Detection of superoxide anion generation by equine spermatozoa

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Objective—To identify the generation of the superoxide anion by equine spermatozoa.

Sample Population—Multiple ejaculates collected from 3 Thoroughbred stallions.

Procedures—Induced superoxide production by reduced nicotinamide adenine dinucleotides (NAD[P]H; ie, reduced nicotinamide adenine dinucleotide [NADH] and reduced nicotinamide adenine dinucleotide phosphate [NADPH]) was measured by use of a nitroblue tetrazolium (NBT) reduction assay on whole spermatozoa and a cytochrome c reduction assay on isolated membrane fractions of spermatozoa. Localization of superoxide generation was determined by use of NBT cytochemistry.

Results—A dose-dependent increase in NBT reduction was found in the presence of NADPH, which was inhibited by superoxide dismutase (SOD). The flavoprotein inhibitor, diphenyleneiodonium (DPI; 5 or 15µM), significantly decreased NBT reduction. Cytochrome c reduction by plasma membranes of spermatozoa was significantly higher in the presence of NADPH than in its absence. Cytochemical staining of equine spermatozoa in the presence of NADPH and NADH revealed diaphorase labeling in the spermatozoon midpiece and head. This staining was inhibited by DPI and SOD.

Conclusions and Clinical Relevance—Results of our study indicate that superoxide generation is associated with a membrane-associated NAD(P)H oxidase present in equine spermatozoa, although mitochondrial generation of superoxide is also detected. This oxidase may play a role in cell signaling or may also contribute to cytopathic effects associated with oxidative stress in equine spermatozoa. (Am J Vet Res 2006;67:701–706)

Increasing evidence exists that ROS serve as physiologically relevant signaling molecules that control normal function of spermatozoa. Several laboratories have shown that spermatozoon capacitation, hyperactivation, acrosomal exocytosis, nuclear condensation, and mitochondrial stability are redox-regulated events. For example, the addition of small amounts of hydrogen peroxide promote capacitation and acrosome reaction while the presence of the enzyme scavenger, catalase, inhibits capacitation. On the other hand, the adverse effects of ROS are well described in male gametes. Excessive ROS generation can overwhelm protective mechanisms, and oxidative damage is an important factor in disruption of spermatozoon function. High concentrations of hydrogen peroxide induce lipid peroxidation and result in cell death. However, seminal plasma is well endowed with an antioxidant defense mechanism to protect spermatozoa and compensate for the low concentration of free-radical scavenging enzymes in spermatozoa.

The primary ROS generated by equine spermatozoa appears to be the superoxide anion, which is rapidly converted to hydrogen peroxide. The mechanisms responsible for the production of ROS by spermatozoa remain controversial. Reactive oxygen species can be generated as a consequence of electron leakage from complex I and II of the mitochondrial electron transport chain, and this source of ROS has been proposed to be important in oxidative damage to spermatozoa. In addition to mitochondrial sources, an enzymatic system for ROS generation located in the plasma membranes of spermatozoa that uses NAD(P)H as a substrate via an NAD(P)H-dependent oxidase has been suggested by several laboratories, although the identity of this enzyme has not yet been determined. The objective of the study reported here was to characterize NAD(P)H-dependent generation of the superoxide anion by equine spermatozoa on the basis of NBT reduction as well as cytochrome c reduction.

Materials and Methods

Animals—Semen was collected from Thoroughbred stallions (n = 3) with the use of an artificial vagina. Horses...
used in this study were maintained under a protocol approved by the University of California-Davis Institutional Animal Care and Use Committee.

