Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition

Eilish T.Donnely1,3, Sheena E.M.Lewis1, William Thompson1 and Usha Chakravarthy2

1Department of Obstetrics and Gynaecology and 2Department of Ophthalmology, The Queen’s University of Belfast, Institute of Clinical Science, Grosvenor Road, Belfast BT12 6BJ, Northern Ireland, UK
3To whom correspondence should be addressed

Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes known as the nitric oxide synthases (NOS). We have recently shown a NOS similar to constitutive brain NOS (bNOS) and endothelial NOS (eNOS) to be present in spermatozoa. The aim of this study is to investigate NO production by human spermatozoa and the effects of stimulation and inhibition of NOS. This was carried out using the Iso–NO, an isolated NO meter and sensor, which provides rapid, accurate and direct measurements of NO. Semen samples with normozoospermic and asthenozoospermic profiles were prepared using a direct swim-up technique. Basal meter and sensor, which provides rapid, accurate and direct measurements of NO. Semen samples with normozoospermic and asthenozoospermic profiles were prepared using a direct swim-up technique. Basal concentrations of NO and stimulated NO production were measured after exposure to the calcium ionophore (A23187; 0.01–10 µM) a potent activator of constitutive NOS. NO production in human spermatozoa was significantly increased by the addition of A23187 30 seconds after stimulation. Furthermore, this response was greatly diminished by pre-incubating the samples with competitive inhibitors of L-arginine, the substrate for NOS, before treatment with calcium ionophore. In the presence of NG-nitro-L-arginine methyl ester (L-NAME), NG-nitro-L-arginine (L-NA) or NG-methyl-L-arginine (L-NMMA; all at 10 µM), NO production was inhibited with a rank order of potency L-NAME > L-NMMA > L-NA which is in accordance with the inhibition of an endothelial type of constitutive NOS.

Key words: human spermatozoa/Iso–NO probe/nitric oxide/NOS inhibitors

Introduction

Nitric oxide (NO) is a highly reactive free radical gas which over the past decade has been shown to possess an extraordinary variety of biological functions. It is a highly lipophilic molecule produced by cells in the brain, vascular endothelium and by phagocytes (Moncada and Higgs, 1993). NO is synthesized from a guanidino nitrogen atom of the essential amino acid L-arginine by a family of isoenzymes known as the nitric oxide synthases (NOS; Marletta, 1993) in the presence of oxygen and the electron donors nicotinic acid adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) with L-citrulline occurring as a by-product.

NO is an important modulator of cellular functions, is a potent vasodilator and neurotransmitter and has been implicated in numerous physiological, pharmacological and pathological processes (for review see Moncada et al., 1991). It is also an essential mediator in the female (Yallampalli et al., 1993; Rosselli et al., 1994) and male reproductive tracts (Adams et al., 1992). NO deficiency has been suggested as a contributory factor in pre-eclampsia (Fickling et al., 1993; Seligman et al., 1994), while the vasodilatory properties of NO are essential for cavernosal smooth muscle action in achieving penile erection (Burnett et al., 1992).

Despite being a free radical itself, NO can act as a free radical scavenger, inactivating (Alvarez et al., 1987; McCall et al., 1989) and even inhibiting production of superoxide anions (O2•−, Clancy et al., 1992) which cause lipid peroxida-

Materials and methods

Collection and preparation of samples

Semen samples were obtained from men with normozoospermic semen profiles (concentration >20×10^6/ml, progressive motility >50%) and asthenozoospermic (concentration >20×10^6/ml, progressive motility <50%; World Health Organization, 1992) individuals currently attending our subfertility clinic following a minimum of 3 days sexual abstinence. All samples were prepared using a direct swim-up technique (Aitken and Clarkson, 1988) in Biggers–Whitten–Whittingham medium (BWW; Biggers et al., 1971) supplemented...
with 0.3% albutein (Alpha Therapeutic UK Ltd., Norfolk, UK) and l-arginine (5 x 10^{-3} M; Sigma Chemical Company, Dorset, UK), the essential substrate for the production of NO. Semen (500 µl) was layered beneath an equal volume of supplemented BWW medium and incubated for 30 min at 37°C in 5% CO₂ at an angle of 45° to increase the surface area into which the spermatozoa could swim. The upper layer containing the motile spermatozoa was then carefully removed and centrifuged at 250 g for 10 min. The supernatant was discarded and the sperm pellet resuspended in a suitable volume of supplemented BWW medium. Post-swim-up count and motility were determined and all samples were analysed using 20 µm depth Microcell counting chambers (Conception Technologies Inc, La Jolla, CA, USA).

