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Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity

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Endogenous nitric oxide (NO) is an important functional mediator in several physiological systems, including the reproductive system. However, when generated in excessive amounts for long periods, mainly during immunological reactions, NO is cytotoxic and cytostatic for invading microbes, as well as for the cells generating it and the tissues present around it. Since infertility associated with urogenital tract infection in males and females is also accompanied by reduced sperm motility and viability, it is possible that reduced fertility in these patients is due to NO-induced sperm toxicity. We therefore evaluated the direct effects of NO, chemically derived from *S*-nitroso-*N*-acetylpenicillamine (SNAP, 0.012–0.6 mM) and sodium nitroprusside (SNP, 0.25–2.5 mM), on the motility and viability of human spermatozoa. Furthermore, we tested whether inhibition of NO synthesis prevents sperm motility and viability by incubating washed total cells present in the semen (spermatozoa, round cells) with *N*-nitro-L-arginine-methyl-ester (L-NAME), a NO synthesis inhibitor. Treatment of purified spermatozoa with SNAP or SNP decreased forward progressive sperm motility and straight line velocity, and also increased the percentage of immotile spermatozoa in a concentration-dependent manner. Furthermore, the percentage of immotile spermatozoa positively correlated with the percentage of dead spermatozoa. In contrast to freshly prepared SNAP, SNAP preincubated for 48 h had no effect on the motility and viability of the spermatozoa. Furthermore, as compared to untreated controls, a significantly higher percentage of forward progressive sperm motility as well as viability ($P < 0.05$) was maintained in washed semen incubated with L-NAME (0.15 mM). Seminal plasma concentrations of nitrite–nitrate (stabile metabolites of NO)/10^6 spermatozoa correlated positively ($P < 0.05$) with the percentage of immotile spermatozoa. Our results suggest that NO can cause sperm toxicity as well as inhibit sperm motility. In conclusion, excessive NO synthesis in response to infection and inflammation could be an important factor contributing to functional change of the spermatozoa, leading to their dysfunction and to infertility.

Key words: human sperm motility/nitric oxide

Introduction

Infertility during several forms of urogenital infection is associated with decreased sperm motility. This is preceded or accompanied by activation of lymphocytes and macrophages as well as the presence of an increased number of white blood cells in the semen (Haney *et al.*, 1983; Anderson and Hill, 1988; Barratt *et al.*, 1990), and depends on the extent of the infection and of the microorganism/spermatozoa ratio (Auroux *et al.*, 1991). Several factors such as highly reactive oxygen free radicals, lymphokines and monokines generated by activated polymorphonuclear cells have been implicated in inducing infertility by causing sperm toxicity and reducing sperm motility (Aitken, 1989; Aitken *et al.*, 1991; Plante *et al.*, 1994). Cytokines, lipopolysaccharide and toxins generated during localized infection are known to activate white blood cells and smooth muscle cells to generate large amounts of nitric oxide (NO) continuously (Moncada *et al.*, 1991; Nussler and Billiar, 1993; Morris and Billiar, 1994). Excessive NO in turn can have a cytotoxic and cytostatic effect on the invading microorganisms as well as the surrounding cells either directly, or in combination with free radicals (Moro *et al.*, 1994; Morris and Billiar, 1994). It is therefore plausible that increased incidence of infertility in subjects with urogenital infection and inflammation is due to NO-induced sperm toxicity as well as reduced sperm motility.

NO is a labile and diffusible molecule which forms stable metabolites, nitrite/nitrate, that can be detected in biological fluids (Ochoa *et al.*, 1991). NO is synthesized in a tightly regulated manner and is well known to be an important mediator in several physiological systems (Moncada *et al.*, 1991; Nussler and Billiar, 1993; Moro *et al.*, 1994; Morris and Billiar, 1994), including the reproductive system (Ellman *et al.*, 1993; Yallampalli *et al.*, 1993; Rosselli *et al.*, 1994). NO is synthesized from a guanidine nitrogen atom of L-arginine by the enzymatic action of the NO synthase system, which can be inhibited by competitive L-arginine analogues like *N*-nitro-L-arginine-methyl-ester (L-NAME) (Moncada *et al.*, 1991). NO synthase exists in three distinct isoforms, two of which are continuously present and termed constitutive NO synthase. The third isoform is inducible and present in macrophages, monocytes and smooth muscle cells; its activation by certain cytokines or microbial products results in a sustained production of NO (Nussler and Billiar, 1993).

