Role of Nitric Oxide Concentrations on Human Sperm Motility

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ABSTRACT: Nitric oxide (NO) is a free radical generated from the oxidation of L-arginine to L-citrulline by 3 isoforms of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthases. Several data suggest a relevant role in sperm cell pathophysiology, but any conclusive data on its role in spermatozoa motility are still lacking. In the present study, we have correlated NO concentration in semen and kinetic features of sperm cells from normozoospermic fertile donors and infertile patients affected by idiopathic asthenozoospermia. Normozoospermic fertile men exhibited NO concentrations that were significantly lower than those of asthenozoospermic infertile men. A significant linear negative correlation was evident between NO concentration and percentage of total sperm motility. A further significant linear negative correlation was found between NO concentration and spermatozoa kinetic characteristics determined by a computerized analysis (curvilinear and straight progressive velocity). These data suggest that the overproduction of this free radical and the consequent excessive exposure to oxidative conditions have a potential pathogenetic implication in the reduction of sperm motility. The positive role played by NO in spermatozoa capacitation leads us to speculate that such paradoxical involvement in both pathologic and physiologic processes depends on the alternative redox state and relative level of NO.

Key words: Male infertility, spermatozoa motility, asthenozoospermia.

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Several studies support the role of nitric oxide (NO) as a messenger in a wide array of biologic processes (Nathan, 1992). NO is involved in neurotransmission (Garthwaite et al, 1988; Peunova and Enikopolov, 1993), regulation of vascular wall tone (Ignarro et al, 1987; Calver et al, 1993), and immune system activity as an effector molecule with bactericidal and antiviral properties (Hibbs, 1991; Karupiah et al, 1993). It was also suggested that NO modulates sexual and reproductive functions in mammalian species (Burnett et al, 1992; Rajfer et al, 1992; Zini et al, 1996).

NO is a free radical generated from the oxidation of L-arginine to L-citrulline by 3 isoforms of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthases (NOS). Both neuronal NOS (nNOS), first described in neurones, and endothelial NOS (eNOS), first identified in endothelial cells, are constitutive Ca^{2+}-dependent isoforms, whereas the macrophage NOS (iNOS) is an inducible Ca^{2+}-independent isoform (Moncada et al, 1991; Fostermann et al, 1994). NOS protein and activity have been detected in both human and rat testis, epididymis, prostate, and seminal vescicles (Ehren et al, 1994; Burnett et al, 1995). Experiments performed with antibodies raised against NOS showed that this enzyme is associated with the acrosome and the tail of mouse spermatozoa (Herrero et al, 1996) and appears to be involved in the fertilization process, including sperm motility and acrosome reaction (Herrero et al, 1997). Furthermore, indirect immunofluorescence assays showed that human spermatozoa express constitutive NOS in the postacrosomal and equatorial segments (Lewis et al, 1996), and spin trapping experiments showed that NO is synthesized by the human male gamete (Herrero et al, 2000).

Low concentrations of NO cause a significant increase in capacitation (Zini et al, 1995) and zona pellucida binding (Sengoku et al, 1998), although the effects of NO on sperm motility and viability remain undefined (Hellstrom et al, 1994; Rosselli et al, 1995; Weinberg et al, 1995; Nobunaga et al, 1996). Together, these data suggest a relevant role of NO in the pathophysiology of sperm cells.

In the present study, we have correlated NO concentration in semen and kinetic features of sperm cells from normal and asthenozoospermic men in order to acquire a deeper insight about the role of NO in the pathophysiology of human spermatozoa.

Patients	NO Concentration (nmol/10⁶ cells)
Normospermic	1.43 ± 0.27
Asthenozoospermic	1.81 ± 0.54*

† NO indicates nitric oxide.
* $P < .01$

Figure 1. A 2-class plot showing single nitric oxide (NO) seminal levels in each donor.

