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Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility

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The aim of this study was to investigate the presence of nitric oxide synthase (NOS) and the production of nitric oxide (NO) by human spermatozoa. Immunoreactivity was examined using a polyclonal antibody raised against porcine cerebellar nitric oxide synthase and monoclonal endothelial (eNOS) and brain (bNOS) antibodies. Using each antibody, NOS was observed localized in the head and midpiece regions of the spermatozoon. Immunofluorescence observed for eNOS and bNOS was more intense in normozoospermic samples. Sperm motility was assessed by computer-assisted semen analysis (CASA) in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME; 10^{-5}M), an NO synthesis inhibitor or tumour necrosis factor (TNF)-α (20 IU/ml), a superoxide inducer. In the presence of L-NAME, percentage progressive motility, average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) were significantly reduced after 30 min. Sperm viability was not decreased by TNFα or L-NAME. The accumulation of nitrite (the stable end-product of the NOS/NO pathway) by spermatozoa was measured using the Griess reaction. After 8 h, nitrite concentrations were lower in asthenozoospermic compared to normozoospermic samples. In the presence of TNFα, nitrite accumulation was significantly reduced in normozoospermic samples. We conclude that NOS is present in human spermatozoa and that eNOS and bNOS are abundant in normozoospermic samples. Nitric oxide (at endogenous concentrations) appears to be necessary for adequate sperm motility.

Key words: L-NAME/nitric oxide synthase/nitric oxide/sperm motility

Introduction

The discovery of nitric oxide as a mammalian metabolic intermediate has exploded into a developing area of research. Nitric oxide is involved in smooth muscle relaxation (Palmer *et al.*, 1987), vasodilation (Calver *et al.*, 1993), immune regulation (Hibbs, 1991) and neurotransmission (Garthwaite *et al.*, 1988). It is also important as a mediator in the female (Yallampalli *et al.*, 1993; Rosselli *et al.*, 1994) and male (Adams *et al.*, 1992) reproductive tracts. Nitric oxide is a free radical but it can inactivate (Alvarez *et al.*, 1987; McCall *et al.*, 1989) and even inhibit the production (Clancy *et al.*, 1992) of superoxide anions which cause lipid peroxidative damage, particularly in spermatozoa. Nitric oxide is also protected by antioxidants (Vallance and Collier, 1994), suggesting a beneficial role.

Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS). Two isoforms of constitutive NOS have been identified: endothelial or eNOS and neural/brain or bNOS. Both enzymes are encoded by unique genes (Sessa *et al.*, 1993), are calcium/calmodulin dependent and are present under basal conditions (Moncada *et al.*, 1991). These isoforms can be detected using indirect immunocytochemistry. Nitric oxide synthase has been demonstrated in epididymis, vas deferens and coagulating gland (Chamness *et al.*, 1995) of the rat but not, to our knowledge, in mature spermatozoa. In this study we provide evidence for the presence of NOS in its different constitutive isoforms in human spermatozoa using polyclonal and monoclonal antibodies.

The effects of tumour necrosis factor (TNF)-α and N^G-nitro-L-arginine methyl ester (L-NAME) on sperm motility were also assessed. TNFα is a strong inducer of superoxide anion which can deactivate nitric oxide (NO) when present in excess. L-NAME is an analogue of L-arginine which competitively blocks the action of the enzyme NOS.

A number of recent in-vitro studies (Rosselli *et al.*, 1995; Weinberg *et al.*, 1995) have examined the effects of exogenous NO donors on sperm function. At millimolar concentrations, NO donors have been shown to have adverse effects which may not be reflective of in-vivo or even routine in-vitro conditions.

