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Is there a relationship between endothelial nitric oxide synthase and erectile dysfunction in Egyptian males?

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ABSTRACT

We investigated the potential association between endothelial nitric oxide synthase (eNOS) gene polymorphism and erectile dysfunction (ED). The study was carried on 60 males with ED. All patients were subjected to medical and sexual history taking including the international index of erectile function (IIEF-5) questionnaire, general and genital examination and combined injection and stimulation test (ICI). Patients with a negative response were evaluated through a penile dynamic duplex to clarify the etiology of ED. eNOS genotype polymorphism was determined by restriction fragment length polymorphism (RFLP). The mean age of patients was 49.7 years and mean ED history duration was 3.45 years. The prevalence of diabetes and hypertension were 51.7% and 16.7%, respectively. The main cause of ED in the studied cases was venoocclusive (43.3%), the least common was the arteriogenic (20%). A psychogenic etiology was present in the remaining 37.7% of patients. The most common eNOS genotype was GG (46.7%), followed by GT (43.3%), then TT (10%). There was significant correlation between the grade of ED and eNOS genotype. eNOS gene polymorphism might contribute to the genetic susceptibility to ED. However, this link needs to be confirmed in larger studies.

KEYWORDS

Endothelial nitric oxide synthase;
Gene polymorphism;
Erectile dysfunction.

INTRODUCTION

Erectile dysfunction (ED), defined as the consistent inability to achieve and maintain penile erection sufficient for adequate sexual relation. ED affects more than 150 million men worldwide and is predicted to double within the next 20 years[1].

Although ED may have a psychogenic etiology, recent research proved that organic etiology is implicated in 80% of ED cases[2]. ED and systemic vascular disease share common risk factors and endothelial dysfunction; this close association identifies ED as a marker for vascular diseases[3-6]. Endothelial cells are the main source of nitric oxide (NO), which with cyclic guanosine monophosphate (cGMP) represents the main signaling cascade controlling penile erection[7-9].

NO is produced from L-arginine by nitric oxide synthase (NOS)[10]. There are 3 constitutive isoforms of NOS; neuronal NOS (nNOS; NOS1), endothelial NOS (eNOS; NOS3), and inducible NOS (iNOS;

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NOS2), each encoded by different genes. Both nNOS and eNOS are coupled to Ca^{2+} and calmodulin playing a principal role in penile erection, while iNOS is independent of Ca^{2+} and calmodulin[11]. Upon sexual stimulation, NO synthesized by nNOS in cavernous nitrergic nerve endings initiates erectile tumescence via cavernous smooth muscle relaxation with subsequent expansion of the sinusoidal spaces of corpora cavernosa This creates mechanical shear stress, which in turn activates eNOS in the cavernous endothelium to produce endothelial-derived NO responsible for maintenance of penile erection[9, 12-15]. Any alteration in NO production may lead to impaired corporal smooth muscle relaxation and subsequent ED[10].

Recent genetic research evidenced a close association of different SNPs with increased risk of developing cardiovascular disorders, endangering the same physiological pathways crucial for penile vasomotor tone regulation[16-18]. Several polymorphisms of the eNOS gene have been identified, among which is the G894T polymorphism in exon 7, which was reported to cause an amino acid substitution (Glu298Asp) of eNOS enzyme, resulting in disturbance of its activity, and correlated with increased risk for essential hypertension, coronary artery diseases (CAD) and myocardial infarction[10, 19, 20]. The association between eNOS G894T gene polymorphism and ED was assessed in a few studies with controversial results[21-23].

We investigated a potential association between eNOS G894T gene polymorphism, ED and related risk factors in Egyptian men.

