vitro* organ-bath experiments, higher concentrations of endogenous vasoactive compounds are required to elicit significant vasodilatory effects. In addition, there is a clear distinction between different *in vitro* methods; androgen-induced coronary vasodilatation *in vivo* was reported after infusing physiological concentrations of T (Webb, et al., 1999).

This study has some limitations without invalidating our findings. First, the functional activity of eNOS was not investigated in the present study by evaluation of eNOS phosphorylation. eNOS activity is regulated by multiple post-translational molecular mechanisms, such as eNOS phosphorylation, eNOS interaction with regulatory proteins and contractile pathways, and actions of reactive oxygen species (Musicki and Burnett, 2006). T induces a rapid relaxation in vascular tissues of different species due to a nongenomic effect of this steroid on vessels (Bucci, et al., 2009). Different mechanisms have been proposed to explain T-induced vasodilatation, but the effective mechanism(s) and the mediators involved are still a matter of debate. Studies on the actions of T are much fewer and inconclusive. The results of *in vitro* studies might not completely reflect all hormonal actions under physiological situation and to further elucidate the hormonal actions, *in vivo* studies are required.

In conclusion, the non-genomic relaxant effects of T on HCC tissues have an auxiliary role to ensure a basal level of perfusion to maintain overall penile function. Our results confirm that T replacement therapy improves erectile function by modulating cGMP/NO signaling pathway in men with hypogonadism. The potentiation of PDE5 inhibitors with T supplementation may improve erectile function (Frajese and Pozzi, 2005). Future cellular and *in vivo* studies are warranted to elucidate the clinical significance and to define threshold levels for T replacement in hypogonadal men suffering from ED.

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FIGURE LEGENDS

Figure 1. Dose-dependent relaxation responses to (a) ACh and (b) EFS in HCC strips in the presence of various concentrations of T. Data represent the mean ± SEM. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$ versus the untreated group (ANOVA, Bonferroni post hoc).

Figure 2. Single-dose relaxation response to (a) sildenafil (10μM) and contractile response to (b) EFS (40 Hz) in HCC strips in the presence of various concentrations of T. Data represent the mean ± SEM. $^{***}P<.001$ versus the untreated group (ANOVA, Bonferroni post hoc).

Figure 3. Western blot analysis from HCC shows expression of nNOS, eNOS and PDE5A enzyme proteins in the presence of various concentrations of T. Please note that densitometry analysis of proteins from HCC tissue in lower panel has been displayed. Data represent the mean ± SEM. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$ versus the untreated group (ANOVA, Bonferroni post hoc).

Figure 4. Immunohistochemical localization of nNOS, eNOS and PDE5 staining (dark brown) in the presence of various concentrations of T in HCC. The negative control section processed without antibodies did not stain (data not shown)

Figure 5. Tissue levels of cGMP (A) and NO (B) in the presence of various concentrations of T in HCC. Data represent the mean ± SEM. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$ versus the untreated group (ANOVA, Bonferroni post hoc).

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Table 1. Demographics especially the underlying disease or comorbidity.

Parameter	Number
Male sex	11
Age (mean ± S.D.), years	51.2 ± 14.0
Age (19-59 years)	6 (54.5%)
Age ≥60	5 (45.5%)
Body mass index (kg/m2)	29.2 ± 2.1
Co-morbidities, *n* (%)*	
Hypertension*	4 (36.3%)
Peyronie's Disease*	2 (18.2%)
Diabetes mellitus*	2 (18.2%)
BPH	2 (18.2%)
No medical history	1 (9.09 %)