Materials and methods

Semen samples were obtained from men of recently proven fertility with normozoospermic semen profiles (sperm number $>20\times10^6$/ml, progressive motility >50%, normal morphology >30%) and from normozoospermic and asthenozoospermic (sperm number $>20\times10^6$/ml, progressive motility <50%, normal morphology >30%; World Health Organization, 1992) individuals currently attending our subfertility clinic. All samples were prepared using a direct swim-up technique (Aitken and Clarkson, 1988) in Biggers–Whitten–

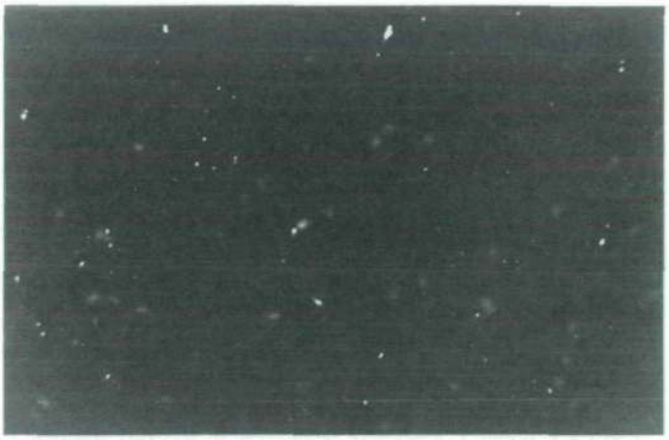

Figure 2. Binding of a polyclonal anti-nitric oxide synthase (NOS) antibody in an asthenozoospermic sample. There was little detectable fluorescence.

Figure 3. Normozoospermic sample exposed to a polyclonal anti-tubulin showing diffuse immunoreactivity.

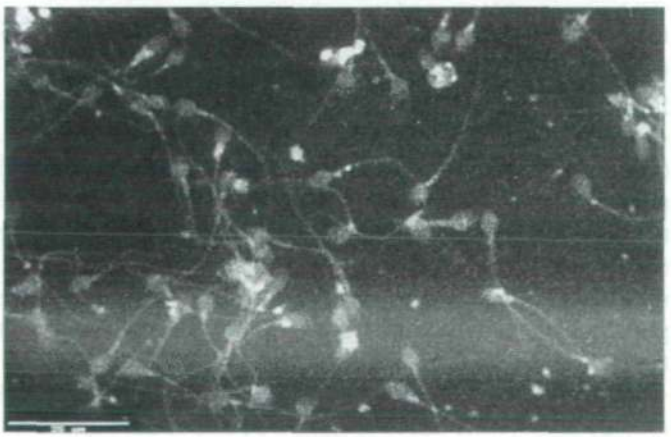

Figure 4. Binding of a monoclonal antibody to brain nitric oxide synthase (bNOS) in a normozoospermic sample, as imaged using confocal microscopy. Fluorescence appears marked in the head and midpiece areas.

The absence of non-specific staining was confirmed by the complete lack of fluorescence in pre-absorbed control samples.

Discussion

For many years the capacity of spermatozoa to produce reactive oxygen species (ROS) has been recognized and superoxide

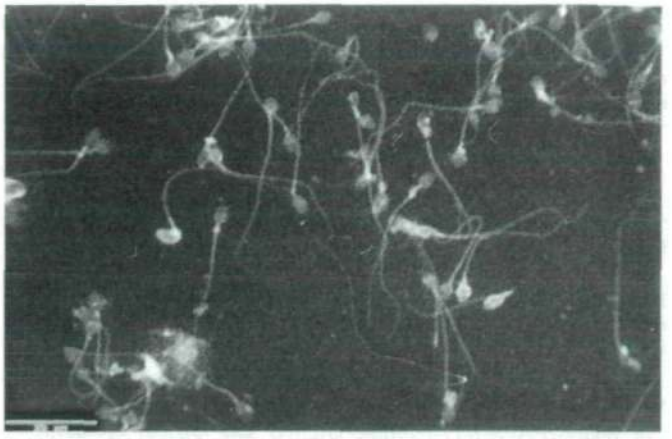

Figure 5. Binding of monoclonal antibody to endothelial nitric oxide synthase (eNOS) in the same normozoospermic sample as in Figure 4, imaged using confocal microscopy. Fluorescence is again marked in the head and midpiece areas and appears to be more intense than that observed for brain nitric oxide synthase (bNOS).