**Evaluation of sperm morphology**

Neat semen (5 µl) was evenly spread along the length of a microscope slide which had been thoroughly cleaned with 70% ethyl alcohol prior to use. The resulting thin smear was allowed to air dry for 20 min before staining which was carried out using a Diff-Quick staining kit (Baxter Dade Diagnostics AG, Dubingen, Switzerland; Kruger et al., 1987; Hall et al., 1995). Stained slides were air-dried for 30 min and coverslips were applied in a fume cupboard using Shandon synthetic tolouene-based mounting medium (Shandon Inc, Pittsburgh, PA, USA). Morphological assessment was performed at ×1000 magnification under oil-immersion. The strict criteria laid down by Kruger et al. (1987) were employed and at least 100 spermatozoa were counted on each slide. Results were expressed as the percentage of normal spermatozoa observed on each slide.

**Measurement of NO using an Iso–NO probe**

The NO sensor is an amperometric sensor with the probe located at the end of a stainless steel sleeve with a 2 mm diameter tip covered by a polymeric membrane. This gas-permeable, polymeric membrane separates the electrode from the sample to be analysed and eliminates any interference from dissolved species, allowing the Iso–NO probe to measure the NO concentration in the sample. The Iso–NO probe is connected to a multichannel recording device and the data collected using a software programme (Duo 18, World Precision Instruments, Sarasota, USA) running on an IBM compatible PC.

**Calibration of the Iso–NO probe**

The Iso–NO probe was calibrated by the chemical generation of NO from NaNO₂ in the presence of H₂SO₄ and KI. A standard series of dilutions of NaNO₂ ranging from 25 nM to 50 µM was prepared from a stock solution (1 µM). A known volume (3 ml) of 0.1 M H₂SO₄ in 0.1 M KI was placed in a small, wide-necked glass container in a constant temperature water bath and continuously stirred using a small magnetic flea. The Iso–NO probe tip was immersed to a depth of 2 mm in this solution and the baseline (mV) recorded. When the current had stabilized, several additions of a known volume of standard solution were made to 3 ml of fresh H₂SO₄/KI solution and the change in current recorded. The current deflections were measured on screen and the amount of NO generated by the sperm samples was calculated from the calibration curve.

**Treatment of spermatozoa with a calcium ionophore**

Individual post-swim-up sperm samples were aliquoted to form replicates and exposed to calcium ionophore (A23187) at concentrations of 0.01–10 µM. NO release following exposure to A23187 was measured at time intervals between 0 and 300 s.

**Treatment of spermatozoa with A23187 plus superoxide dismutase (SOD)**

Post-swim-up sperm samples were aliquoted to form replicates and exposed to A23187 at a concentration of 1.0 µM plus SOD at a final concentration of 40 IU/ml. NO release following exposure to A23187 plus SOD was measured after a 30 s incubation period.

**Treatment of spermatozoa with competitive inhibitors of NOS**

Sperm replicates were incubated with either N⁵⁻-nitro-l-arginine methyl ester (l-NAME), N⁵⁻-nitro-l-arginine (l-NA) or N⁵⁻-methyl-l-arginine (l-NMMA; Sigma Chemical Company, Dorset, UK) at a concentration of 10 mM for 2 h at 37°C. Each sperm sample was divided into three aliquots to form replicates, controls were included which had not been exposed to inhibitor.

**Treatment of spermatozoa with Methylene Blue (a NO scavenger)**

Post-swim-up samples were divided into aliquots to form replicates and incubated with a range of concentrations of Methylene Blue (10, 25, 50 and 100 µM). NO release was measured at time intervals of 0.5, 2, 5 and 10 min.

