The association of 4a4b polymorphism of endothelial nitric oxide synthase (eNOS) gene with the sperm morphology in Korean infertile men

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Objective: To investigate the association between three polymorphism sites of endothelial nitric oxide synthase (eNOS; −786T>C, 4a4b, and 894G>T) with nonobstructive male infertility.
Design: Prospective case-control study.
Setting: University-based hospital.
Patient(s): Three hundred seventy-one nonobstructive infertile men in azoospermia (n = 184) or ejaculate semen (n = 187) group were enrolled in this study. Two hundred twenty fertile men who had at least one child without any history of requiring assisted reproductive technology were included as the nationwide control group.
Intervention(s): Semen analysis according to the World Health Organization guidelines and cytogenetic and Y chromosome microdeletion assay.
Main Outcome Measure(s): Three eNOS polymorphisms were investigated to assess its association with male infertility by pyrosequencing and gel electrophoresis.
Result(s): The statistical analysis of three eNOS polymorphisms showed no significant association between the polymorphisms of the control and infertile group. We investigated the sperm parameters depending on the genotypes of the ejaculate semen group. The sperm morphology was found to be significantly associated with the 4a4b polymorphism of eNOS.
Conclusion(s): Endothelial nitric oxide synthase may be important to sperm morphology in infertile men. (Fertil Steril® 2008;90:1126–31. ©2008 by American Society for Reproductive Medicine.)

Key Words: eNOS, male infertility, single nucleotide polymorphism, sperm parameter, smoking, pyrosequencing

Nitric oxide (NO) is implicated in numerous physiologic and pathologic processes (1). Three isoforms of nitric oxide synthase [NOS: neuronal NOS, inducible NOS, and endothelial NOS (eNOS)] can synthesize nitric oxide (NO) through the oxidation of L-arginine to L-citrulline. Human endothelium-derived nitric oxide (NO) was derived from eNOS (2). Recent studies support that eNOS plays a role related to reproductive functions and spermatogenesis (3, 4). Activity of eNOS has been detected in Leydig cells, Sertoli cells, spermatocytes, and spermatids of male reproductive organs (5–7). Immunofluorescence experiments have shown that this enzyme can be detected from the acrosome and tail of mouse sperm (8, 9). The eNOS transcripts were expressed in low-motility sperm populations but not in high-motility normal sperm (8–11). Balercia et al. showed that normozoospermic men have significantly lower NO concentrations than asthenozoospermic infertile men (12). Ratnasooriya et al. showed that high levels of an NOS substrate, L-arginine, can affect the fertility of male rats (13, 14). High levels of NO can induce germ cell–specific apoptosis of sperm, which may indicate impaired spermatogenesis(13). Therefore, it is interesting to investigate the association of the genotype of eNOS with male infertility, because there may be a direct association between spermatogenesis and NO synthesis.

Three polymorphisms of the eNOS gene have been identified, including a −786T>C polymorphism in the promoter region, a variable number of tandem 4a4b repeats in intron 4, and a 894G>T polymorphism in exon 7. Previous results showed that each polymorphism can influence the expression or functional activity of the eNOS enzyme (15–18). To date, eNOS genes have been reported to be associated with various diseases, including cardiovascular disease and ischemic stroke (19–21), but a relationship between eNOS polymorphism and male infertility has not been reported previously. In the present study, we investigated three polymorphisms of the eNOS gene with nonobstructive male infertility, especially focusing on sperm parameters.

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MATERIALS AND METHODS

Study Population

This case-control study included 220 fertile and 371 infertile subjects. Fertile men with at least one child and no history of

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requiring assisted reproductive technology (ART) were included as the nationwide control group. These fertile men were consecutively enrolled from the Division of Genome Resources, National Genome Research Institute, National Institute of Health, Republic of Korea.

