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Seminal plasma nitrite/nitrate and intratesticular Doppler flow in fertile and infertile subjects

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The objective of the present study was prospectively to evaluate the role of nitric oxide (NO) in modulating intratesticular blood flow and sperm function. A total of 56 males, undergoing assisted reproduction, were divided into three groups according to semen analysis: (i) normozoospermic (n = 16); (ii) oligozoospermic (n = 21); and (iii) azoospermic (n = 19). All the subjects were submitted to hormone analysis [luteinizing hormone, follicle stimulating hormone (FSH), growth hormone, testosterone, androstenedione, insulin], and to ultrasonographic (testicular volume) and Doppler (transmediastinal artery) evaluations. Plasma and seminal plasma nitrite/nitrate concentrations, and plasma insulin-like growth factor-I were assayed. All 56 patients completed the study. In normozoospermic patients, significantly greater testicular volume, lower transmediastinal resistances, and higher seminal plasma nitrite/nitrate concentrations were observed in comparison with both oligo- and azoospermic subjects. Testicular volume was inversely correlated with plasma FSH (r = –0.589; P = 0.005) and pulsatility index of transmediastinal artery (r = –0.402; P = 0.049). Furthermore, the seminal plasma nitrite/nitrate concentrations were inversely correlated with pulsatility index of transmediastinal artery (r = –0.511; P = 0.015). It was concluded that NO is involved in vascular modulation of testicular vessels and ultimately in sperm output.

Key words: Doppler/infertility/nitric oxide/spermatozoa/ultrasonography

Introduction

The demand for infertility investigations and treatment is progressively increasing in Western countries. This is due to different factors: women are delaying childbearing; improvements in assisted reproductive techniques; increased awareness of such possibilities. The prevalence of infertile couples is ~10–15% of all couples and the causes of infertility can be divided into four major categories: pure male factor, pure female factor, anomalies detected in both partners, and unexplained infertility [European Society of Human Reproduction and Embryology (ESHRE), 1996].

Assessment of male fertility is based on history, physical examination and at least one semen analysis which should be performed according to the World Health Organization-recommended procedure (WHO, 1992). If the first semen analysis is normal, there is no need to repeat the analysis. In the case of pathological findings (oligo-, astheno-, terato-, azoospermia) an additional semen sample should be examined after ~3 months and ancillary tests should be considered. Between these, the assessment of testicular volume, by palpation or ultrasonography, could be important (seminiferous tubules represent ~80–85% of the testicular mass) because a small testicular volume (<15 ml) may suggest an impairment of seminiferous tubules and may be associated with a raised plasma concentration of FSH (Forti and Krausz, 1998). High plasma FSH concentrations, probably due to decreased secretion of inhibin by Sertoli cells, are considered reliable indicators of germinal epithelial damage and are usually associated with azoospermia or severe oligozoospermia ($<5\times10^6$ spermatozoa/ml) (Bergmann *et al*., 1994). However, other than FSH, testicular paracrine [i.e. inhibin, activin and nitric oxide (NO)] products may be implicated in abnormal sperm function (Martin-du-Pan and Bischof, 1995).

NO is a labile and diffusible molecule which forms stable metabolites (nitrite/nitrate; NO_2^-/NO_3^-) detectable in many biological fluids. NO is synthesized from a guanidine nitrogen atom of L-arginine either by a constitutive calcium-dependent, or a pro-inflammatory cytokine-inducible, NO-synthase (NOS) (Moncada *et al*., 1991). The precise role of NO has not been elucidated but it has been thought to be involved in a wide array of biological processes, including neuronal transmission (Bredt and Snyder, 1992), immune modulation (Mills, 1991), vascular wall contractility (Ignarro *et al*., 1987), hormone production (Welch *et al*., 1995; Sharma *et al*., 1998), cell differentiation (Magrinat *et al*., 1992), apoptosis (Blanco *et al*., 1995) and protection from oxygen radicals (O'Bryan *et al*., 1998). NO has also been implicated as a mediator of penile erection (Burnett *et al*., 1992) and its localization at the level of male testes, epididymides, prostate and seminal vesicles suggests a role in vascular modulation of reproductive organs, spermatogenesis and sperm maturation (Burnett *et al*., 1995; Zini *et al*., 1995, 1996). Furthermore, a correlation between increased nitrite/nitrate concentrations in seminal plasma and decreased percentage of rapid progressive motile spermatozoa has been demonstrated, suggesting that the increases in NO synthesis *in vivo* can significantly affect sperm viability (Rosselli *et al*., 1995).