**Determination of sperm motility parameters**

Sperm motility parameters were analysed at a number of different time intervals following incubation with Methylene Blue and A23187. Spermatozoa were incubated with Methylene Blue at a concentration of either 10, 25, 50 or 100 µM for 0.5, 2, 5 or 10 min and with A23187 at a concentration of 0.01, 0.1, 1.0 or 10 µM for the same time intervals. Sperm motility parameters were measured using a Hamilton Thorn Integrated Visual Optical System (IVOS) sperm analyser (Version 10.7; Hamilton Thorn Research, Beverley, USA). The settings employed for analysis were from acquisition rate (Hz) 30; minimum contrast 7; minimum size 6; low-size gate, 0.4; high-gate size, 1.6; low-intensity gate, 0.4; high intensity gate, 1.6; magnification factor, 2.04. Cells were counted as progressively motile if the average path velocity (VAP) was >25 µm/s. The motility
Table I. Basal nitric oxide (NO) production in normozoospermic and asthenozoospermic samples and those with normal and poor morphology. Values are medians with interquartile ranges

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>NO production (nmoles NO/10^6 motile spermatozoa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermic samples</td>
<td>16.00</td>
</tr>
<tr>
<td>(&gt;50% progressive motility)</td>
<td>(7.45–35.50) (n = 24)</td>
</tr>
<tr>
<td>Asthenozoospermic samples</td>
<td>13.00</td>
</tr>
<tr>
<td>(&lt;50% progressive motility)</td>
<td>(3.80–23.00) (n = 37)</td>
</tr>
<tr>
<td>≥14% normal morphology</td>
<td>10.00</td>
</tr>
<tr>
<td>(&lt;14% normal morphology)</td>
<td>(3.80–24.00) (n = 23)</td>
</tr>
</tbody>
</table>

parameters measured were: progressive motility (i.e. those spermatozoa which exhibit an actual space-gain motility), straight line velocity (VSL; the straight line distance from beginning to end of a sperm track divided by the time taken), VAP, curvilinear velocity (VCL; a measure of the total distance travelled by a given spermatozoon divided by the time elapsed), sperm head movements, i.e. the amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation) and beat cross frequency (BCF; the frequency of the sperm head crossing the sperm average path), percentage motility (i.e. the number of spermatozoa which exhibit an actual space-gain motility), VAP, curvilinear velocity (VCL; a measure of the total distance travelled by a given spermatozoon divided by the time elapsed), sperm head movements, i.e. the amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation) and beat cross frequency (BCF; the frequency of the sperm head crossing the sperm average path). Percentage hyperactivation (HA) was also measured and was defined as VCL>100, LIN<65, ALH>7.5.

Statistical analysis

In view of the non-Gaussian distribution of data, the non-parametric Wilcoxon matched pairs test was employed. The analysis was carried out using Statistica 5.0 (Statsoft Europe, Hamburg, Germany).

Results

Basal synthesis of NO by spermatozoa

Aliquots (100 µl) of BWW media were added as a control to the H2SO4/KI solution in the vicinity of the probe and there was no detectable change in the baseline current which remained steady. Additions of 100 µl of sperm suspension caused an immediate but small deflection in the baseline current ranging from 0 to 3.8 mV. Intra-sample variation was extremely low (<5%) whereas there was considerable variation in the responses obtained from different samples. Basal release of NO by spermatozoa from normozoospermic samples (n = 24, Table I) was not significantly different from asthenozoospermic sperm samples (n = 37, Table I).

Basal NO production by spermatozoa as classified by morphology

As there was considerable variation in the responses obtained from different samples, the possible effect of sperm morphology on the high variability was considered. Thus sperm samples were classified into two distinct groups on the basis of their strict morphology results (Kruger et al., 1987) (i.e. group 1: ≥14% normal spermatozoa; n = 23, group 2: <14% normal spermatozoa; n = 38). No significant differences were observed in baseline NO concentrations (Table I).

Effect of exposure to calcium ionophore (A23187) on NO production

Samples incubated with 10 µM A23187 showed a significant increase (P ≤0.005) in NO production (NO burst) (Table II). NO synthesis peaked at 30 s post-stimulation and further incubation with the ionophore did not cause any increase in signal after this time point (Figure 1a), suggesting an ‘all or nothing’ response by spermatozoa to A23187. Measurements in subsequent experiments involving the use of varying concentrations of ionophore were therefore made at 30 s. In normozoospermic samples (n = 23) exposure to calcium ionophore at a concentration of 10 µM resulted in an immediate NO burst which was recorded at the working electrode. This increase ranged from a 50–100% increase in NO release and occurred at 30 s post-stimulation (P ≤0.005). NO synthesis by asthenozoospermic samples (n = 36) was also significantly increased (P ≤0.005) by exposure to 10 µM A23187 for 30 s (Table II). This increase in NO was 50–200% over basal values. Incubation of spermatozoa with lower concentrations of A23187 (0.01–1.0 µM) also caused a significant increase in NO production (P ≤0.005). These observed increases ranged from a 10–200% increase in NO release and again occurred at 30 s post-stimulation (Figure 1b).