Measurement of NAD(P)H-induced ROS production—

The NAD(P)H-induced superoxide production was measured by use of an NBT reduction assay and a cytochrome c reduction assay, as described by Miller and Griendling. The NBT assay for superoxide activity is based on the ability of superoxide to reduce NBT to the blue formazan, which is monitored by a change in absorbance at 550 nm. The effects of NADPH or NADP H in the presence or absence of SOD or a flavoprotein inhibitor, DPI, were evaluated by the reduction of NBT in air-dried spermatozoa. Briefly, spermatozoa separated on a Percoll gradient were washed (in Triton X-100) and resuspended in TALP-PVA. Spermatozoa (1.25 X 10^5/well) were air dried in a 96-well microplate and incubated with the following: NADPH, NADH, NADP+, or NAD+ in combination with SOD (0 or 200 U/mL) or DPI (0, 1, 5, and 15 µM). The DPI was added to spermatozoa prior to addition of NADPH; NBT (0.75 mM) was added last, plates were then incubated at 37°C, and absorbance (550 nm) was determined via a microplate reader.

Cytochrome c reduction—Spermatozoa separated on a Percoll gradient were washed in TALP-PVA, centrifuged twice at 300 X g for 10 minutes, and submitted to hypotonic shock in 10 mM potassium phosphate buffer containing 1 mM EDTA and protease inhibitors (pepsin, leupeptin, and antipain; 0°C for 60 minutes). Spermatozoa were then sonicated for 5 seconds 3 to 4 times. The suspension of spermatozoa was centrifuged (10,000 X g, 20 minutes, 4°C); the pellet was resuspended in TALP-PVA and protease inhibitors and stored at -20°C. The supernatant containing the plasma membrane was centrifuged (100,000 X g, 90 minutes, 4°C), and the plasma membrane pellet was resuspended in TALP-PVA containing protease inhibitors, 2 mM MgSO4, and 1.25 mM EGTA. In a 96-well plate, flavin adenine dinucleotide (0.011 mM), acetylated cytochrome c (0.1 mM), and membrane sample (65 to 100 µg) were added. The SOD (200 U/mL) was included in half the samples. After a 2-minute incubation at 20°C, sodium dodecyl sulfate (0.1 mM) was added. After 3 minutes, NADPH (0.5 mM) was added and absorbance at 550 nm was measured every 18 seconds for 15 minutes. An acetylated cytochrome c was used because it is much less susceptible to tissue oxidases and reductases. The change in absorbance at 550 nm was determined by the difference in cytochrome c reduction between mixtures containing membrane sample with or without SOD. Superoxide formation was expressed in nanomoles per minute per milligram of protein.

RESULTS

Measurement of ROS generation by equine spermatozoa—On the basis of the results of the NBT assay, generation of superoxide significantly increased in a time- and NADPH concentration-dependent manner, and the generation of superoxide was significantly inhibited by the addition of SOD (Figures 1 and 2). Likewise, DPI significantly inhibited superoxide generation in a dose-dependent fashion in the presence of NADPH (Figure 3). Reduced nicotinamide adenine dinucleotide (NADH) significantly increased generation of superoxide, whereas NAD(P)H did not increase NBT reduction (Figure 4).

In membrane fractions of equine spermatozoa, SOD-inhibitable superoxide generation was significantly increased in the presence of 500 µM NADPH (superoxide formation, 506.8 ± 81.9 nmol/min/mg of protein), compared with the absence of NADPH (superoxide formation, 122.2 ± 37.9 nmol/min/mg of protein); values were determined by the difference in cytochrome c reduction between membrane samples with and without SOD (n = 9). Results of this experiment reveal the presence of NAD(P)H oxidase in the purified membrane fractions. These fractions were free from mitochondrial contamination as determined

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Figure 1—Generation of superoxide as determined by NBT reduction in a microplate assay. Spermatozoa (1.25 X 10^5) were added to each well of a 96-well plate, air dried, and incubated with NADPH (0 µM [circles], 250 µM [squares], 500 µM [triangles], and 1,000 µM [diamonds]), SOD (0 U/mL [closed symbols] and 200 U/mL [open symbols]), and NBT. Plates were incubated at 37°C and reduction of NBT determined over 16 hours by measuring absorbance at 590 nm. Generation of superoxide increased (P< 0.05) with time and with increasing concentrations of NADPH, whereas the addition of SOD decreased (P< 0.05) reduction of NBT (n = 3 replicates). OD = Optical density.
by the use of the succinate dehydrogenase assay to measure reduced INT. Mitochondrial fractions and membrane fractions had reduced INT concentrations of 765.9 ± 124.1 µmol/mg of protein and ≤ 73.1 ± 32.0 µmol/mg of protein, respectively (n = 5).