Colour Doppler ultrasonography is a recent advance in

sonographic imaging that provides simultaneous display of tissue morphology in grey scale and blood flow in colour. Recent reports showed that colour Doppler facilitates the detection of small intratesticular vessels and allows the measurement of impedance to flow in this vascular tree (Bader *et al*., 1997; Foresta *et al*., 1998).

The aim of the present study was to evaluate, in fertile and infertile males, a possible role of NO in modulating intratesticular vascular parameters and ultimately sperm output.

Materials and methods

Study population

The study protocol was approved by the local ethics review committee. Fifty-six males attending the infertility clinic participated in the study after giving informed consent. On the basis of two previous semen analyses, performed according to the WHO-recommended procedure (WHO, 1996), the subjects were divided into normozoospermic (group I, $n = 16$); oligozoospermic (sperm concentration $<10\times10^6$/ml; group II, $n = 21$); azoospermic (no spermatozoa in the ejaculate; group III, $n = 19$). Excluded from the study were patients with: varicocele, drug or alcohol abuse, ongoing medical treatments (anabolic steroids, gonadotrophins, cancer chemotherapy), heavy smokers (>10 cigarettes/day), diabetes, hypertension (systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg), testicular injury, cord injury, orchitis, surgery for varicocele or cryptorchidism, leukocytospermia ($>1^6$ leukocyte/ml), ultrasonographic abnormalities of scrotal content, unilateral testicular atrophy.

All patients underwent semen analysis and ultrasound and colour Doppler scanning of the testes. NO concentration was assayed in seminal plasma. Furthermore, on the same day, peripheral blood was obtained from all patients between 08.00 and 11.00 h, after an overnight fast, and different hormonal and biochemical parameters were analysed.

Semen analysis and seminal plasma NO assay

After 2–3 days of sexual abstinence, semen samples were produced by masturbation, collected into sterile specimen cups and allowed to liquefy at room temperature. Semen volume, pH, sperm concentration, motility and morphology were determined following the WHO guidelines for semen analysis (WHO, 1992).

For assessment of NO concentration in the seminal plasma, the whole semen was centrifuged at 1500 *g* for 5 min, the seminal plasma removed and stored at –70°C until bioassay. NO concentrations were assessed by monitoring seminal plasma concentrations of stable oxidation products of NO metabolism (NO_2^-/NO_3^-). Since very little or no NO_2^- is normally found in the plasma, no attempt was made to differentiate between the respective amounts of NO_2^- and NO_3^- and therefore results are reported as NO. The concentrations of NO_2^-/NO_3^- were assayed with the Greiss reaction with procedures previously described (Facchinetti *et al*., 1997; Battaglia *et al*., 1999).

Hormonal and biochemical assay

Peripheral blood samples, obtained from an antecubital vein, were immediately centrifuged, the plasma was separated and stored at –20°C, and subsequently assayed as previously reported (Battaglia *et al*., 1999). Plasma concentrations of LH, FSH, testosterone and androstenedione were determined by radioimmunoassay (Radim, Pomezia, Italy). Serum growth hormone (GH) concentrations were similarly measured by radioimmunoassay (Sorin Biomedica, Saluggio, Italy). The concentrations of serum insulin and insulin-like growth factor (IGF-I) were determined by double-antibody radioimmunoassay (Amersham, Milan, Italy, and Immuno Nuclear Corp., Stillwater, CA, USA respectively). The NO_2^-/NO_3^- plasma concentrations were determined with the same methods used for seminal plasma assays.

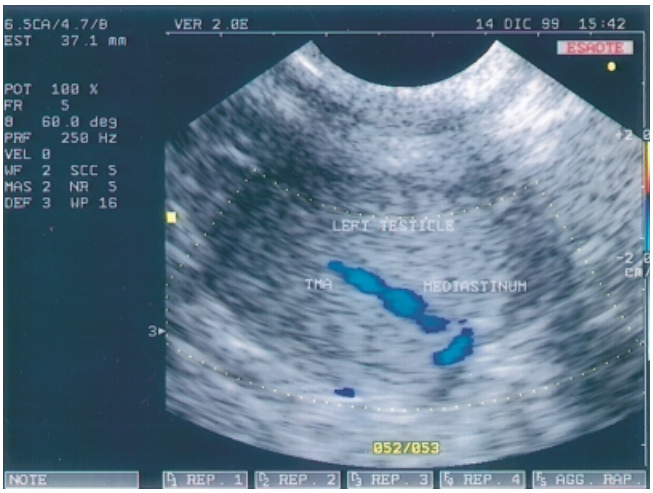

Figure 1. Colour flow image of the transmediastinal artery (TMA). TMA is sampled, in a longitudinal plane, at the level of testicular mediastinum.