Table II. Nitric oxide (NO) production in normozoospermic and asthenozoospermic samples after treatment with A23187 and NO synthesis inhibitors. Values are medians with interquartile ranges

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>NO production (% of basal value) a</th>
</tr>
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<tbody>
<tr>
<td>Normozoospermic samples</td>
<td></td>
</tr>
<tr>
<td>(&gt;50% progressive motility)</td>
<td></td>
</tr>
<tr>
<td>+ A23187 (10 µM)</td>
<td>143.75 b (87.5–200.00)</td>
</tr>
<tr>
<td>+ l-NAME (10 µM)</td>
<td>13.75 c (6.87–20.62)</td>
</tr>
<tr>
<td>+ A23187 (10 µM)</td>
<td>75.00 c (43.75–125.00)</td>
</tr>
<tr>
<td>+ l-NMMA (10 µM)</td>
<td>134.62 (2.50–33.00)</td>
</tr>
<tr>
<td>+ A23187 (10µM)</td>
<td>84.61 (78.75–93.75)</td>
</tr>
<tr>
<td>+ l-NA (10 µM)</td>
<td>19.23–253.85 (n = 5)</td>
</tr>
<tr>
<td>Asthenozoospermic samples</td>
<td></td>
</tr>
<tr>
<td>(&lt;50% progressive motility)</td>
<td></td>
</tr>
<tr>
<td>+ A23187 (10 µM)</td>
<td>203.85 b (92.30–469.92)</td>
</tr>
<tr>
<td>+ l-NAME (10 µM)</td>
<td>9.61 b (0–23.07)</td>
</tr>
<tr>
<td>+ A23187 (10 µM)</td>
<td>143.75 b (23.00–245.85)</td>
</tr>
<tr>
<td>+ l-NMMA (10 µM)</td>
<td>84.61 (78.75–93.75)</td>
</tr>
<tr>
<td>+ A23187 (10 µM)</td>
<td>19.23–253.85 (n = 8)</td>
</tr>
</tbody>
</table>

aAbsolute control values were 16 and 13 nmoles NO/10^6 motile spermatozoa for normozoospermic and asthenozoospermic samples respectively (see Table I).

bSignificantly greater than corresponding control value (P ≤0.05; Wilcoxon matched pairs test).

bSignificantly lower than corresponding control value (P >0.005; Wilcoxon matched pairs test).

Exposure of spermatozoa to A23187 plus SOD

Treatment of spermatozoa with 1.0 µM A23187 plus 40 IU/ml SOD caused an even greater increase in NO production than that recorded for samples treated with 1.0 µM A23187 alone (Figure 1b). Exposure of spermatozoa to A23187 plus SOD caused NO concentrations to rise to greater than 3.5 times the basal value which was significantly greater than the 98% increase observed after incubation with 1.0 µM A23187 (P ≤0.005).
Figure 1. (a) Effects of incubation with A23187 on nitric oxide (NO) production by human spermatozoa showing control values (–○–) and following treatment with 10 µM A23187 (–■–) over a 5 min period. (b) Effects of various concentrations of A23187 on NO production by spermatozoa. Values are medians (□) with interquartile ranges (25–75%) and outliers (◦) and are expressed as a percentage of the basal value. **Significantly lower than corresponding control value (P < 0.01), ***Significantly lower than corresponding control value (P < 0.005), †Significantly different from other treatment groups (P < 0.01; Wilcoxon matched pairs test).