PATIENTS AND METHODS

A total of 60 ED patients (group A) and 50 healthy controls (group B) were enrolled in our study. All subjects were Egyptian and had an active sexual life with a regular sexual partner for more than 6 months before enrolment. They were all recruited from the Andrology and Sexology outpatient clinic of Cairo University hospitals after institutional review board (IRB) approval. All cases signed a written informed consent to participate in the study, completed medical, surgical, and psychosexual histories and underwent detailed physical examination. Patients with ED were defined as those who subjectively had problems of being unable to achieve or maintain a sufficient erection for sexual intercourse since at least 6 months[24]. All cases completed the validated Arabic translation of the International Index of Erectile Function (IIEF-5) questionnaire[25]. Patients with ED were diagnosed as those having an IIEF-5 score less than 21[23, 26]. Group A cases (ED patients) were evaluated by combined injection and stimulation test (CIS) using an intra-cavernous injection (ICI) of 20 μg prostaglandin E1 (PGE1). A positive erectile response was defined as the achievement of a sufficient erection for vaginal penetration which is maintained for at least 20 min. Patients were then evaluated by color Doppler ultrasound (CDU) study using a 7.5 MHz linear array transducer with a color flow mapping capability (*Esaote Biomedical AU3, Italy*). Vascular risk factors for ED were reviewed. Hypertension, ischemic heart diseases (angina pectoris and/or previous myocardial infarction) or diabetes mellitus (DM) were defined as cardiovascular comorbidities. Exclusion criteria for group A cases included diminished sexual desire, hypogonadism, hyperprolactinemia as well as ED due to anatomical penile deformities, spinal cord injury, post-radical prostatectomy; or any substance abuse disorders, as well as medical psychiatric histories with or without medication. Regarding group B, 50 healthy males without ED or cardiovascular comorbidities were included as control subjects.

Serum samples from all cases were separated for biochemical analysis and endocrine profiling (prolactin and testosterone). Blood samples (3 ml) for the assay of eNOS G894T allele status were collected in EDTA and immediately stored at -80°C. Then, DNA was extracted from peripheral whole blood using the QIAMP DNA Extraction Kit (*Qiagen, Valencia, California*). The concentration of the extracted DNA was determined by using spectrophotometer at wave length 260 nm. Gene amplification was performed by polymerase chain reaction (PCR). Two specific sets of primers were used. The sequence of primers used for amplification of eNOS exon 7 was 5'-GACCCTGGAGATGAAG GCAGGAGA (G894T forward) and 5'-ACCACCAG GATGTTGTAGCGG-TGA (G894T reverse), as described in previous studies[22, 23]. The PCR mixture (total volume 50 μl) was formed of 5 μl of 10X reaction buffer with $MgCl_2$ (*Amersham Pharmacia Biotech, Piscataway, NJ, USA*), 50 ρmol of each primer (for-

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ward and reverse), 100 µmol each of dNTPs (*Perkin-Elmer Corporation, Foster City, CA, USA*), 2 units Taq DNA polymerase (*Amersham Pharmacia Biotech, Piscataway, NJ, USA*) and 100 ng genomic DNA template. The cycling condition was denaturation at 95°C for 5 min, followed by 35 cycles under the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, then final extension cycle of 72°C for 7 min. The resulting 517bp amplification product was incubated at 37°C for at least 20 h with 8 U of the restriction enzyme *Ban-II* (*New England Biolabs Inc.*). *Ban-II* digestion of amplified DNA derived from individuals who are homozygous for the G allele resulted in no cleavage. DNA from heterozygous individuals yielded 3 fragments of 517bp, 346bp and 171bp and individuals homozygous for the T allele yielded 2 fragments of 346bp and 171bp, as shown in Figure 1.

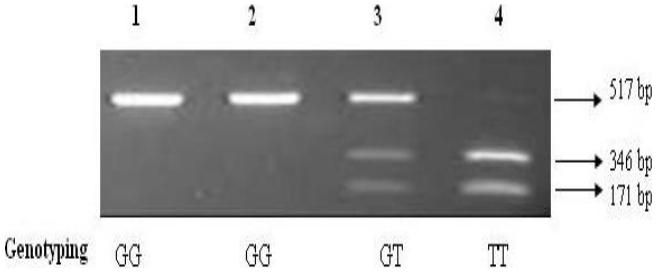

Figure 1 : An Agarose gel electrophoresis showing eNOS gene polymorphism, where Lanes 1 & 2 show homozygous GG (517bp), Lane 3 shows heterozygous GT (517, 346 and 171bp), and Lane 4 show homozygous TT (346 and 171bp) genotypes.

Statistical analysis was performed using the chi-square test and the independent t test. A 2-sided $P < 0.05$ was considered significant.