*One or more conditions are possible

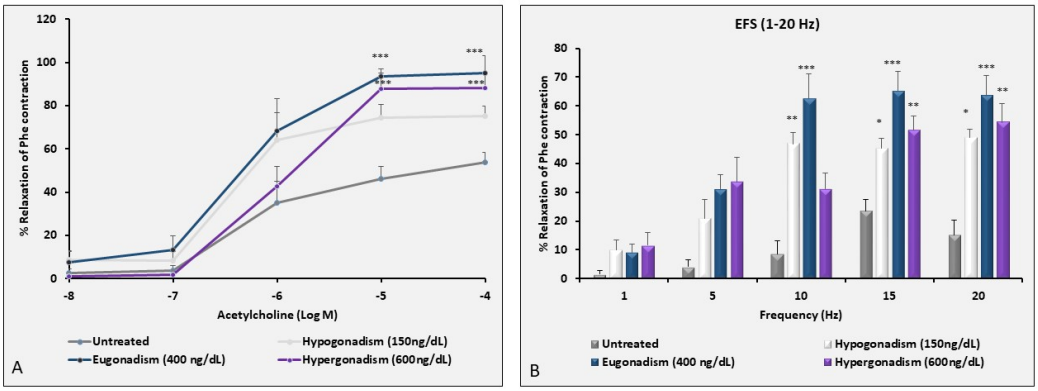

Figure 1

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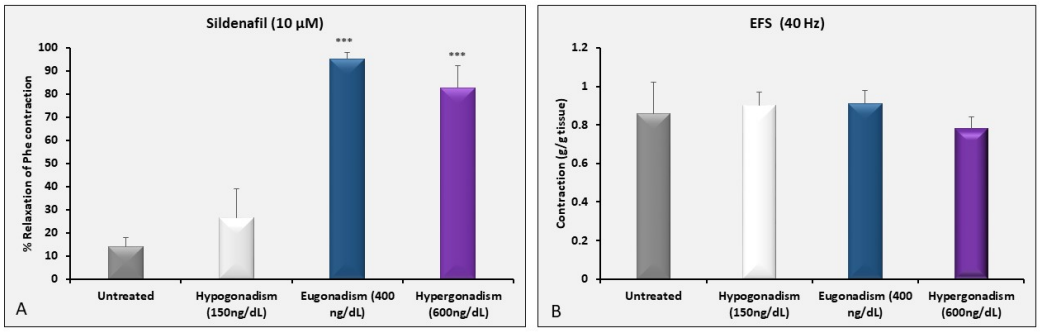

Figure 2

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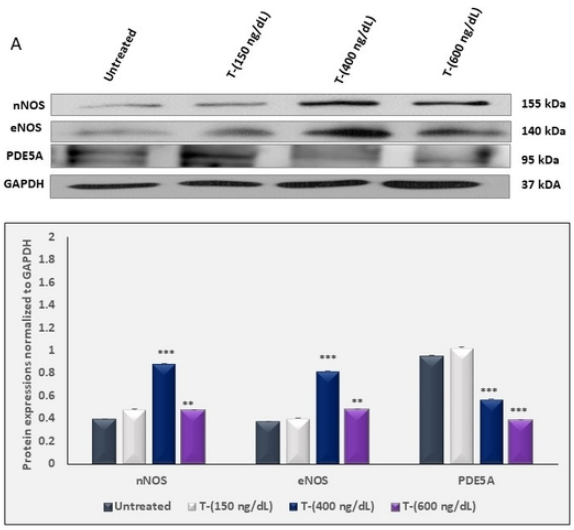

Figure 3

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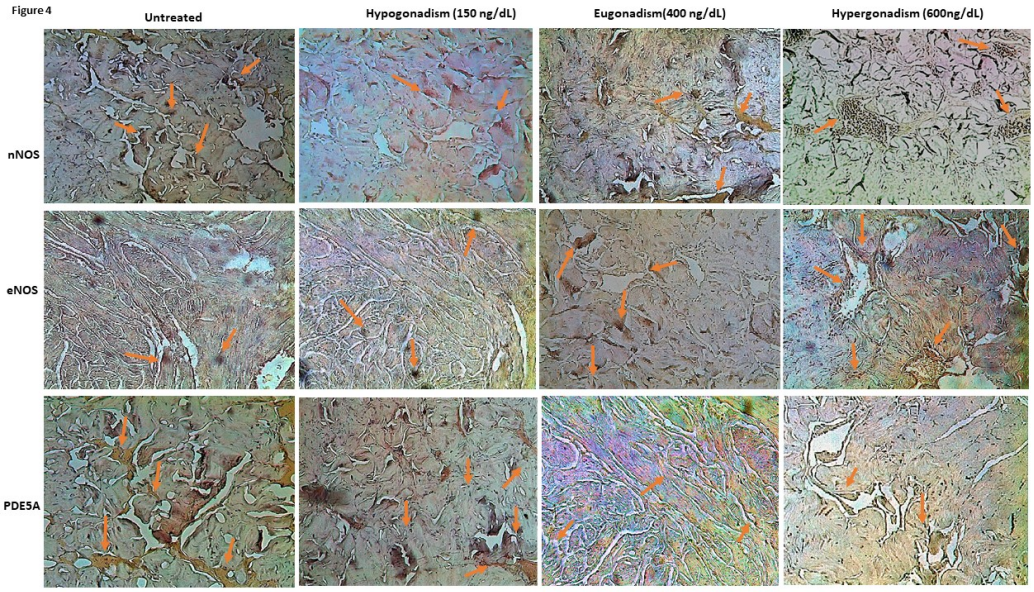

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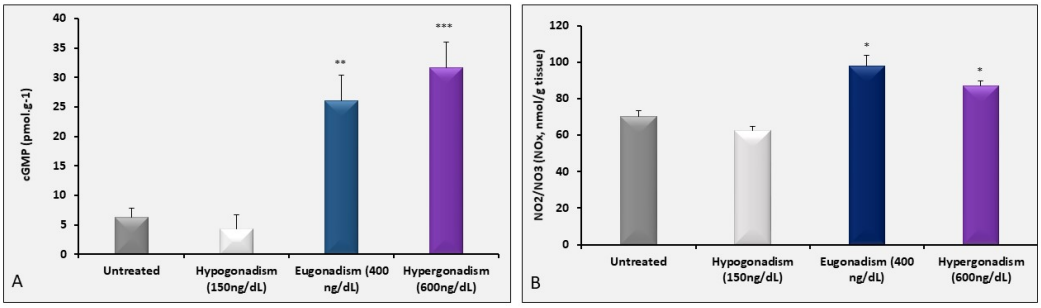

Figure 5

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