The primary mechanism of NO-induced cytostaticity and cytotoxicity includes direct inhibition of mitochondrial respiration and DNA synthesis (Hibbs *et al.*, 1990). Furthermore, NO toxicity is also mediated indirectly through its interaction with other molecules, mainly superoxide anions (O_2^-) and formation of peroxynitrite anion ($ONOO^-$), which when protonated, decomposes to form $^{\bullet}OH$ and NO_2, both of which are

tissue-damaging agents (Beckman *et al.*, 1990; Ischiropoulos *et al.*, 1992; Moro *et al.*, 1994). Compared to NO and superoxide free radical alone, peroxynitrite (their reactive product) is several times more toxic (Moro *et al.*, 1994). Based on the above facts, we postulate that excessive NO generation and/or concentration causes cytotoxicity to spermatozoa and decreases sperm motility. Furthermore, in infertility associated with microbial infection, excessive production of NO may mainly contribute to the sperm dysfunction.

In the present study, we evaluated the direct effects of NO, chemically generated from SNAP (*S*-nitroso-*N*-acetylpenicillamine) or SNP (sodium nitroprusside), on the motility and viability of purified human spermatozoa. Additionally, using a non-purified sperm preparation (containing round cells) the direct evidence for NO-mediated sperm toxicity was evaluated by testing the effect of NO synthase inhibition by L-NAME on sperm motility and viability. Further, to confirm whether changes in endogenous NO concentrations are also accompanied by parallel changes in sperm functionality, we directly measured the concentrations of nitrite–nitrate in the seminal plasma obtained from different patients and we correlated its concentration with sperm motility.

Materials and methods

Semen collection

Sperm samples were obtained from healthy subjects who underwent a semen analysis for couple infertility. Specimens depicting normozoospermia (rapid forward + non-rapid forward motile spermatozoa >50%) in accordance with the World Health Organization guidelines (WHO laboratory manual, 1987) or light asthenozoospermia (rapid forward + non-rapid forward motile spermatozoa >38%) were used for the study.

Effect of SNP and SNAP on sperm motility

Semen samples (n = 5) for each set of experiments were layered on a pre-established two-step 90, 47% Percoll (Pharmacia, Uppsula, Sweden) gradient buffered with Ham's F10 medium (Sigma, Basel, Switzerland). Gradients were centrifuged for 20 min at 300 *g*. Spermatozoa present in the pellet were resuspended at 30 ± 10×10^6 spermatozoa/ml in incubation medium [Ham's F10 with 1% fetal calf serum (FCS; Gibco, Basel, Switzerland), 25 mM L-(+)-sodium lactate (Sigma), 0.33 mM sodium pyruvate (Sigma)]. Aliquot (200 µl) of spermatozoa suspension treated with or without SNP (0.25, 1.25, 2.5 mM; Sigma) or SNAP (0.012, 0.12, 0.6 mM; Alexis Corporation, Läufelfingen, Switzerland) or 0.12 mM SNAP preincubated at room temperature for 48 h were kept at room temperature under sterile conditions. Motility was assessed with a computer-assisted sperm motility analyser (Motility Analyzer; Mika Medical GmbH, Germany) after overnight incubation.

Percentages of rapid forward, non-rapid forward (local) and immotile spermatozoa were classified according to WHO (1987) guidelines. Straight line velocity (VSL) was obtained from all the motile spermatozoa.

A vitality test was performed using the eosin vital live–dead staining method (Berthelsen, 1981). All data represent the mean ± SE of the means of five different experiments.

Effect of L-NAME on sperm motility

Semen specimens (n = 7) were diluted 1:4 with incubation medium [Ham's F10 with 1% FCS, 25 mM L-(+)-sodium lactate, 0.33 mM sodium pyruvate] and centrifuged for 10 min at 300 *g*. Pellets containing spermatozoa and other cells present in the ejaculate were resuspended in 5 ml of incubation medium and recentrifuged for 10 min at 300 *g*. The final pellets were resuspended in incubation medium at 30 ± 10×10^6 spermatozoa/ml. Aliquots of sperm suspension (200 µl) were incubated under sterile conditions, at room temperature, in the absence (control) or presence of 0.150 mM L-NAME (Calbiochem, Läufelingen, Switzerland). Motility was assayed with a computer-assisted sperm motility analyser after 4 h incubation.