From January 2000 to August 2003, nonobstructive infertile men enrolled in the study after physical examinations and hormone assays (LH, FSH, T, and PRL). Our Institutional Review Board approved the study, and informed consent was required from each subject before participation. Three hundred seventy-one nonobstructive infertile men whose partners had not conceived in at least one year were included as infertile men. Patients who were determined to have cryptorchidism and varicocele via physical examination and clinical testing were excluded. Semen analysis was performed with infertile patients strictly according to the World Health Organization (WHO) guidelines (22) as well as the criteria of Kruger et al. (23).

The nonobstructive infertile men were classified into two groups: azoospermia group (no spermatozoa in the ejaculate; n = 184) and ejaculate semen group (spermatozoa in the ejaculate; n = 187). The diagnosis of azoospermia and ejaculate semen was made on the basis of two semen analyses. The ejaculate semen group included patients having at least one of sperm abnormalities: concentration ($<20 \times 10^6$), motility (<50%), or morphology (<14%). Nine had oligozoospermia (sperm concentration $<20 \times 10^6$ motility >50%, morphology >14%), 7 asthenozoospermia (sperm concentration $>20 \times 10^6$, motility < 0%, morphology >14%), 17 teratozoospermia (sperm concentration $>20 \times 10^6$, motility >50%, morphology <14%), 25 oligoasthenozoospermia (sperm concentration $<20 \times 10^6$, motility <50%, morphology >14%), 3 oligoteratozoospermia (sperm concentration $<20 \times 10^6$, motility >50%, morphology <14%), 63 asthenoteratozoospermia (sperm concentration $>20 \times 10^6$, motility <50%, morphology <14%), and 63 oligoasthenoteratozoospermia (sperm concentration $<20 \times 10^6$, motility <50%, morphology <14%). The experiments were performed at the Christian Humanism Academia General Hospital, Seoul, Republic of Korea.

Cytogenetic and Y Chromosome Microdeletion Assay

Three hundred seventy-one infertile men without any chromosome abnormalities were selected after cytogenetic and Y-chromosomal microdeletion analyses.

Cytogenetic analysis was performed on the metaphase spreads of cultured lymphocytes. Patients with abnormal chromosomes, which included Yq deletion, inversion, translocation, derivate, XXY, and chromosomal mosaicism, were excluded from this single nucleotide polymorphism (SNP) study (24, 25).

For Yq deletion studies, DNA was extracted from peripheral blood and amplified by a multiplex-PCR kit with primers to 15 loci on the Y chromosome, including one SRY and 14 sequence-tagged sites according to the instructions of the manufacturer (AZF-a region: sY84, sY86; AZF-b region: sY124, sY127, sY130, sY138; AZF-c region: sY152, sY147, sY254, sY255, SPGY1, sY157, sY242, sY158; Genocheck, Ansan, Korea).

Pyrosequencing Assay of eNOS −786T > C and 894G > T Polymorphisms and PCR Length Polymorphism (LP) for 4a4b Polymorphism

The pyrosequencing assay for eNOS −786T>C and 894G>T polymorphism was performed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Pyrosequencing Primer SNP Design Version 1.01 software (26–28). The sequences for pyrosequencing primers are shown in Table 1. Genotyping for SNPs in eNOS (−786T>C and 894G>T) was performed by pyrosequencing. The analysis of the 4a4b polymorphism was performed according to the method indicated by Fatini et al. (29).

TABLE 1

The primer sequences for pyrosequencing assay for −786T > C and 894G > T sites of endothelial nitric oxide synthase (eNOS).

eNOS polymorphism	Primer sequence	Tm	Pyrosequencing primer
−786T>C	F 5′(biotin)-CCTGCATTCTG GGAACTGTA-3′ R 5′- CGCAGGTCAGCAGA GAGACT-3′	62°C	5′-GCTGAGGCAGGGTC-3′
894G>T	F 5′(biotin)-ACTCCCCACAG CTCTGCAT-3′ R 5′-GGGGCAGAAGGA AGAGTTC-3′	64°C	5′-GGAAGAGTTCTGGGGG-3′

Note: biotin = 5′ biotin labeling; F = forward primer; R = reverse primer.

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