Patients and Methods

Patient Selection

We evaluated 21 normozoospermic fertile healthy men (control group, aged 26–34 years) and 23 asthenozoospermic patients (sperm concentration $>20 \times 10^6$/mL; forward motility grade a + b $<50\%$; and normal viability percent) referred to the Andrology Unit of the Division of Endocrinology, Umberto I Hospital, University of Ancona, Italy, for infertility (of at least 18 months). All subjects included in the study exhibited a normal sperm morphology greater than 30% and a seminal white blood cell count less than 1×10^6/mL. The sperm culture was negative, including *Chlamydia* and *Mycoplasma ureoliticum* detection, as well as the Mar-test. The hormonal serum profile (gonadotropins, testosterone, estradiol, and prolactin) was normal. There was no evidence of anatomical abnormalities of the genital tract, including varicocele (after Doppler sonography), nor was there a history of criptorchidism, testicular torsion, or genital tract infection. The absence of systemic diseases or treatment with other drugs within 3 months before enrollment in the present study; the absence of smoking, alcohol, and drug addiction; and the absence of occupational chemical exposure were also verified. Since no possible causes for motility reduction have been discovered, asthenozoospermic patients have been thought to be affected by idiopathic asthenozoospermia. This study was approved by the Institutional Review Board of the University of Ancona, Umberto I Hospital. All patients provided their written informed consent.
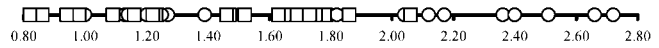
Semen Analysis

Semen samples were collected after 3 days of sexual abstinence. After liquification at room temperature for 30 minutes, standard seminal parameters were analyzed according to World Health Organization (1999) guidelines. Computer-assisted sperm analysis (CASA) for sperm motility assay was additionally performed, as previously reported (Balercia et al, 2003). One semen aliquot (3 μL) was placed in a 20-μm-deep cell VU chamber (Conception Technologies, La Jolla, Calif). Two chambers were loaded, 6 different fields per chamber were randomly examined, and at least 200 spermatozoa for each field of chambers were scored. Movement characteristics were analyzed using an automated analyzer (CellTrack VP110, Motion Analysis Corp, Palo Alto, Calif). Kinetic characteristics were evaluated only for motile sperm and expressed as mean values considering: total sperm motility (percent), curvilinear velocity (VCL, μm/s), straight progressive velocity (VSL, μm/s) and lateral head displacement (ALH, μm).