Nitrite–nitrate analysis

Seminal plasma were obtained from different subjects (n = 13) after centrifugation of the semen at 300 *g* for 10 min. Before nitrite–nitrate analysis, seminal plasma was recentrifuged at 8000 *g* for 15 min. Nitrite–nitrate concentrations were then measured using the Greiss reagent as described before (Green *et al.*, 1982; Ochoa *et al.* 1991). Briefly, seminal plasma aliquots (250 µl) were diluted with ultrapure water (500 µl; Seromed Biochrom, Berlin, Germany) and incubated at room temperature with 250 µl of substrate buffer (imidazole 0.1 mol/l, NADPH 210 µmol/l, flavine adenine dinucleotide 3.8 µmol/l; pH 7.6) in the presence of nitrate-reductase (*Aspergillus niger*, 70 IU/l; Boehringer Mannheim, Rotkreuz, Switzerland) for 45 min, to convert nitrate to nitrite. Total nitrite was then analysed by reacting the samples with Greiss reagent (1% sulfanilamide, 0.1% naphthaleneethylene diamine dihydrochloride in 5% H_3PO_4, Spectroquant; Merck, Darmstadt, Germany). Reacted samples were treated with 500 µl of trichloroacetic acid (20%), centrifuged for 15 min at 8000 *g* and the absorbence of the supernatant was measured spectrophotometrically at 520 nm. Amounts of nitrite–nitrate in seminal plasma were estimated from a standard curve of $NaNO_2$ obtained by enzymatic conversion of $NaNO_3$ (0–32 nmol/ml; Merck). The measured concentration was normalized to the number of 10^6 spermatozoa/ml.

Statistics

Comparison between the untreated and treated samples was made using Student's t-test and a P value of <0.05 was considered significant. Linear regression analysis and Spearman's rank correlation coefficient were used to determine the correlation between the concentrations of nitrite–nitrate/10^6 spermatozoa and the percentage of immotile spermatozoa; $P < 0.05$ was considered significant.

Results

Effects of chemically derived NO on sperm motility and viability

SNAP releases large amounts of NO spontaneously in aqueous media, whereas SNP releases NO either spontaneously or by intracellular catalysed reaction (Ignarro *et al.*, 1981; Marks *et al.*, 1991; Kowaluk *et al.*, 1992), although the exact mechanism still remains unclear. The effects of NO-generating compounds on sperm motility are shown in Figure 1A and B. Treatment of spermatozoa with SNAP or SNP significantly decreased the percentage of spermatozoa expressing rapid forward motility as well as an increase in the percentage of immotile spermatozoa in a concentration-dependent manner. The threshold concentration at which these NO donors had a significant effect on sperm motility was 0.12 mM for SNAP and 0.25 mM for SNP.

After vital staining we could observe that the profile of decreasing spermatozoa vitality in response to SNAP or SNP

