Nitric oxide level in seminal plasma of fertile and infertile males and its correlation with sperm parameters

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ABSTRACT

Nitric oxide (NO) is a free radical molecule, produced by most cells and tissues in the body. The effect of NO on cells is concentration dependent. Low concentration of NO is essential in biology and physiology of most of cells, but high amounts of NO is toxic and has detrimental effects on cells. The role of NO in biology of male and female genital systems is under investigation.

In the present study, the nitric oxide concentration was measured in the seminal plasma of both fertile and infertile males and compared with spermatogram parameters. For this purpose**,** semen samples were collected from 45 patients and 70 healthy donors. After analysis of samples, the stable metabolites of nitric oxide (nitrite and nitrate) were measured by Griess assay. The results indicated that the nitric oxide concentration in the seminal plasma of infertile males was significantly higher than controls. There was a significant negative correlation between the nitric oxide concentration and sperm motility and viability in infertile males.

In conclusion, this study demonstrated that the level of nitric oxide in seminal plasma of infertile men was higher than that of fertile men. The increasing level of nitric oxide concentration in seminal plasma leads to the decrease in sperm motility and viability and affects fertility.

Keywords: Nitric Oxide, Sperm, Infertility.

INTRODUCTION

Nitric oxide (NO) is a free radical molecule which is synthesized through the enzymatic conversation of L-arginine to L-citroline by a family of isoenzymes known as the nitric oxide synthase (NOS) and mediates a number of biological functions. The NOS isoforms include the neuronal (nNOS), the endothelial (eNOS), and the inducible (iNOS) types. Whereas the nNOS and eNOS are constitutively expressed enzymes, which are stimulated by increased Ca^{2+} concentration, iNOS expression is induced by inflammatory cytokines and toxins and leads to production of much higher amounts of NO compared to the constitutive enzymes (1). Since the discovery of nitric oxide as an endogenously formed radical, its effect on numerous physiological processes has been intensively investigated. The physiological role of NO in a varity of reproductive process such as folliculo-genesis and spermatogenesis has been established (2). Various kinds of NOS were detected in both male and female human genital tracts (3-11). Several investigations have been carried out to identify the role of NO in male infertility and results indicated that nitric oxide strongly affects human sperm functions, such as motility, viability and metabolism (12-16). In vitro studies have shown that the effects of nitric oxide on sperm functions is concentration- dependent, being positive at low but detrimental at higher concent-ration (17-24).

Contradictory results have been published on the concentration of NO in seminal plasma and its correlation with sperm parameters (22-28).

For example, the positive correlation between the concentrations of NO in seminal plasma with the proportion of immotile spermatozoa has been shown(17).In another report, it has been shown that nitric oxide concentration in oligo-and or astheno-zoospermic patients with varicocele was signi-ficantly higher than healthy males or oligo and asthenozoospermic males without varicocel (25). On the contrary, it has been reported that the concentration of NO in seminal plasma does not correlate with sperm concentration, sperm motility and leukocytespermia (24) and there are no significant differences in NO concentration and correlation between its concentration and sperm concentration and motility in seminal plasma of

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normal and abnormal subjects. However, a positive correlation between seminal plasma NO and sperm morphology has been reported (28).

According to these contradictory results, more investigation is necessary to identify the correlation between seminal plasma nitric oxide concentration and male infertility.

In the present study, the nitrite and nitrate concentration of the seminal plasma of both fertile and infertile males were measured and compared with spermatogram parameters and viability.

MATERIALS AND METHODS

Collection and evaluation of sperm sample

Semen samples were collected by masturbation after 3-5 days of abstinence from men (n = 45) attending the fatemieh infertility clinic with a history of infertility of at least one years duration. Subjects who had $<20 \times 10^6$ /mL sperm concentration, < 50% motility or < 30 % normal forms as assessed by the world health organization (WHO, 1999) guidelines, were considered to have abnormal semen parameters. Controls consisted of samples obtained from 70 donors with normal sperm parameters.

The exclusion criteria were the presence of $< 10 \times 10^6$ / mL total motile spermatozoa in the original (post – liquefaction) samples, azoospermia and severe oligospermia. No subjects in either group were smokers, on medication, had a history of exposure to chemotherapy or radiation, or a varicocele.