Determination of NO Levels in Semen

For NO level evaluation, individual sperm samples were diluted to 5×10^6/mL with Dulbecco phosphate-buffered saline (PBS) (20 mM, pH 7.4), aliquoted to form 2 replicates and stored at $-80°C$ in sterile tubes until nitrite measurements were performed within 15 days. Each sample was then suspended in a substrate buffer (HEPES 25 mM, NaCl 140 mM, KCl 5.4 mM, CaCl₂ 1 mM, and MgCl₂ 1 mM, pH 7.4) in the presence of 1.44 mM of NADPH and 20 mU of nitrate reductase and incubated at room temperature for 1 hour in order to convert all available nitrate
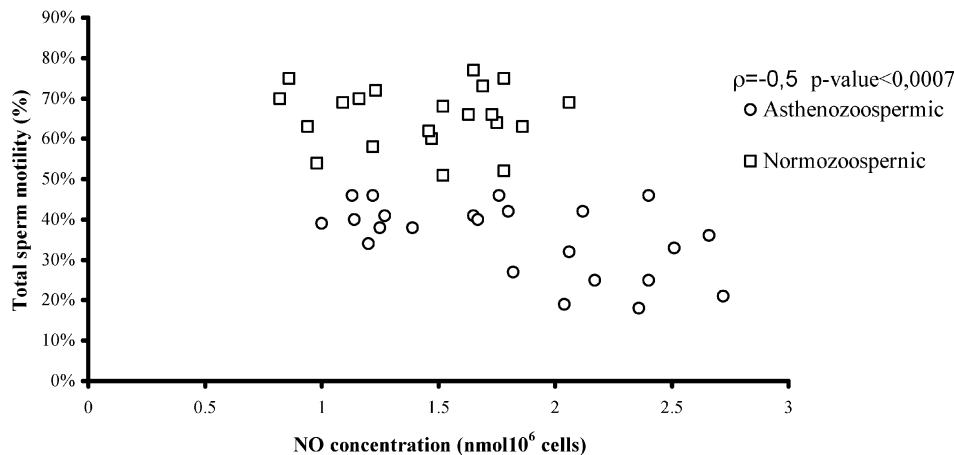
Figure 2. Negative linear correlations between nitric oxide (NO) concentration and total sperm motility in asthenozoospermic and normozoospermic donors.
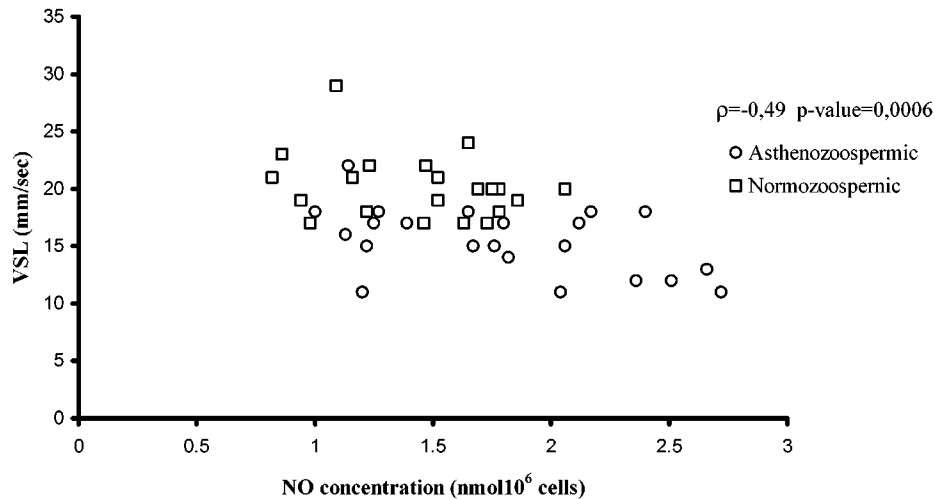
Figure 3. Negative linear correlations between nitric oxide (NO) concentration and straight progressive velocity (VSL) in asthenozoospermic and normozoospermic donors.

to nitrite by the enzyme. The reaction was stopped by freeze-thawing the samples, which were then sonicated and centrifuged at $1500 \times g$ for 15 minutes. NO concentration, which is related to nitrite and nitrate levels, was determined using a spectrophotometric assay based on the formation of a colored azo dye product when a Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 2.5% H_3PO_4) was mixed in equal volumes with the sample, as first described by Chen and Mehta (1996) and then modified by Camilletti et al (2001). The chromophore absorption was then read at 543 nm, and nitrite concentrations, expressed in nmol NO/10^6 cells, were determined from a standard curve generated by using known concentrations of sodium nitrite.

Western Blot Analysis

Washed spermatozoa were lysed in RIPA buffer ($1\times$ PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl

sulfate [SDS], 10 mg/mL of PMSF, Aprotinin, 100 mM of sodium orthovanadate, and 4% protease inhibitor cocktails) through a microcentrifugation at $10\,000 \times g$ for 10 minutes at 4°C. The supernatants were collected and treated with an equal volume of sample application buffer (125 mmol/L Tris HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol). Forty micrograms of protein was loaded and separated by 8% SDS-polyacrylamide gel electrophoresis with a set of molecular-weight markers (Sigma-Broad range). After electrophoresis, samples were transferred to polyvinylidine difluoride membranes. The blots were blocked with 5% nonfat milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20 and incubated with eNOS and iNOS rabbit polyclonal antibodies (both from Sigma Chemical Co, St Louis, Mo) diluted at 1:200 in 5% milk PBS/Tween for 1 hour at room temperature. Positive controls were included in all experiments as provided by the manufacturer to confirm antibody specificity. As an internal control, blots were
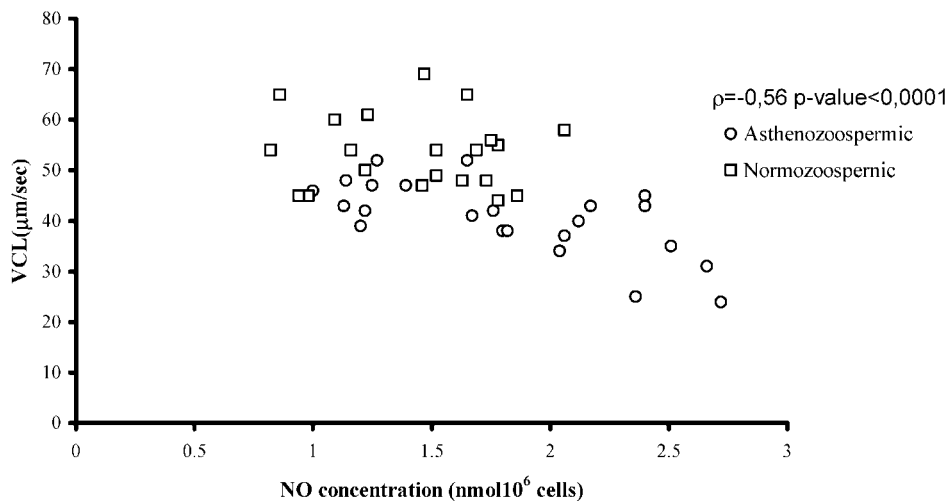

Figure 4. Negative linear correlations between nitric oxide (NO) concentration and curvilinear velocity (VCL) in asthenozoospermic and normozoospermic donors.
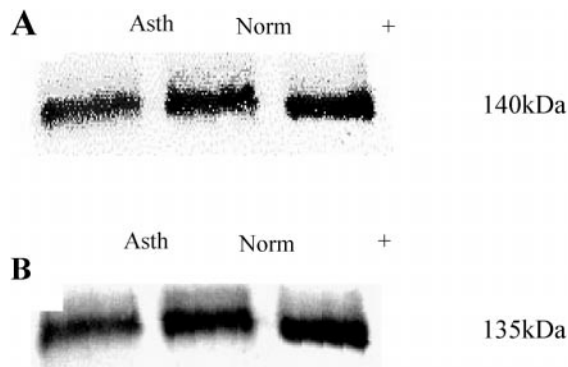
Figure 5. Western blot analysis of iNOS **(A)** and eNOS **(B)** protein expression in both asthenozoospermic (Asth) and normozoospermic (Norm) men. + indicates positive control.

reprobed with an anti–β-actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Blots were then washed with TTBS (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20) and incubated with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (IgG) (1:5000; Sigma) for 1 hour at room temperature. Peroxidase activity was detected using 3,3′-diaminobenzidine (Sigma) as a substrate.

Statistical Analysis

Statistical analysis was performed using the SAS statistical package (Statistical Analysis Systems Institute, Cary, NC). Results are reported as mean value plus or minus standard deviation. Differences among the groups were evaluated by *t* tests, and the Kolmogorov-Smirnov test was used to determine whether the data were random samples from a normal distribution. Finally, the linear dependence was measured using Pearson's correlation coefficient.

Results

The mean value of the seminal NO concentration of all subjects included in the study was 1.67 ± 0.38 nmol/10^6 cells. The Kolmogorov-Smirnov test allowed the acceptance of the null hypothesis concerning the normal distribution of the values in the 2 groups, and the subsequent statistical analysis showed that normozoospermic fertile men exhibited NO concentrations that were significantly lower than those of asthenozoospermic infertile men (1.43 ± 0.27 vs 1.81 ± 0.54 nmol/10^6 cells, $P < .01$) (Table). Single NO seminal levels of each donor are shown in a 2-class plot in Figure 1.