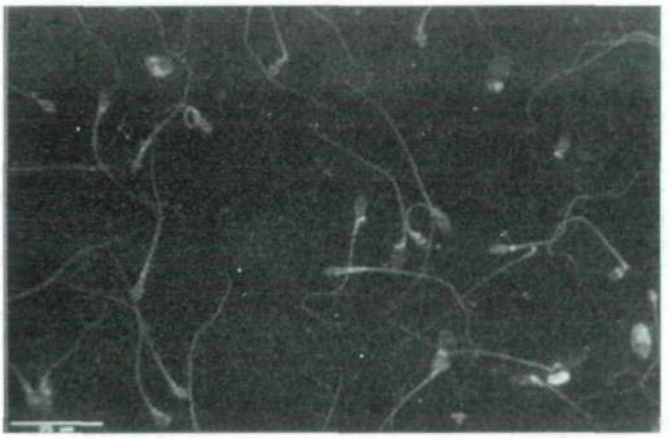

Figure 6. Binding of monoclonal antibody to endothelial nitric oxide synthase (eNOS) in an asthenozoospermic sample, as imaged using confocal microscopy. Fluorescence is significantly less intense than that observed for the normozoospermic sample.

anions, hydroxyl radicals and NO have been either shown or suggested to play a role in sperm physiology (Aitken and Fisher, 1994). Although ROS may be necessary for sperm capacitation (de Lamirande and Gagnon, 1995), they themselves can induce peroxidative damage and a subsequent reduction in sperm motility. NO, despite being a free radical itself, can act as an 'acceptor' and inactivate superoxide. Thus, a protective role for NO has been proposed in many situations including ischaemic reperfusion injury. The presence of NOS in cells is taken to represent an ability to synthesize NO and the enzyme has been demonstrated in testicular tissue, but as yet there has been no evidence to suggest that the spermatozoon itself may be the source of NO. The present study has provided compelling evidence to show that the spermatozoa are the source of NO and that constitutive NOS is present in two isoforms similar to those present in both eNOS and bNOS cells (Figures 4 and 5). This suggests a physiological role for NO in maintenance of sperm motility.

In the present study, nitrite, the stable end-product of the NOS/NO pathway, was detected in the medium after incubation

with spermatozoa for 30 min and 8 h. The initial leukocyte concentration was $<1\times10^6$/ml in each sample and the final concentration was lower again. Hence, it is unlikely that a significant contribution to nitrite concentrations could have come from neutrophil cells which are the only other likely contaminating cell type capable of NO production. Also, the continued accumulation of nitrite in the incubation medium over a period of several hours (Table II) provided additional evidence that the spermatozoa themselves were the source of NO. A more sensitive method of NO detection is currently being used to investigate NO concentrations in spermatozoa. Here, we have also shown the localization of NOS to the head and midpiece of the spermatozoon by immunostaining (Figures 1, 4 and 5). Thus, the results imply the presence of NOS in spermatozoa which is most likely to be a constitutive isoform by virtue of its presence under basal, unstimulated conditions. The presence of immunostaining to both anti-eNOS and bNOS suggests that the isoforms of NOS seen in spermatozoa share similar topes to those seen in brain and endothelium.

There are two conflicting opinions on the effects of NO on sperm motility. Hellstrom *et al.* (1994) found that NO improves sperm motility, whereas Rosselli *et al.* (1995) and Weinberg *et al.* (1995) observed a NO-induced inhibition of motility. Our results support the former belief. Our explanation for the disparity of results is that in the studies by Weinberg *et al.* (1995) and Rosselli *et al.* (1995) millimolar concentrations of exogenous NO donors have been added to sperm preparations, possibly resulting in a burst of high concentrations of NO which results in damage. In our study we measured the nitric oxide produced by the spermatozoa themselves with time, and have shown that the basal concentration of NO is lower (Tables I and II, range 1.6–2.9 $\mu M/10^6$ viable spermatozoa). Hence, we would suggest that the concentrations of nitric oxide must be regulated closely to be beneficial to spermatozoa.