Cytochemical localization of NADH and NADPH diaphorases in equine spermatozoa—After a 24-hour incubation, a positive reaction (ie, blue formazan deposits [reduced tetrazolium salts]) was observed as heavy diaphorase labeling of the middle piece in the presence of NADH and NADPH. Also, strong diaphorase labeling of the head was found in the presence of NADPH and NADH, which appeared slightly darker in the presence of NADH (Figure 5). Addition of SOD and DPI reduced NBT cytochemical staining in the presence of NAD(P)H.

Discussion

In our study, we confirmed that equine spermatozoa use NAD(P)H as a substrate for superoxide generation. It is important to consider the type of assay used. On the basis of the results of the various methods of detection used, the ability of spermatozoa to generate an NADPH-dependent signal has been controversial.28,29 For example, no ROS production was detected from human30 or equine31 spermatozoa in contrast to a high amount of ROS production from activated neutrophils. In our study, we were unable to measure detectable superoxide anion production in intact spermatozoa (data not shown); however, when spermatozoa were air dried, measurable amounts of superoxide anion were detected with the NBT assay. It is possible that the NAD(P)H binding sites were not exposed in intact spermatozoa and are internally located on the plasma membrane, as is the case in neutrophils.32 The structure of NADPH oxidase in phagocytic cells has been described as a membrane-bound electron transport complex in which the carboxy-terminal end of the large subunit houses the NADPH and flavin adenine dinucleotide binding sites and is located on the cytoplasmic side of the membrane to allow access to the NADPH substrate.33 In the neutrophil oxidase complex, cytosolic components combine with the membrane-associated components to activate the complex. Smith et al32 provided evidence that 1 of the cytosolic components is the NADPH binding site and that the NADPH binding subunit of the oxidase complex exists in a slowly dissociating complex with 1 or more cytosolic components.

Generation of ROS by whole spermatozoa was reduced by the flavoprotein inhibitor DPI, consistent with an NAD(P)H oxidase as the principal oxidant source in these nonphagocytic cells. The DPI is a potent arylation lipophilic reagent, and an efficient inhibitor of the production of superoxide by the activated NAD(P)H oxidase. However, it is reported that iodo-
nium salts react with a number of redox systems, including the superoxide generating NAD(P)H oxidase, nitric oxide synthase, xanthine oxidase, cytochrome P450 reductase, and NADH ubiquinone oxidoreductase.34-37 Despite its broad spectrum of action, DPI has been used extensively in recent years to block NAD(P)H oxidase activity in nonphagocytic cells. Electrons are transported through the flavin moieties of the NAD(P)H oxidase complex and cause reduction of DPI to its radical form, followed by irreversible phenylation of either the flavin or heme groups.38 Although the equine testis NAD(P)H oxidase has not yet been fully identified, the DPI inhibitory effect suggests a flavin-heme component in the NAD(P)H oxidase, as in the neutrophil NAD(P)H oxidase.

Generation of ROS is frequently considered to be a byproduct of mitochondrial respiration. We have shown that the single source of superoxide production from NAD(P)H resides in the membrane fraction, which was relatively free from mitochondrial components on the basis of the results of the succinate dehydrogenase assay. Findings in our study provide evidence for the role and the presence of an NAD(P)H oxidase on the membranes of equine spermatozoa but does not exclude other possible sources of ROS. This is consistent with Vernet et al’s findings,21 which suggested a plasma membrane NADPH oxidase in epididymal rat spermatozoa. Vernet et al addressed this question in a series of experiments that attempted to distinguish between the membrane and mitochondrial source of ROS production. These authors used various mitochondrial inhibitors and concluded that ROS generation in mammalian spermatozoa involves 2 independent mechanisms: an enzymatic system located in the plasma membranes of spermatozoa that uses NADPH as a substrate and a second system involving the mitochondrial electron transport chain, as they observed in rat spermatozoa but not in human spermatozoa.11,21