Ultrasound and Doppler examinations

Scrotal ultrasonographic assessments of testicular volume were performed, in a warm room with the patients in the supine position and with the penis resting on the lower abdomen, using a 6.5 MHz digit probe (AU4 Idea; Easote, Milan, Italy). Each testicle was measured in three dimensions and the volume electronically calculated by machine (Kim and Lipshultz, 1996). No significant differences between the volumes of the left and right testicles were observed, and therefore the average value of testicular volumes was used.

Doppler flow measurements of transmediastinal arteries (TMA) were performed in each testis with a trans-scrotal approach using a 6.5 MHz (AU4 Idea) colour Doppler system. All the patients were studied between 08.00 and 11.00 h to exclude the effects of circadian rhythmicity on blood flow (Zaidi *et al*., 1995). They rested for at least 15 min before being scanned, and completely emptied the bladder to minimize any external effects on blood flow (Battaglia *et al*., 1994). A 50 Hz filter was used to eliminate low frequency signals originating from vessel wall movements. The maximum ultrasonographic energy was <80 mW/cm^2. The intensity is within the safety limits suggested by the American Institute for Ultrasound in Medicine (Lizzi and Mortimer, 1988). Colour flow images of the transmediastinal artery were sampled, in a longitudinal plane, at the level of testicular mediastinum (Figure 1) (Middleton and Bell, 1993). As previously reported, the transmediastinal arteries presented blood flow directed away from the mediastinum. The angle of insonation was altered to obtain the maximum colour intensity. When good colour signals were obtained, blood flow velocity waveforms were recorded by placing the sample volume across the vessel and entering the pulsed Doppler mode. The pulsatility index (PI = S - D/mean), defined as the difference between peak systolic (S) and end diastolic (D) flow velocity divided by the mean flow velocity, was calculated electronically by the machine. The PI has been shown to reflect blood flow impedance downstream from the point of sampling and may be used when the end diastolic shift is absent or reversed. For each examination the mean value of three consecutive waveforms was obtained. No significant differences between the PI of left and right transmediastinal arteries were observed. Therefore, the average value of both arteries was used. An indication of the within-patient precision

Table I. Hormonal and biochemical parameters in both fertile and infertile men

	Normozoospermic (n = 16)	Oligzoospermic (n = 21)	Azoospermic (n = 19)	Normal range[a]
LH (IU/l)	3.8 ± 1.8	2.9 ± 1.2	4.5 ± 2.0	1.4–8.9
GH (μg/l)	0.5 ± 1.1	0.3 ± 0.5	0.2 ± 0.7	0.1–6.0
Testosterone (ng/100 ml)	353 ± 69	331 ± 112	362 ± 87	300–900
Andostenedione (ng/100 ml)	114 ± 51	157 ± 62	170 ± 138	30–310
IGF-I (ng/ml)	133 ± 31	145 ± 31	161 ± 52	101–303
Insulin (μg/l)	23 ± 2	21 ± 3	19 ± 7	5–25

Values are mean ± SD.
[a]The normal range is derived from 50 normozoospermic patients conceived by assisted reproductive techniques.
GH = growth hormone; IGF-I = insulin-like growth factor-I.

of the Doppler evaluation of TMA was obtained by analysing the flow-velocity waveforms recorded, at a single setting, on three occasions at 1 min intervals. An analysis of variance of results gave a mean PI coefficient of variation of 7.8%. There was no significant difference between replicate analyses. The correlation between PI and heart rate was tested by using linear regression analysis. A weak and statistically non-significant inverse correlation was found. Therefore, in the results the PI have not been corrected for heart rate.

Ultrasound and colour Doppler analyses were performed by a single examiner (C.B.) who was unaware of the seminal, hormonal and biochemical status of the scanned patients.