A significant linear negative correlation was evident between NO concentration and percentage of total sperm motility ($\rho = -.50$; $P = .0007$) (Figure 2). Furthermore, the CASA evaluation of sperm kinetic characteristics revealed a significant linear negative correlation between NO concentration and VSL ($\rho = -.49$; $P = .0006$) (Figure 3) and VCL ($\rho = -.56$; $P < .0001$) (Figure 4). These

data become more interesting from the statistical point of view, in consideration of the reduced numerical size of the sample. Finally, no correlation was evident between NO concentration and ALH (data not shown).

iNOS and eNOS expression was examined by Western blot in both normozoospermic and asthenozoospermic men to evaluate the NOS isoforms that are responsible of the observed NO levels. As shown in Figure 5, both iNOS and eNOS proteins were expressed in the 2 groups of considered patients.

Discussion

Several in vitro studies have investigated the effects of exogenous NO donors on sperm function, namely motility and viability, with controversial results (Hellstrom et al, 1994; Rosselli et al, 1995; Weinberg et al, 1995; Nobunaga et al, 1996; Zhang and Zheng, 1996). There is some evidence that low concentrations of NO increase human sperm capacitation without affecting sperm motility (Zini et al, 1995). In addition, the results obtained by Aitken et al (1995) suggest that the stimulation of NO generation is associated with the enhancement of tyrosine phosphorylation of sperm proteins and that this activity is an essential component of the cascade of biochemical changes leading to sperm capacitation.

Herrero et al (2000) have reported that NO is capable of regulating cyclic adenosine monophosphate (cAMP) concentration and, consequently, capacitation via stimulation of adenyl cyclase activity. This modulation could act directly by targeting the enzyme or by altering the action of a distinct regulatory protein. It has been speculated on the sensitivity of sulfhydryl groups of proteins to both nitrosative and oxidative events, which in turn may elicit distinct functional changes. However, this type of regulation remains to be elucidated in the male gamete.

Even though cAMP is the best-established messenger in human sperm capacitation, it is possible that NO has other targets for modulating this process. As demonstrated in other tissues, NO could modulate cyclic guanosine monophosphate levels (Murad, 1994). Alternatively, the mechanism by which NO leads to phosphotyrosine accumulation could conceivably involve the stimulation of tyrosine kinase activity, the inhibition of tyrosine phosphatase activity, or a combination of both of these effects.

In the present study, nitrite, the stable endproduct of the NOS/NO pathway, has been detected in the semen of a control group of normozoospermic fertile men and a group of idiopathic asthenozoospermic infertile patients. Our results provide evidence that NO concentration is significantly lower in the former than in the latter. Furthermore, a significant negative linear correlation between NO concentration and sperm motility is evident, as well

as other kinetic characteristics of spermatozoa such as VCL and VSL. Since the leukocyte concentration was less than 1×10^6/mL in each sample, it is unlikely that a significant contribution to nitrite concentrations could have come from neutrophils, the only other contaminating cell type capable of NO production.

Our findings suggest that high concentrations of NO play a deleterious effect on spermatozoa kinetic characteristics. As a possible explanation, it has been reported that NO may react with superoxide or hydrogen peroxide, resulting in the formation of peroxinitrite, hydroxyl radical, NO_2, or singlet oxygen, which cause oxidation of sperm membrane lipids and thiol proteins (Stamler et al, 1992). NO also may inhibit cellular respiration by nitrosylation of heme in mitochondrial enzymes, aconitase, and glyceraldehyde phosphate dehydrogenase, leading to a depletion of adenosine triphosphate and a consequent loss of motility by spermatozoa.

In conclusion, the main features of the present study are that the NO concentrations in the semen samples of infertile patients affected by asthenozoospermia are significantly higher than those in normozoospermic fertile subjects. The present data suggest that the overproduction of this free radical and the consequent excessive exposure to oxidative conditions have a potential pathogenetic role in the reduction of sperm motility. The role played by NO in spermatozoa capacitation (Aitken et al, 1995; Zini et al, 1995) leads us to speculate that such paradoxical involvement in both pathologic and physiologic processes depends on the alternative redox state and relative level of NO.

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