The same group of investigators77 presented evidence in human spermatozoa of multiple pathways for regulating electron flux near the plasma membrane, 1 superficially located and 1 bound to the membrane. On the basis of the results of our experiments, we can confirm that superoxide formation in equine spermatozoa measured by cytochrome c reduction in the presence of NADPH was detectable in the membranes of equine spermatozoa. However, we cannot exclude that ROS generation as measured with the NBT assay may also involve leakage of electrons from the mitochondrial electron transport chain, as in rabbit spermatozoa.10 It is suggested that NADPH-dependent signals may also involve a ubiquitous NADPH-dependent enzyme system, including cytochrome P450 reductase in rat epididymal spermatozoa,41 rather than a specific NADPH oxidase acting alone. This may explain the discrepancies in findings among various studies28,29 that used different detection probes.

Results of our study indicate that a significant difference in NBT reduction does not exist between NADPH and NADH. Results of other studies39,42 indicate that a preference for the phosphorylated nicotinamide exists over the nonphosphorylated NADH. The NADH and NADPH serve as reducing agents for NADPH oxidase, but enzymologic considerations have resulted in the identification of NADPH as the electron donor in vivo.15 We compared diaphorase activity of equine spermatozoa in the presence of NADPH with that of NADH. Diaphorase generally defines a group of flavoenzymes including NADPH oxidase, which catalyze the reduction of specific electron acceptors. In our study, the pattern of NADH diaphorase staining in equine spermatozoa was not different from that of NADPH diaphorase staining. Heavy diaphorase labeling was found along the entire length of the midpiece as well as at the head in the presence of NADH or NADPH. Results of a study by Zini et al also reveal different NADPH- and NADH-dependent diaphorase localization, although NADPH-dependent diaphorase was detected in the postacrosomal region in human spermatozoa. Diaphorase labeling of the midpiece indicates that mitochondrial oxidoreductase localization is generally related to the role of these enzymes in energy and spermatozoon motility. Like other flavoenzymes, NADPH oxidase express diaphorase activity, passing electrons to an artificial electron acceptor such as NBT or dichlorophenolindophenol.40 The translocation of hydrogen to NBT leads to formazan deposits. Diaphorase labeling of the spermatozoon head in the presence of NADH and NADPH suggests that equine spermatozoa contain NAD(P)H-dependent enzymes that could be associated with the membrane or with residual cytoplasm as other oxidoreductase enzymes. Spermatozoa are known to possess diaphorase activity as an enzymatic activity located in the cytosol.27,47 In addition, defective spermatozoon function has been correlated with the retention of residual cytoplasm and ROS production; NADPH diaphorase staining has been used to monitor the retention of residual spermatozoon cytoplasm.27,16 Although diaphorase labeling does not provide direct

Figure 5—Photomicrographs of equine spermatozoa incubated for 24 hours with NADH (A1, A2, and A3; 500 µM), NADPH (B1, B2, and B3; 500 µM), and NBT in the absence or presence of DPI (A2 and B2; 15 or SOD (A3 and B3; 200 U/mL) to determine diaphorase activity. C = Photomicrograph of control spermatozoa treated with NBT only. Bar = 10 µm.
evidence of membrane localization, it confirms the inhibitory effect of SOD and DPI.

In conclusion, results of our study indicate that equine spermatozoa possess a membrane-bound NADPH oxidase that may share some similarities with the neutrophil NADPH oxidase, but the generation of reactive oxygen in much lower amounts in spermatozoa is a unique strategy distinct from the known host-defense system in phagocytes. This suggests a different role for reactive oxygen generated in spermatozoa, including cellular signaling. Further studies are needed to investigate its role in equine spermatozoa signaling and identify various components of the membrane-bound NADPH oxidase in equine spermatozoa.

References