All samples for evaluation, were allowed to liquefy for at least 30 minutes at 37 °C, then evaluated for sperm concentration, motility, and morphology and leukocyte concentration.

Cell viability was determined in each sample using eosin-staining method. The liquefied semens were centrifuged at 300g for 10 minutes. After centrifugation, the supernatant was withdrawn and stored at -70 °C until used.

Nitrite Assay

NO concentration was assessed according to the reported method by monitoring seminal plasma concentration of stable oxidation products of NO metabolites (NO_2^-/NO_3^-) (18, 22, 24 and 29).

Nitrite was determined spectrophotometrically by a stepwise Griess reaction. Nitrate was detected after reduction to nitrite using nitrate reductase. For nitrate reduction, the samples were centrifuged at 1000g for 15 minutes at room temperature to remove cells and particles. Then, 150 µl of samples were incubated for 15 minutes at 30 °C in the presence of 0.1 unit/mL nitrate reductase (from aspergillus, sigma), 5µm FAD (sigma), 30 mM NADPH (sigma) in final volume of 160 µL. When, nitrate reduction was completed, NADPH (up to 0.3 mM) was oxidized to avoid interference with the following nitrite determination. For this purpose, the samples were incubated with 10 units/mL lactate dehydrogenase (from rabbit muscle, sigma) and 10 mM sodium pyravate (sigma) for 5 minute at 37 °C in a final volume of 170 µL. Total nitrite was then determined spectrophotometrically using Griess reaction (17) and by addition of 1% sulfanilamide and 0.1% naphthylethlenediamine dihydrochloride in 2% phosphoric acid and recording absorbance at 540 nm with a spectrophotometer after 10 minutes incubation at 37 °C in the dark. Nitrite concentration was expressed as µmol/mL. Linear regression was used to determine NO concentration from standard curve of $NaNO_2$.

STATISTICAL ANALYSES

Data are reported as mean ±SD. The comparisons between two groups were made by t-test using SPSS10. Correlation between two continues outcomes were evaluated using Pearson correlation coefficients. P values of 0.05 or less were considered statistically significant.

RESULT AND DISCUSSION

The characteristics of the participants in the two groups are shown in Table I. There were no significant differences in the mean of age of participants and morphology of sperms in two groups but the means of sperm concentration, motility and viability in fertile males were significantly higher than infertile males. The mean of NO concentration in the seminal plasma of the 45 infertile males (figure 1) was significantly higher than that of the 70 control males (5.47 ± 1.01 vs. 3.88 ± 0.53 µmol/L, $P < 0.001$). Among the sperm parameters, there were significant negative correlations between NO concentration and total sperm motility (R = –. 402; P < 0.01), forward motion (R=-.407; P<. 01) and sperm viability (R = –392; P < .01) in the infertile group. These correlations were not found in the fertile group (figures 2-4).

However, the data indicated that 12 of the infertile cases with leukocytospermia had high concentration of NO in their seminal plasma but there was not a significant correlation between the leukocytes concentration and seminal plasma NO concentration (fig5).

Contrary to a report (28), the present results show that NO concentration in seminal plasma of infertile males is significantly higher than that of healthy males. Also contrary to other reports (27, 32) and in agreement with some reports (17, 19, 28), in the present study a significant negative correlation was found between NO concentration and sperm motility in the infertile males.

Table 1. Characteristics of the study population. There were no significant differences in the mean age of participants and morphology of sperms in two groups but the means of sperm concentration, motility and viability in fertile males are significantly higher than infertile males.