Figure 2. The effect of calcium ionophore (A23187) on sperm motility over a 10 min period showing control values (–○–) and following incubation with 0.01 (–■–), 0.1 (–□–), 1.0 (–△–) and 10 µM (–□–) A23187. Values are medians.
**Direct measurement of nitric oxide in spermatozoa**

**Figure 3.** Effects of arginine analogues on nitric oxide (NO) production in (a) normozoospermic and (b) asthenozoospermic samples. Values are medians (□) with interquartile ranges (25–75%) and are expressed as a percentage of the basal value. *Significantly lower than corresponding control value (P < 0.05; Wilcoxon matched pairs test). Absolute control values are shown in Table II. L-NAME = N^G-nitro-L-arginine methyl ester; L-NMMA = N^G-methyl-L-arginine; L-NA = N^G-nitro-L-arginine.

**Effect of incubation with A23187 on sperm motility**

A23187 at concentrations of 1.0 and 10 µM caused significant reductions in sperm progressive motility after just 30 s incubation (P < 0.005; Figure 2). Decreases in VAP, VCL and VSL were recorded after 1 min incubation with A23187 while LIN was significantly reduced after 5 min (data not shown). The lower concentrations of A23187 used (0.01 and 0.1 µM) did not appear to have any significant effect on progressive motility until after 5 min incubation at 37°C (Figure 2; P < 0.05), at which time VAP, VCL and VSL were also significantly reduced in these samples (P < 0.05). Head movements (i.e. ALH and BCF) appeared to be unaffected by incubation with A23187 and there was no significant change in percentage hyperactivation following any of the treatments with this calcium ionophore (data not shown).

**Effect of incubation with substrate analogues of arginine**

The basal NO signal obtained from normozoospermic samples was significantly reduced (P < 0.05) following 2 h of incubation with either L-NAME or L-NMMA (Figure 3a; Table II). L-NA proved to be the least effective as basal NO concentrations were not significantly reduced following 2 h incubation with this arginine analogue (Figure 3a; Table II). Similarly, basal NO production by asthenozoospermic samples was significantly reduced by incubation with the same two arginine analogues (Figure 3b). Exposure of normozoospermic and asthenozoospermic samples to A23187 in the presence of each of these arginine analogues produced an NO burst which was considerably lower than that observed in samples incubated without the substrate analogues in the case of L-NAME (P < 0.05, Table II) but was only significant for normozoospermic samples in the case of L-NMMA (P < 0.005, Table II).

**Effect of incubation with Methylene Blue on NO production**

Samples incubated with 10–100 µM Methylene Blue showed a significant decrease (P < 0.005) in NO production (Figure 4). These inhibitory responses appeared to be time- and dose-dependent. Exposure of spermatozoa to a concentration of 10 µM for 0.5 min showed a decrease of >20% in NO concentrations while samples incubated with 100 µM Methylene Blue for the same time period had NO concentrations that were <50% of basal values.

**Effect of incubation with Methylene Blue (a NO scavenger) on sperm motility**

Methylene Blue inhibited sperm progressive motility and caused reductions in VAP, VCL, VSL (Figure 5). ALH and.
LIN were also decreased, although BCF was unaffected (data not shown). These inhibitory responses were again time- and dose-dependent with a low concentration of Methylene Blue (i.e. 10 µM) having lesser effects on sperm motility parameters than concentrations of >10 µM, and the observed reductions in VAP, VCL, VSL and ALH were less marked after 2 min incubation than after a 10 min incubation period with this NO scavenger. Sperm viability remained unchanged following treatment with Methylene Blue (data not shown), illustrating that it was not toxic to the spermatozoa, despite the decrease in motility.

Discussion

NO is a molecule of great biological significance and has long been considered to play an important role in sperm physiology (Aitken and Fisher, 1994). Our recent study provided compelling evidence to show that the spermatozoon itself is the source of NO and that constitutive NOS is present in two isoforms similar to those present in both endothelial (ecNOS) and brain (bNOS) cells (Lewis et al., 1996). NO itself is a highly reactive, short-lived, lipophilic molecule with a half-life of just a few seconds (Ignarro et al., 1989) which makes it difficult to measure. In this study the use of a sensitive, isolated NO meter and sensor (Iso–NO) allowed us to directly measure minute quantities of NO in human spermatozoa from semen samples as it was produced.