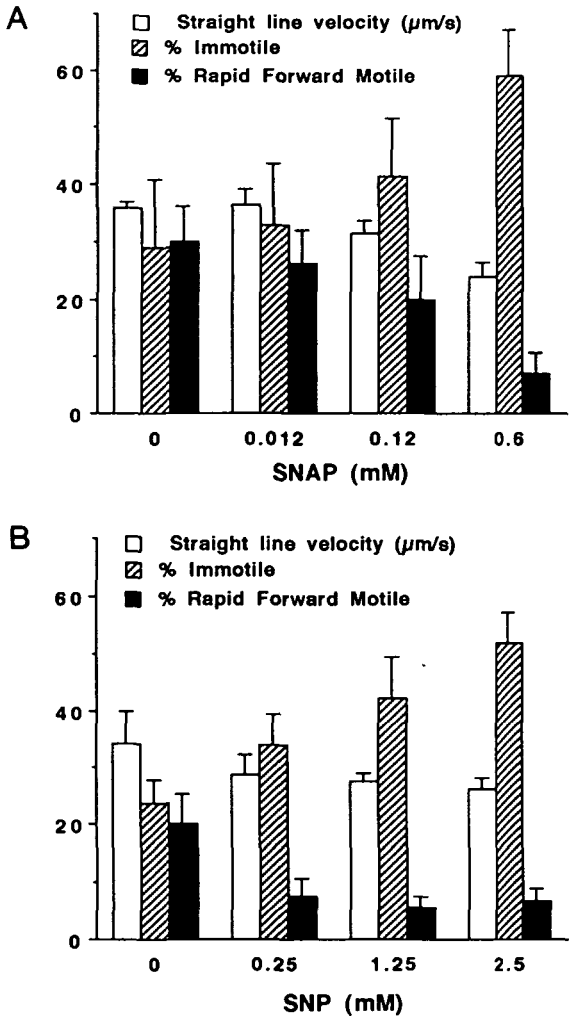

Figure 1. The effect of (**A**) *S*-nitroso-*N*-acetylpenicillamine (SNAP) and (**B**) sodium nitroprusside (SNP) on sperm motility. Treatment of human spermatozoa with SNAP or SNP significantly decreases rapid forward sperm motility (progression) in a concentration-dependent manner and also increases the number of immotile spermatozoa in a concentration-dependent manner. Furthermore, treatment of human spermotozoa with SNAP or SNP decreases straight line velocity (VSL) in a concentration-dependent manner.

was similar and parallel to the increase in the number of immotile spermatozoa.

Since the motility of spermatozoa has been characterized based on their velocity and pattern of movement, the effects of these compounds were also evaluated on the straight line velocity (VSL) of the total motile spermatozoa. Treatment of spermatozoa with SNAP or SNP reduced the VSL of the spermatozoa in a concentration dependent manner (Figure 1A and B). Of the two chemically dissimilar NO-generating compounds, SNAP, which generates more NO than SNP, was more potent in decreasing the VSL of the spermatozoa population.

To confirm that the inhibitory effect was due to NO and not due to the compounds themselves, the effect of freshly prepared SNAP on spermatozoa motility was compared with that of SNAP that had been preincubated for 48 h. No significant difference was observed between the untreated spermatozoa

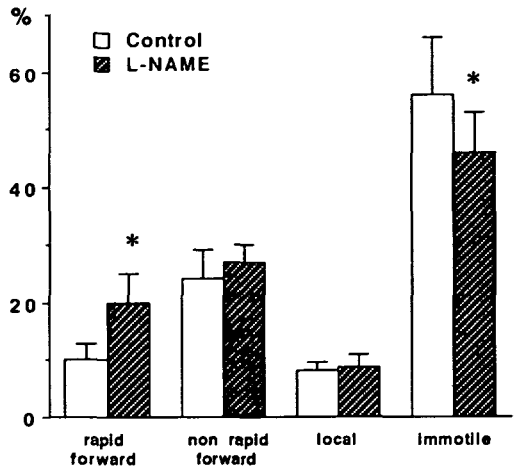

Figure 2. The effect of *N*-nitro-L-arginine-methyl-ester (L-NAME; 0.150 mM) on human sperm rapid forward motility, non-rapid forward motility, local motility and immotility. * = significantly different ($P < 0.05$). Control represents the motility of the washed semen but in the absence of L-NAME.

and the spermatozoa treated with 0.12 mM SNAP preincubated for 48 h.

Effect of a NO synthesis inhibitor (L-NAME) on spermatozoa motility and vitality

To study the effect of L-NAME on spermatozoa motility and vitality, we used a sperm preparation from which contaminating round cells (polymorphonuclear cells and germ cells) and bacteria were not eliminated. The effect of L-NAME is shown in Figure 2. When compared with untreated sperm samples, the rapid forward motility of the spermatozoa was significantly ($P < 0.05$) maintained in spermatozoa incubated for 4 h with 0.150 mM L-NAME. No significant changes were observed between the non-rapid forward motile spermatozoa as well as the local motile spermatozoa. Furthermore, the immotile spermatozoa population significantly decreased in the presence of L-NAME ($P < 0.05$).