RESULTS

eNOS G894T gene polymorphism

The genotypic distribution of eNOS G894T gene polymorphism among both groups, and according to the presence or absence of cardiovascular co-morbidities in groups A cases and in ED patients with vasculogenic etiology is displayed in TABLE 1. The frequency distribution of the GG, GT, and TT genotypes did not differ significantly between ED patients and healthy controls, as they represented 28 (47%), 26 (43%), and 6 cases (10%) in group A (ED patients), while it was 19 (38%), 28 (56%), and 3 cases (6%) in group B healthy controls, respectivel. However, 66.7 % of homozygous TT genotype cases (6/9) were among ED group. The prevalence of homozygous TT genotype carriers was very low (9 subjects in the study population), representing 10% of ED patients (10%) and 6% of controls. Accordingly, we combined cases with homozygous TT and heterozygous GT alleles to represent the T allele carriers.

TABLE 1 : Distribution of eNOS Genotypes in Controls, ED patients, and patients with Vasculogenic ED

	No	GENOTYPE			
NOS3 Genotype		**GG**	**GT**	**TT**	**GT+TT**
Controls, n (%)	50	19 (38)	28 (56)	3 (6)	31(62)
All ED Patients, n (%)	60	28 (46.7)	26 (43.3)	6 (10)	32 (53.3)
No Cardiovascular Co-morbidities	25	11 (44)	11 (44)	3 (12)	14 (56)
Cardiovascular Co-morbidities	35	17 (48.6)	15 (42.9)	3 (8.5)	18 (51.4)
Organic ED Patients, n (%)	45	23 (51.1)	17 (37.8)	5 (11.1)	23 (48.9)
No Cardiovascular Co-morbidities	15	7 (46.7)	6 (40)	2 (13.3)	8 (53.3)
Cardiovascular Co-morbidities	30	16 (53.3)	11 (36.7)	3 (10)	14 (46.7)

The mean age (SD) of healthy controls was 44.7 (2.8) compared with 49.7 years (9.7) in ED patients, displaying a significant difference, which was more pronounced on comparing it to the mean age (SD) of 51.1 years (9.2) in ED patients with vasculogenic etiology *($p < 0.01$)*. The mean age (SD) of ED patients in relation to eNOS gene polymorphism was 48.8 (10.4) in GG, 50.9 (8.1) in GT and 48.7 years (13.3) in TT genotype. As for the control group, their mean age (SD) was 44.7 (2.7) in GG, 44.3 (2.8) in GT and 48.3 years (0.6) in TT allele carriers *($p = 0.06$)*. The mean age of onset of ED did not show any significant difference between different eNOS genotypes; however T allele carriers (TT+GT) in the control group were younger than those in the ED group (44.6 vs 48.8 years, $p < 0.05$). Accordingly, it may be suggested that T allele carriers in the control group might be at a higher risk to develop ED with aging.

Etiological classification of ED patients showed that 25% (15/60) were psychogenic while 75% (45/60) were

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vasculogenic, among them 48.3% (29/60) were due to veno-occlusive dysfunction while 26.7% were arteriogenic (16/60). Data from patients with vasculogenic ED was selectively analyzed to assess the role of eNOS polymorphism in penile pathophysiology. The etiology of ED was independent of the eNOS genotypes, as the frequency distribution of GG, GT and TT genotypes was comparable between patients with various ED etiologies, being in 8 (28.6%), 6 (23.1%) and 2 (33.3%) of arteriogenic, and 10 (35.7%), 15 (57.7%) and 4 (66.7%) of veno-occlusive dysfunction cases, respectively. However, among psychogenic cases 10 (35.7%) had GG, 5 (19.2%) had GT and none of them had TT genotype.

Although 10 of GG (35.7%) and 5 of GT (19.2%) genotypes had ED of psychogenic etiology, none of the homozygous TT genotype were among psychogenic cases.

Smokers represented 43.3% (26/60) of ED patients and 32% (16/50) of controls; however, 62% of smokers were among the ED group. A significant association between smoking and ED, where 75% of smokers with GG genotype had ED, compared with only 42% of non-smoker GG carriers *($p = 0.018$)*. The mean age (SD) of non-smokers and smokers did not differ among controls [44.9 (2.7) vs 44.1 years (2.9), $p > 0.05$], however, a highly significant difference was detected on comparing non-smokers to smokers among vasculogenic ED patients [52.1 (9.0) vs 49.6 years (9.5), $p < 0.01$].