There are two schools of thought as to the role of NO in the maintenance of sperm motility. Detrimental inhibitory effects have been reported by Rosselli et al. (1995) and Weinberg et al. (1995) when millimolar concentrations of exogenous NO donors were added to sperm samples. The present study showed that basal release of NO by spermatozoa from normozoospermic samples tended to be greater than that from asthenozoospermic samples, suggesting a physiological and beneficial role for endogenous NO in the preservation of sperm motility. This is in accordance with our previous observations that normozoospermic spermatozoa express more NOS and generate more nitrite than asthenozoospermic spermatozoa (Lewis et al., 1996). These observations are also in agreement with those of Hellstrom et al. (1994) who have shown that exogenous treatment of human spermatozoa with sodium nitroprusside was associated with enhanced post-thaw motility and viability while reducing lipid peroxidative damage to cellular membranes.

This study has demonstrated that there was little difference in basal NO production between samples with a good (>14% normal) or poor (<14% normal) morphology. This suggests that poor quality semen is not associated with elevated basal NO production and that the effects of reactive oxygen species (ROS) on sperm membranes, which depress sperm function (Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992) are not mediated by basal NO per se.

Calcium is an important regulator of sperm physiology and is involved in several stages of sperm maturation, with its influence on sperm motility, capacitation and acrosome reaction being the subjects of numerous studies (Hong et al., 1984; Lindemann et al., 1987; Prien et al., 1992). A23187 is a divalent calcium ionophore which causes an influx of intracellular Ca²⁺ (Babcock et al., 1976) and induces hyperactivated motility in

Figure 5. The effect of Methylene Blue on sperm motility over a 10 min period showing control values(–○–) and following treatment with 10 (–■–), 25 (–□–), 50 (–▲–) and 100 µM (–△–) Methylene Blue. Values are medians.
spermatozoa (Pilikian et al., 1991). The likely mechanism of action is that cytoplasmic Ca\(^{2+}\) activates the Ca\(^{2+}\)-dependent adenylate cyclase, resulting in an increase in cAMP which is responsible for the accelerated flagellar beating observed in hyperactivation (Feng et al., 1988). The increase in intracellular Ca\(^{2+}\) induced by A23187 also provokes a reaction which is structurally identical to the physiological acrosome reaction in spermatozoa (Russell et al., 1979; Aitken et al., 1984). Constitutive NOS (cNOS) which is present in human spermatozoa is known to be Ca\(^{2+}/\)calmodulin dependent (Forsterman et al., 1987). Hence, the influx of Ca\(^{2+}\) induced by A23187 is likely to cause an immediate up-regulation of cNOS activity. This is produced by optimal use of calcium binding sites, rather than by cNOS synthesis since spermatozoa contain few or no mechanisms for transcription, and results in an increase in NO production. Our results substantiate this, as an immediate increase in basal concentrations of NO was observed for both normozoospermic and asthenozoospermic samples on the addition of A23187 for just 30 s.

We found that the optimal incubation period for maximum NO synthesis after A23187 treatment was just 30 s and longer incubation periods resulted in the measurement of NO concentrations which were similar to basal values. Recent findings (Griveau et al., 1995) show that the addition of A23187 to spermatozoa for longer periods of 1 h increased the production of superoxide by spermatozoa 4–5-fold. NO synthesis is known to decrease superoxide (O\(_2^•\)-) formation, thus excluding the required substrate for the powerful oxidant peroxynitrite (ONOO\(^{-}\); Clancy et al., 1992). However, when excess amounts of O\(_2^•\)- are present it will interact with NO, favouring the generation of hydroxyperoxyl radicals or peroxynitrite which will decompose to produce the hydroxyl radical (Halliwell and Gutteridge, 1992). In order to investigate the proposed role of O\(_2^•\)- in inhibiting the increased production of NO, via the formation of ONOO\(^{-}\), we incubated spermatozoa with A23187 + superoxide dismutase (SOD) and measured NO production directly. We found that the addition of SOD along with A23187 caused a significant additional increase in NO synthesis due to the decreased presence of O\(_2^•\)- which reduced the possibility of interaction of this molecule with NO. Therefore the presence of O\(_2^•\)-, e.g. in the testis as a result of inflammation, is likely to significantly reduce NO values and have a direct effect on sperm motility—a situation which may arise in asthenozoospermic individuals.