Nitrite–nitrate concentrations in seminal plasma

The concentrations of nitrite–nitrate analysed in seminal plasma ($n = 13$) of different subjects ranged from 16 to 50 nmol/ml. A significant positive correlation ($r = 0.740$, $P < 0.01$; Spearman rank coefficient = 0.67, $P < 0.05$) was observed between seminal plasma concentrations of nitrite–nitrate (nmol/10^6 spermatozoa) and the percentage of immotile spermatozoa in the different semen samples (Figure 3).

Discussion

In the present study we provide the first evidence that NO can induce toxicity as well as decrease forward progressive motility in human spermatozoa. NO is known to be an important mediator in several physiological systems (Moncada *et al.*, 1991) and has beneficial biological effects when generated within physiological limits. When generated in large quantities for long periods, mainly during immunological reactions, NO is cytostatic and cytotoxic for invading microorganisms and

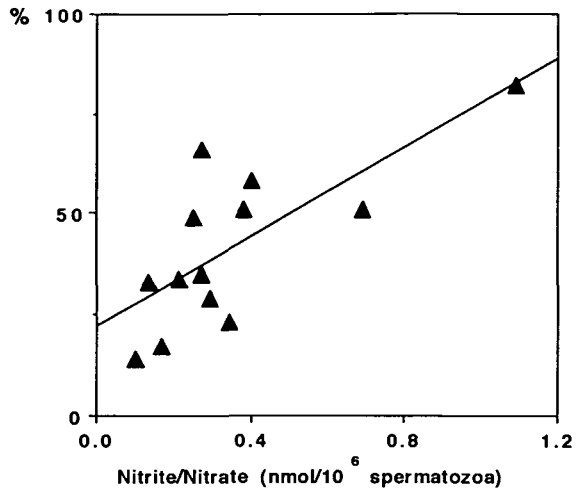

Figure 3. The correlation between the nitrite–nitrate concentrations (nmol/10^6 spermatozoa) in seminal plasma and the percentage of immotile spermatozoa ($r = 0.74$, $P < 0.01$; Spearman rank coefficient = 0.677, $P < 0.05$).

might also damage the tissues that generate it (Morris and Billiar, 1994; Nussler and Billiar, 1993). Together with the observation that clinical studies have shown that in humans, female and male, infection of the reproductive tract is associated with increased incidence of infertility (Haney *et al.*, 1983; Barratt *et al.*, 1990; Glezerman and Bartoov, 1993), we postulate that pathologically increased NO synthesis might, at least in part, be responsible for causing infertility in infected patients, possibly by causing spermatozoa death and by decreasing sperm motility.

In the present study this hypothesis was tested by studying the effects of NO generated from SNAP and SNP on sperm motility and viability. Furthermore, the possibility that inhibition of NO synthesis by L-NAME improves sperm motility and maintains sperm viability was also investigated.

Our observation that both SNP and SNAP decreased sperm motility in a concentration-dependent manner suggests that increased NO concentrations are toxic and decrease sperm viability. Indeed, the observed increases in the percentage of immotile spermatozoa were also accompanied by a parallel change in the percentage of dead spermatozoa. Compared to SNP, SNAP was more potent in decreasing sperm velocity. Differences in toxic potency between these compounds may be largely attributable to differences in the mechanisms by which they generate NO. SNAP releases NO spontaneously in aqueous media (Ignarro *et al.*, 1991; Marks *et al.*, 1991; Kowaluk *et al.*, 1992; Dubey, 1994), whereas the mechanism by which SNP generates NO still remains undefined. Previously it was assumed that SNP releases NO by spontaneous chemical degradation. However, more recently it has been shown that generation of NO from SNP requires an intracellular or membrane catalysed metabolic reaction (Ignarro *et al.*, 1981; Marks *et al.*, 1991; Kowulak *et al.*, 1992).

The observation that SNP and SNAP decrease sperm motility and viability could only suggest the involvement of NO. However, the possibility that the toxic effects were caused by the chemicals themselves could not be ruled out from these experiments. Hence, the role of NO in mediating the toxic effect was confirmed by comparing the effect of freshly prepared SNAP on sperm motility with that of SNAP that had been preincubated for 48 h. Our observation that sperm motility decreases in response to freshly prepared SNAP but not to preincubated SNAP further substantiates the evidence that the toxic effects were not due to the compound itself, but rather to a consequence of NO generation.