Statistical analysis

A statistical analysis (SPSS software; SPSS Inc., Illinois, IL, USA) was performed using the Mann-Whitney test and one-way analysis of variance. The relationship between the parameters analysed was assessed using the stepwise linear regression method. $P \leqslant 0.05$ was considered to be the limit of statistical significance. Data are presented as mean ± SD unless otherwise indicated.

Results

All 56 patients completed the study. The seminal analysis confirmed the diagnosis at entrance. The mean age was 37.6 ± 6.3 years and no significant differences were observed between the groups.

Plasma LH, GH, testosterone, androstenedione, insulin and IGF-I did not differ between the groups (Table I). Plasma FSH was significantly higher in the azoospermic group (10.2 ± 6.5 IU/l; range 3.4–22.0) than in both the oligozoospermic (4.8 ± 1.9 IU/l, P = 0.04; range 1.9–9.1) and normozoospermic (4.4 ± 2.0 IU/l, P = 0.011; range 1.4–6.6) patients.

The assessment of ultrasonographic volume showed significantly smaller testes in azoospermic patients (group III, 12.4 ± 3.1 ml; range 7–23) than in oligozoospermic (group II, 15.3 ± 4.3 ml, P = 0.049; range 9–25 ml) and normozoospermic (group I, 19.7 ± 4.1 ml, P = 0.009; range 15–29 ml) patients respectively. Furthermore, the testis volume of group I was greater than in group II (P = 0.029). On Doppler analysis, TMA were observed in at least one testicle in 100% of the cases and bilaterally in 91% of the cases. Higher resistances were observed in TMA of azoospermic (PI = 1.47 ± 0.2; range 1.22–1.74) than in oligozoospermic (PI = 1.37 ± 0.21, P = 0.058; range 0.82–1.55) and normozoospermic (PI = 1.19 ± 0.25, P = 0.038; range 0.70–1.42) patients. A weak significant difference was also observed between group I and group II (P = 0.049) men.

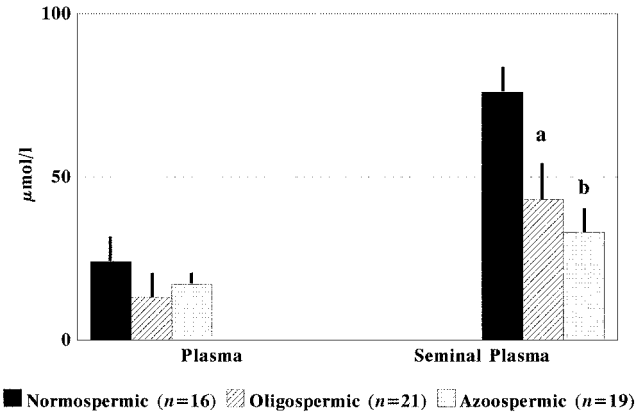

Figure 2. Nitric oxide concentrations in plasma and seminal plasma of fertile and infertile subjects. [a]Normozoospermic versus oligozoospermic patients: P = 0.03. [b]Normozoospermic versus azoospermic patients: P = 0.001.

The NO_2^-/NO_3^- plasma concentrations were similar in the analysed groups (Figure 2). Seminal plasma concentrations were significantly higher in normozoospermic men than in patients of other groups (Figure 2). Normalizing the NO_2^-/NO_3^- seminal plasma concentrations to testes volume, NO_2^-/NO_3^- seminal concentrations of 3.85 ± 0.22 μmol/ml testis, 2.81 ± 0.19 μmol/ml testis ($P < 0.01$) and 2.41 ± 0.15 μmol/ml testis were obtained ($P < 0.019$) respectively in normo-, oligo- and azoospermic patients. In addition, the NO_2^-/NO_3^- plasma concentrations, in normo- and oligozoospermic patients were significantly lower ($P < 0.001$) than in the corresponding seminal plasma. No significant differences were observed in the azoospermic group.

In the study population, testicular volume was inversely correlated with plasma FSH (r = –0.589; P = 0.005) and TMA PI (r = –0.402; P = 0.049). Furthermore, the NO_2^-/NO_3^- seminal plasma concentrations was inversely correlated with TMA PI (r = –0.511; P = 0.015).

Discussion

Recent reports on progressive decrease of sperm count and increase of sexually transmitted infections and developmental

anomalies of male reproductive tract have supported the hypothesis of declining male fertility in Western countries. However, despite the explosion of research on testicular physiology, the aetiology and pathophysiology of defective sperm function remain poorly understood.