	Fertile (Mean ±SD)	Infertile (Mean ±SD)	P-value (Mean ±SD)
Mean Age (yrs)	31.4±4.6	33.2±5.5	NS
Sperm concentration (* 10^6)	63.8±16.7	39.7±26.3	<0.001
Sperm motility (%)	53±5	29.8±13.7	<0.001
Sperm Morphology (%)	25±6	13.48±5.6	NS
Viability (%)	59.4±5.4	36±13.4	<0.001

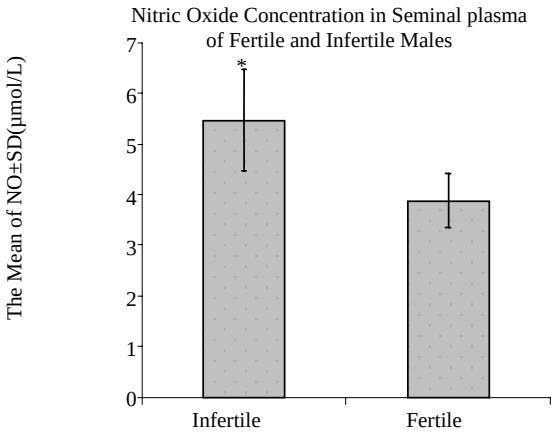

Figure1. The mean of NO concentration in fertile and infertile groups. *There is a significant difference between two groups (p<. 001)

A negative correlation between seminal plasma NO concentration and sperm viability has been identified in this study for the first time. However, the findings suggest that high concentrations of NO plays a deleterious effect on spermatozoa viability and kinetic characteristics, but in contrast to the reported findings (28) these results do not confirm any positive correlation between NO and sperm morphology. It seems that the controversial results achieved in this and other studies may be due to different study populations and etiology of infertility in the subjects.

The beneficial effects of NO on sperm function have been previously reported (26-28). Results of an investigation suggested that stimulation of NO generation is associated with enhancement of tyrosine phosphorylation of sperm proteins and this activity is an essential component of the cascade of biochemical changes leading to sperm capacitation (31). On the other hand, it has been reported that NO has ability of regulation of cyclic adenosine monophosphate (cAMP) concentration and, consequently, capacitation via stimulation of adenyl cyclase activity (33). This modulation could act directly by targeting the enzyme or by altering the action of a distinct regulatory protein (34). The NO which is produced at supra-physiological concentration can freely diffuses across membranes and exerts action through its biologically activated molecules at different levels. The harmful effects of NO are mediated by biologically activated molecules produced by the reaction of NO with the superoxide anion yielding $ONOO^-$ and peroxy-nitrous acid (ONOOH). The resulting molecules are strong oxidant that can causes molecular damages to a variety of tissues (35). The acid ONOOH reacts with the cysteine residues of proteins or glutathione, forming S-nitrosothiols (36). S-nitrosylation causes disregulation of cellular signal transduction processes has also harmful effects on cellular energetic through inhibition of complex I in mitochondrial respiration promotion of DNA damage and/or apoptosis (29,37).

NO also may inhibits cellular respiration by nitrosylation of heme in mitochondrial enzymes, aconitase, and glyceraldehyde phosphate dehydro-genase, leading to a depletion of adenosine triphosphate and a consequent loss of motility by spermatozoa.

However, the sources of overproduction of NO in infertile males are unknown but some studies suggest that it may be produced by induced genital tract cells such as laydig cells (38), epididimal or vas deferan epithelial cells or spermatozoa itself (4,5,7) and finally in some cases, induced leukocytes are the source of high concentration of NO in seminal plasma. Since in the present study, the leukocyte concentration in some of the samples was high, a significant contribution to nitrite concentrations might be due to leukocyte

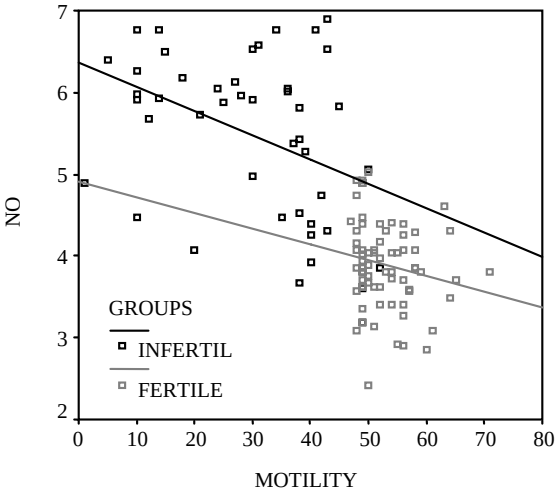

Figure 2. Negative correlations between nitric oxide (NO) concentration and total sperm motility in infertile (R=-.402, p<. 01). The correlation in fertile (control) group is not significant.