Cardiovascular co-morbidities prevailed in 58.3% (35/60) of ED patients, where 51.7% (31/60) were diabetics and 16.7% (10/60) were hypertensive. The distribution of eNOS genotypes in ED patients with cardiovascular co-morbidities showed their presence in 60.7% (17/28) of GG, 57.7% (15/26) of GT, and 50% (3/6) of TT genotypes and in 56.2% (18/32) of the T allele carriers (GT/TT). On excluding psychogenic cases, these co-morbidities showed more pronounced prevalence among patients with vasculogenic ED, as it increased to 69.6% (16/23) in GG, 64.7% (11/17) in GT, 60% (3/5) in TT genotypes, and 60.9% (14/23) in T allele carriers (GT/TT).

The mean peak systolic velocity (PSV) on right and left cavernosal arteries was evaluated reflecting systolic blood flow in the corpora cavernosa of ED patients after ICI of PGE1, and revealed diminished flow in men with the homozygous T allele carriers (TT), and even more decline was elicited in patients with cardiovascular co-morbidities, as shown in TABLE 2. However, these differences failed to attain statistical significance, possibly due to reduced sample size.

TABLE 2 : eNOS Genotypes in ED patients according to Penile Duplex Findings & IIEF-5

	No	GENOTYPE			
NOS3 Genotype		***GG***	***GT***	***TT***	***GT+TT***
All ED Patients, n	60	28	26	6	32
Right PSV (cm/sec)	39.12	39.25	40.04	34.5	39
Left PSV (cm/sec)	38.30	39.14	38.42	33.83	37.56
IIEF-5	13.4	13.04	12.96	17	13.72
No Cardiovascular Co-morbidities, n	25	11	11	3	14
Right PSV (cm/sec)	44.24	45.27	44.27	40.33	43.43
Left PSV (cm/sec)	45.08	46.73	45.09	39.00	43.79
IIEF-5	15.4	15.36	14.64	18.33	15.43
Cardiovascular Co-morbidities, n	35	17	15	3	18
Right PSV (cm/sec)	35.46	35.35	36.93	28.67	35.56
Left PSV (cm/sec)	33.46	34.24	33.53	28.67	32.72
IIEF-5	11.97	11.53	11.73	15.67*	12.39

IIEF-5 = The five-item version of the international index of erectile function; PSV = peak systolic velocity; *P = 0.036, T genotype Carriers.

DISCUSSION

Penile erection is a complex neurovascular process that necessitates increased arterial inflow and restricted venous outflow from the penis[27].

In the present study, we examined the G894T polymorphism of the eNOS gene, a G to T substitution, subsequently leading to a change from aspartate to glutamate (Glu298Asp) in the eNOS, resulting in disturbance of its activity[10]. Reduction of eNOS activity has also been associated with aging, smoking, DM and hypercholesterolemia[28-31].

To date, there have been few studies on the association between the eNOS G894T gene polymorphisms and ED resulting in limited and conflicting data. In 1999, Park et al. failed to detect any correlation between genotypes of the fourth intron of eNOS gene (4 VNTR) in patients with vasculogenic ED[32]. A finding that was again reported in ED patients who showed a significant higher prevalence of DM and coronary artery disease

(CAD)[33]. In 2007, Peskircioglu et al. detected a better response to sildenafil in ED patients with the "A" allele of eNOS gene intron 4 variable number of tandem repeats (VNTR)[34].

On analyzing the eNOS G894T gene polymorphism, Eisenhardt and Siffert (2003) did not find any increased risk of ED among different eNOS genotype carriers, while Eisenhardt et al. (2003) reported an association between eNOS G894T genotypes and response to sildenafil in ED patients[21, 35].

Other investigators showed that T allele carriers are at greater risk to develop ED both in prevalence and severity. Accordingly, eNOS G894T gene polymorphism, together with DM, hypertension, cardiac disease and cigarette smoking were identified as independent risk factors for ED[22, 23].

In our study, eNOS genotypes not differ significantly between ED patients and healthy controls, a finding similar to those of Eisenhardt and Siffert[21]. We detected only 9 subjects with homozygous TT genotype among the whole study population (n = 110) representing 0.08%, and 66.7% of them (6/9) were among the ED group, suggesting a dosage-cumulative effect previously shown by others[22, 23].