In the present study, in contrast to Lenz *et al*. (1993), who found the right testis ~1 ml larger than the left, no significant differences were observed between ultrasonographic volumes of left and right testes in both fertile and infertile patients. It is suggested therefore that, in the absence of specific unilateral pathologies, the size should be similar in both testes and that ultrasound-derived measurements of testicular volume are accurate and more reproducible than those obtained by physical examination and comparison with Prader's orchidometer. In addition, data reported here showed a progressive decrease of testicular volume, from normozoospermic to azoospermic patients, associated with increased plasma FSH values. This resulted in an inverse correlation between FSH and testicular volume. Lenz *et al*. (1994), in accordance with previous studies on sperm quality and orchidometer measurements of the testes, found a positive correlation between sperm count and ultrasonographic testicular volume. As the seminiferous tubules represent ~80–85% of testicular mass, a small testicular volume may suggest a significant impairment of the seminiferous tubules with subsequent testicular fibrosis and damaged spermatogenesis. Although elevated serum FSH concentrations are usually considered to be associated with severe damage of spermatogenesis, there remain a considerable number of patients who do not match any specific hormonal pattern by showing rather low sperm counts and normal serum FSH concentrations and *vice versa* (Bablock *et al*., 1978; Fredricsson *et al*., 1989; Nieschlag, 1993).

The above considerations allow the supposition that factors other than FSH are involved in the modulation of spermatogenesis and spermiogenesis. Paracrine molecules may be involved in both physiological and pathological processes of spermatozoa. In the last few years there has been mounting evidence in support of a role for NO in sperm function and dysfunction. It has been shown that, *in vitro*, high concentrations of NO have a deleterious effect on sperm motility (Weinberg *et al*., 1995), whereas low NO concentrations increase post-thaw sperm motility and viability (Hellstrom *et al*., 1994). The data obtained *in vitro* on NO involvement in sperm function have been clinically supported with the findings of constitutive NOS expression in human spermatozoa suggesting the local release of controlled amounts of NO (Schaad *et al*., 1995).

In the present study, significantly higher NO concentrations were found in seminal plasma than in blood serum of both normo- and oligozoospermic patients. No significant differences were observed in azoospermic men. Furthermore, the NO_2^-/NO_3^- seminal plasma concentrations normalized to testes volume showed higher values in normozoospermic than in oligo- and azoospermic patients. This may confirm a sperm release of NO and allows speculation that in seminal plasma the NO concentrations are related to the number of spermatozoa. However, Middendorf *et al*. (1997) showed that NOS is also present in Leydig cells and in peritubular lamina propria, Sertoli, and blood vessel cells, suggesting a production and a local activity in such structures. From a functional point of view, our data demonstrated an inverse correlation between NO and PI of TMA. Given that there was no significant change in the heart rate or vessel diameter, the observed higher PI in oligo- and azoospermic compared with fertile men is suspected to reflect, in an infertile population, an increased resistance in the small vessels distal to the TMA. Although it is not possible to exclude the possibility that the increased TMA vascular resistances in subjects with oligo- and azoospermia may be due to vaso-occlusive disorders, the above considerations lend support to the hypothesis that testicular vasculature is a site of NO production and activity and that NO acts locally to regulate the distribution of hormones and nutrients by testicular vessels and, by influencing the permeability of lamina propria, contributes to their (hormones and nutrients) transport into the tubular lumen and thereby modulates sperm production and function. Furthermore, because NO can freely diffuse across membranes, it cannot be excluded that sperm production of NO occurs at the level of seminiferous tubules, and, through the lamina propria, subsequent action occurs at the level of the neighbouring vascular smooth muscle cells.

Unlike Middleton *et al*. (1989), who affirmed that 'it is unusual for arteries to pass directly through the mediastinum of the testis', and who subsequently visualized TMA in only about one-half of normal tests, this study showed TMA in at least one testis in 100% of the cases and bilaterally in 91% of the cases. It seems that the progressive improvements of colour Doppler machines allow an easy and reproducible visualization of testicular vessels.

It is concluded that NO is involved in vascular modulation of testicular vessels and that Doppler analysis of TMA is an accurate and easy-to-perform technique. Furthermore, Doppler studies of the TMA seem capable of giving specific physiological and pathophysiological information for the assessment of intratesticular vascularization. Further extensive studies are necessary for a better understanding of the relationship between NO, testicular vascularization and sperm production and function in both fertile and infertile men.

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