In this study, A23187 inhibited CASA sperm motility parameters with time. This again concurs with our belief that only basal NO in tightly regulated concentrations is beneficial to sperm motility. The NO burst (induced by the addition of A23187) caused sustained reductions in sperm motility over a period of 10 min, despite our findings that NO concentrations had returned to basal values after 2 min incubation with A23187. So, although the increase in NO is only transient it is sufficient to initiate the cascade of events leading to peroxidative damage, one of the first indicators of which is a decrease in sperm motility. This may account for the adverse effects of exogenous NO donors at mM concentrations on sperm motility observed by Rosselli et al. (1995) and Weinberg et al. (1995).

The influence of competitive inhibitors of NO production (the arginine analogues L-NAME, L-NMMA, L-NA) was analysed. These analogues are antagonists of NO generation as they compete with L-arginine for a limited number of binding sites on the enzyme NOS. L-NAME has been found to significantly inhibit nitrate accumulation in a murine breast cancer cell line (Cendan et al., 1996) and is known to abolish the beneficial effects of bradykinin on ischaemic hearts during post-ischaemic myocardial recovery (Zhu et al., 1995). L-NMMA was the most potent of the inhibitors studied while L-NA was found to be least effective at blocking NO generation. L-NMMA is known to inhibit NO generation by vascular endothelial cells (Palmer et al., 1988; Sakuma et al., 1988) and the guanidino-substituted analogue l-NA produces a similar L-arginine-reversible inhibition of endothelium-dependent vasodilation (Moore et al., 1990). In cerebellar supernatant preparations L-NA is a more potent inhibitor of NOS activity then l-NMMA (Dwyer et al., 1991). In rabbit aortic tissue, l-NA was found to be six times more effective at blocking vasodilation than l-NMMA (Moore et al., 1990) while other studies have found l-NA to have 100-fold greater potency than l-NMMA in blocking endothelial cell NO synthesis (Gross et al., 1990). In contrast l-NA is substantially less effective than L-NMMA at inhibiting NO generation by macrophage (Gross et al., 1990). l-NMMA has been reported to be more effective than l-NA in reducing basal NO release in canine femoral circulation (Kirkeboen et al., 1992). NO production by both normozoospermic and asthenozoospermic samples was inhibited by arginine analogues with a rank order of potency L-NAME > L-NMMA > l-NA, suggesting that the isoform of NOS present in human spermatozoa is similar to an endothelial-type constitutive NOS.

Methylene Blue (a NO scavenger) inhibited sperm motility (but not viability) and NO production markedly within 0.5 min. This would suggest that it acted by rapidly reducing the endogenous NO concentrations available to spermatozoa thereby reducing motility, rather than by a more gradual toxic effect. This agrees with our previous study (Lewis et al., 1996) where motility parameters were also consistently depressed by the addition of L-NAME. Human seminal plasma is known to inhibit bNOS activity (Schaad et al., 1996) and it has also been shown that seminal plasma decreases sperm motility (de Lamirande et al., 1984), possibly due to the inhibition of bNOS. This again suggests an important physiological role for endogenous NO in the maintenance of sperm motility. In contrast, Weinberg et al. (1995) found that incubation of spermatozoa with haemoglobin (a quencher of NO) maintained sperm motility when added in the presence of 3-morpholininosyndonimine (SIN-1; another donor of NO).

There has recently been considerable debate as to the role of NO in hyperactivation of human spermatozoa and there have been several studies with conflicting results. Hyperactivated sperm motility, i.e. high amplitude flagellar waves with an accompanying increase in velocity and reduction in progressive motility, has been described in detail in numerous studies (Mortimer et al., 1983; Burkman, 1984). An investigation involving mouse spermatozoa (Herrero et al., 1994) found that adding sodium nitroprusside (SNP; a classical donor of NO) to spermatozoa at a concentration of 300 µM caused a decrease in motility and a significant increase in hyperactivated cells. While we agree...
with their decreased motility results, we did not observe any change in hyperactivation. Our results concur with those of Zini et al. (1995) who found that hyperactivation remained unchanged even with the addition of mM concentrations of NO-releasing compounds.

In summary, NO is produced directly by human spermatozoa. Endogenous NO appears to have an important role in the maintenance of sperm motility, if carefully regulated. Any increase in endogenous NO, such as that induced by A23187, inhibits sperm motility. Thus, it may be useful as another indicator of fertility potential and could have numerous clinical applications in the field of assisted reproduction.

References


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