Human seminal plasma has recently been shown to inhibit bNOS activity (Schaad *et al.*, 1996). Seminal plasma can also inhibit sperm motility (de Lamirande *et al.*, 1984) so this inhibition of motility may be mediated by an inhibition of bNOS and hence NO production. Here, motility parameters (Table I) were consistently depressed by the addition of the NO synthesis inhibitor L-NAME. In addition, in asthenozoospermic samples classified by their motility, NO concentrations are lower than in samples with acceptable motility by World Health Organization standards (WHO, 1992). These results substantiate our claim that NO is necessary for adequate motility. The NO produced by spermatozoa (per 10^6 viable cells) is similar to that produced by somatic cells such as retinal microvascular endothelial cells (Chakravarthy *et al.*, 1994). In the study by Rosselli *et al.* (1995), NO values in nmolar range were observed in seminal plasma. These results may be explained by the presence of significant antioxidants in seminal plasma (Lewis *et al.*, 1995) which would alter the concentrations of NO, although in our experience, such low concentrations cannot be accurately determined using the Griess reaction, which is able to measure nitrite concentrations only in the micromolar range. Table II shows that the endogenous production by both normozoospermic and asthenozoospermic human spermatozoa is at the lowest end of the sensitivity of this assay. In addition, although the variability of nitrite production within sperm samples is very low, the between sample variation is wide. We are currently investigating a more sensitive method for the measurement of NO production by human spermatozoa.

Normozoospermic samples showed significantly greater concentrations of nitrite than those from asthenozoospermic individuals, implying that NO can improve or maintain sperm motility. These results are in accord with the observations of Hellstrom *et al.* (1994) who have shown that exogenous treatment of human spermatozoa with sodium nitroprusside (a donor of NO at nanomolar concentrations) was associated with enhanced post-thaw motility and viability while reducing lipid peroxidative damage to cellular membranes.

It is noteworthy that, in normozoospermic samples, treatment with TNFα caused a reduction in the accumulation of nitrites in the medium of incubation. Under normal conditions, NO interacts with molecular O_2 to produce nitrites and nitrates which can be detected in solution by the Griess reaction. TNFα is a potent inducer of the superoxide anion. NO synthesis has recently been shown to decrease superoxide formation, thereby eliminating the necessary substrate for the potent oxidant peroxynitrate (ONNO-; Clancy *et al.*, 1992) and may be the cause of the reduction in lipid peroxidation in sodium nitroprusside-treated sperm samples (Hellstrom *et al.*, 1994). However, when superoxide is produced in excess (as occurs after stimulation with TNFα) the interaction between NO and O^{2-} favours the formation of hydroxyperoxyl radicals or peroxynitrite which decompose to produce the hydroxyl radical (OH) and initiate peroxidation in inherently susceptible sperm membranes (Halliwell and Gutteridge, 1992). This may explain the findings of Buch *et al.* (1994) where TNFα was shown to cause a massive increase in lipid peroxidation in spermatozoa. This shunting of NO into an alternative pathway could explain the reduction in nitrite accumulation within the incubation medium which was seen in TNFα-stimulated samples in the present study. It is therefore possible that the quenching of NO by superoxide generated as a consequence of inflammatory disease of the testis or epididymis is responsible for the reduced sperm motility observed in asthenozoospermia.

Sperm progressive motility also correlates with superoxide dismutase (SOD) activity (Kurpisz *et al.*, 1996). As SOD reduces superoxide anion activity in the seminal plasma, this would allow nitric oxide to predominate and sperm motility to be enhanced. A more detailed understanding of the interactions of ROS is particularly pertinent in view of the findings of Sukcharoen *et al.* (1996) that the incidence of ROS is highly predictive of fertilization potential *in vitro*.

It is also known that constitutive isoforms of NOS are down-regulated in the presence of cytokines (Lamas *et al.*, 1991). It is however, unlikely that transcriptional down-regulation of NOS could have played a role in TNFα-induced reduction in nitrite production as spermatozoa contain few or no mechanisms for transcription.

In summary, NO appears to have an important role in the maintenance of sperm motility. Thus, it may be useful as another indicator of fertility potential. Studies are under way

to further elucidate the isoforms of NOS active in human spermatozoa and the biochemical functions of NO in these cells.

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