Since leukocytes and macrophages as well as bacteria are present in human seminal plasma (Anderson and Hill, 1988) and macrophages are capable of generating large amounts of NO during an immune reaction (Nussler and Billiar, 1993), we evaluated whether inhibition of NO synthesis by L-NAME improves sperm motility. This set of experiments was conducted without removing the contaminating cells, i.e. macrophages, leukocytes and bacteria. Our observation that motility and viability were significantly maintained in samples treated with L-NAME as compared to untreated spermatozoa strongly suggests that increased synthesis of NO causes sperm damage and may be also one of the factors contributing to infertility.

The notion that NO can induce sperm toxicity was further confirmed by our observation that in the native semen collected from different subjects the concentrations of nitrite–nitrate (stable metabolites of NO) positively correlated with the percentage of immotile spermatozoa. Furthermore, a correlation between increased nitrite–nitrate concentrations in seminal plasma and a decreased percentage of rapid progressive motile spermatozoa was also observed (data not shown). Hence, these results suggest that the increases in NO synthesis *in vivo* can also affect sperm viability.

It is well established that in several pathological situations related to systemic as well as non-systemic infection there is a continuous release of excessive amounts of NO (Morris and Billiar, 1994). The increase in NO synthesis is mediated via inflammatory factors such as endotoxins and cytokines capable of inducing NO synthesis activity (Moncada *et al.*, 1991). NO synthase activity is also localized in the oviduct (Rosselli *et al.*, 1994), the conduit for the gametes to reach the oocyte and the environment where fertilization occurs. Apart from the effort of the spermatozoa, the rhythmic contraction of the oviduct also promotes and contributes to the process of sperm movement and fertilization. Hence, pathological conditions such as infection of the oviduct or endometriosis might also result in generation of excessive amounts of NO, which not only would impair the normal contractility of the oviduct, but also damage the movement of the spermatozoa, causing sperm toxicity and consequently resulting in infertility.

In contrast to our observations, Hellstrom *et al.* (1994) recently reported that NO generated from SNP improves sperm motility and viability and suggested that these protective effects of NO are due to its capability to react with superoxide free radicals, which are known to inflict cell toxicity (Hill *et al.*, 1987; Plante *et al.*, 1994), and hence NO would neutralize the cytotoxic effects of the free radicals on spermatozoa. Although attractive, their explanation is flawed by the documented fact that within the biological system NO effectively reacts with superoxide anion (O^{2-}), forming a highly toxic peroxynitrite anion ($ONOO^-$) (Beckman *et al.*, 1990; Ischiropoulos *et al.*,

1992; Moro *et al.*, 1994). This powerful oxidant, when protonated, decomposes rapidly, resulting in the formation of $OH^{\bullet}$ and NO_2 both of which are tissue damaging agents (Beckman *et al.*, 1990). Another explanation to the difference between this study and that reported by Hellstrom *et al.* (Hellstrom *et al.*, 1994) could be the differences in concentration of SNP used and the duration of the incubation.

The mechanism by which NO induces its cytotoxic effect on human spermatozoa can only be speculated. Since NO is known to activate guanylate cyclase activity (Moncada *et al.*, 1991) and increase cGMP formation, it is possible that the increased cGMP synthesis could be inducing these effects. Indeed, it has been demonstrated that non-physiological concentrations of cGMP decrease human sperm motility (Chan *et al.*, 1990). Furthermore, it is conceivable that increased formation of NO and O_2^- under pathological conditions consequently leads to the formation of $ONOO^-$, which could directly interact with sperm membranes leading to sperm dysfunction and toxicity.

In conclusion, we provide evidence that NO can decrease sperm motility as well as viability, an effect that can be abrogated by inhibiting NO synthesis by L-NAME. Based on our finding it could be speculated that NO generated in non-physiological/excessive amounts by macrophages in response to invading organisms could be one of the factors which induce dysfunction in spermatozoa and consequently increase infertility. The exact mechanism of such a process needs to be further investigated.

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