Age-related ED is attributed to dysregulation of eNOS phosphorylation in the penis and accordingly inactivation of eNOS, or age associated decline of androgen concentration which impair eNOS expression in the penis[14, 31, 36]. Our study showed that healthy controls were significantly younger than ED patients, particularly those with vasculogenic ED. The present data support the results of Lee et al. (2007)[23].

In 2007, Imamura proved that smoking impairs eNOS activity and cavernosal smooth muscle relaxation by upregulating asymmetric dimethylarginine (ADMA) content, an endogenous eNOS inhibitor, and increase dimethylarginine dimethylaminohydrolase (DDAH) activity[37]. A significant association between smoking and increased prevalence of ED was elicited in our study, particularly in patients with vasculogenic ED.

Currently, advanced molecular analysis revealed an association between SNPs and the risk for ED, as well as closely linked cardiovascular co-morbidities such as coronary heart disease, hypertension, hypercholesterolemia and DM[38].

DM alters endothelial NO production in the corpora cavernosa due to down-regulation of eNOS protein expression and activity, decreased shear stress-induced NO release, diminished penile arginine levels and increased oxidative stress through hyperglycemia-induced mitochondrial overproduction of superoxide[31, 39-44].

In the present study, the prevalence of cardiovascular co-morbidities was higher in various eNOS genotypes among vasculogenic ED patients. The close association of eNOS genotypes and endothelial dysfunction was evidenced once again by detection of a greater decline of systolic blood flow in the corpora cavernosa of T allele carrier ED patients, which was more pronounced in cases with cardiovascular co-morbidities. These findings are in concordance with data presented by Rosas-Vargas et al. (2004) and Lee et al. (2007) who identified eNOS G894T polymorphism, together with DM, hypertension, cardiac disease and cigarette smoking as independent risk factors for ED[22, 23].

Psychogenic cases of ED were excluded from the study of Rosas-Vargas et al. (2004)[22]. In our study, none of psychogenic ED patients had homozygous TT genotype, a finding that highlights the importance of excluding psychogenic cases of ED when investigating the association of eNOS polymorphism and ED to avoid any bias that may lead to failure to attain significance in previous studies in which psychogenic ED patients were included.

Reduced androgen concentration result in impaired expression of eNOS in cavernous tissue[45, 46]. That is why we excluded ED patients with hypogonadism.

Regarding the control group, we only included healthy males without ED or cardiovascular co-morbidities, in order to avoid the cumulative effect of these co-morbidities in investigating the potential association between eNOS G894T gene polymorphism and ED. Similarly, Eisenhardt et al. (2008) considered the presence of cardiovascular co-morbidities as one of the exclusion criteria of the control group[35].

In 2009, Cherney et al. showed that the T allele of eNOS G894T polymorphism is associated with a blunted response to L-arginine, decreased NO bioactivity and vascular complications, suggesting this polymorphism is a functional variant in humans that worth studying in a large cohort[47].

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Several explanations were postulated for the down regulation of eNOS activity in eNOS T allele carriers. The eNOS G894T polymorphism results in glutamate to aspartate amino acid substitution at position 298, where this position of amino acid substitution on the surface of the enzyme represents a potential site for selective enzymatic cleavage, elaborating cleaved fragments that lack NOS activity and consequently reducing vascular NO generation[23]. Secondly, T allele carriers showed an enhanced responsiveness to α-adrenergic sympathetic stimulation which favors rapid detumescence after erection[48]. A third explanation is the gene-environment interaction, where T allele influence the response of cardiovascular system to various environmental and noxious substances as shown by Wang et al. (2000) who displayed genotype-dependent cigarette-specific deleterious effects on eNOS expression and activity[49]. Finally, the gene-gene interaction theory should be considered, where the coexistence of 2 or more functional polymorphisms, each making a minor contribution to a common trait, are capable of significant interaction that may increase the risk of various vascular diseases and ED as well.

In the present study, although we did not find a significant difference in the frequency distribution of eNOS genotypes between ED patients and healthy controls, however, the presence of T allele increased genetic susceptibility for vasculogenic ED, particularly in co-existence of cigarette smoking, aging, as well as various cardiovascular co-morbidities.

Although recent advances have led to better understanding of the pathophysiology of ED, we still need more functional and expression investigations to unmask different signal transduction pathways that clarify the underlying molecular mechanisms that confer genetic susceptibility to ED.

Conflict of Interest: None.

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