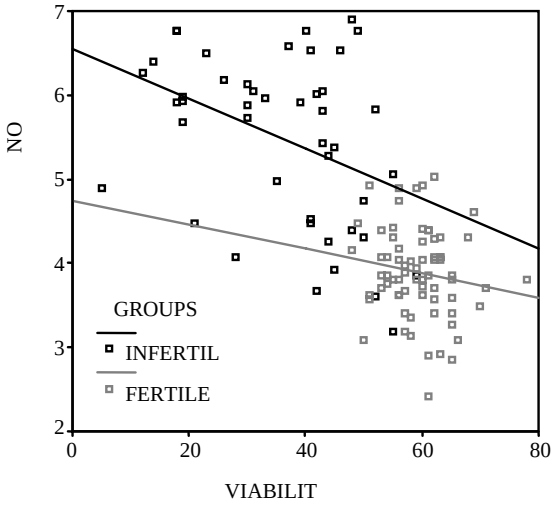

Figure 4. Negative correlations between nitric oxide (NO) concentration and sperm viability in infertile (R=-.407, p<. 01). The correlation in fertile (control) group is not significant.

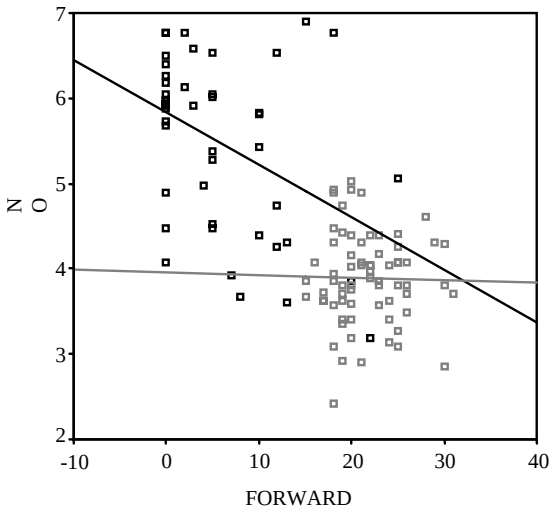

Figure 3. Negative correlations between nitric oxide (NO) concentration and forward progressive sperm motion in infertile (R=- .407, p<. 01). The correlation in fertile (control) group is not significant.

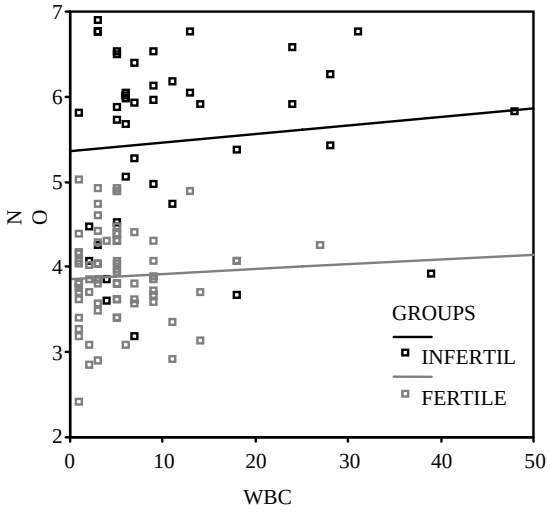

Figure 5. The figure shows that there is no correlation between nitric oxide (NO) concentration and leukocyte concentration in infertile and fertile (control) groups.

concentration but in other cases, the source of NO is unknown and it may be produced by macrophages in response to infection, or from steady secretion from multiple sources such as testis and structures of male reproductive tract (28).

However, this study established negative effect of NO in high concentration on sperm motility but it is not the main factor of sperm immotility in all of the infertile males. It should be considered that in the case of severely impaired sperm motility, factors other than the nitric oxide and other free radicals (e.g. the intrinsic structure of sperm flagellum) might results in sperm damages.

CONCLUSION

This study demonstrates that NO concentrations in the semen samples of the infertile patients are significantly higher than those in normo-zoospermic fertile subjects. The present data suggest that overproduction of this free radical and the consequent excessive exposure to oxidative conditions have a potential pathogenetic role in the reduction of sperm motility.

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