Significance of Mitochondrial Reactive Oxygen Species in the Generation of Oxidative Stress in Spermatozoa

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Context: Male infertility has been linked with the excessive generation of reactive oxygen species (ROS) by defective spermatozoa. However, the subcellular origins of this activity are unclear.

Objective: The objective of this study was to determine the importance of sperm mitochondria in creating the oxidative stress associated with defective sperm function.

Method: Intracellular measurement of mitochondrial ROS generation and lipid peroxidation was performed using the fluorescent probes MitoSOX red and BODIPY C_{11} in conjunction with flow cytometry. Effects on sperm movement were measured by computer-assisted sperm analysis.

Results: Disruption of mitochondrial electron transport flow in human spermatozoa resulted in generation of ROS from complex I (rotenone sensitive) or III (myxothiazol, antimycin A sensitive) via mechanisms that were independent of mitochondrial membrane potential. Activation of ROS generation at complex III led to the rapid release of hydrogen peroxide into the extracellular space, but no detectable peroxidative damage. Conversely, the induction of ROS on the matrix side of the inner mitochondrial membrane at complex I resulted in peroxidative damage to the midpiece and a loss of sperm movement that could be prevented by the concomitant presence of α-tocopherol. Defective human spermatozoa spontaneously generated mitochondrial ROS in a manner that was negatively correlated with motility. Simultaneous measurement of general cellular ROS generation with dihydroethidium indicated that 68% of the variability in such measurements could be explained by differences in mitochondrial ROS production.

Conclusion: We conclude that the sperm mitochondria make a significant contribution to the oxidative stress experienced by defective human spermatozoa. **(*J Clin Endocrinol Metab* 93: 3199–3207, 2008)**

Reactive oxygen species (ROS) are conventionally considered as detrimental by-products of cellular metabolism or xenobiotic exposure, which generate a state of oxidative stress in susceptible cells (1). Spermatozoa are particularly vulnerable to such stress because they are richly endowed with polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (2, 3). Furthermore, the limited volume and restricted location of their cytoplasmic space place constraints on the availability of intracellular antioxidant enzymes in these cells, which may be further compromised by the effects of aging (3, 4). This inherent vulnerability is exacerbated by the tendency of defective spermatozoa to generate abnormally high quantities of ROS, with the result that extensive peroxidative damage is commonly observed in the spermatozoa of infertile patients (2, 5–8). Notwithstanding the significance of ROS generation in both the etiology of male infertility and the physiological regulation of sperm capacitation (9–12), neither the subcellular origin nor the biochemical basis for this activity has been established.

The major source of ROS generation in somatic cells is thought to involve electron leakage from the mitochondrial elec-

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Abbreviations: BWW, Biggers-Whitten-Whittingham; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; DHE, dihydroethidium; DMSO, dimethylsulfoxide; ETC, electron transport chain; MSR, MitoSOX Red; PUFA, polyunsaturated fatty acid; PVA, polyvinyl alcohol; ROS, reactive oxygen species; $O_2^{-\bullet}$, superoxide anion; QH_2, ubiquinol; $Q^{-\bullet}$, ubisemiquinone.

tron transport chain (ETC) during cellular respiration, with an estimated 2% of consumed oxygen being converted to superoxide anion ($O_2^{-\bullet}$) via this route (13). Usually, this activity is balanced by antioxidant factors within the intermembrane space and mitochondrial matrix (14). However, mitochondrial ROS production may overwhelm these defense mechanisms, creating a source of oxidative stress that has been implicated in numerous pathological conditions, including both Alzheimer's (15) and Parkinson's disease (16). Currently, evidence exists for the generation of ROS by rabbit and rat sperm mitochondria, but no equivalent data are available for human gametes (17–19). In view of our poor understanding of mitochondrial function in spermatozoa in general, and the potential importance of these organelles as a source of oxidative stress in the male germ line in particular, we have analyzed the ability of human sperm mitochondria to generate ROS and evaluated the significance of this activity in the etiology of defective sperm function.

Materials and Methods

Chemicals

Unless stated otherwise all chemicals/reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of media

Biggers-Whitten-Whittingham (BWW) medium consisted of 95 mM NaCl, 44 mM sodium lactate, 25 mM $NaHCO_3$, 20 mM HEPES, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.27 mM sodium pyruvate, 0.3% (wt/vol) BSA, 5 U/ml penicillin, and 5 mg/ml streptomycin. Where indicated, BWW/polyvinyl alcohol (PVA) was also used, which consisted of 0.1% (wt/vol) PVA in place of BSA.

Oxygen consumption

Oxygen consumption was measured using an Apollo 4000 free radical analyzer (World Precision Instruments, Inc., Sarasota, FL) in a sealed 0.8-ml reaction chamber maintained at 37 C. Spermatozoa were incubated for 120 min in the presence or absence of mitochondrial inhibitors. Respiratory activity was defined as nmoles of oxygen consumed/min per 10^6 cells, calculated using the ideal gas equation. Cell concentration used was 100×10^6 cells per ml.

Preparation of human spermatozoa

Institutional and state government ethical approval was secured for the use of human semen samples for the purposes of this research. Samples from unselected donors were collected into sterile containers before immediate transportation to the laboratory. After initial inspection for liquefaction, consistency, debris, and volume, assessments of cell count and motility were conducted, and cell viability was measured using the eosin exclusion test (20). After allowing at least 30 min for liquefaction to occur, the spermatozoa were fractionated on a discontinuous two-step Percoll gradient, as described (21). Spermatozoa were ultimately recovered from the base of the high-density portion of the gradient as well as the low- to high-density Percoll interface. Spermatozoa from the Percoll gradients were ultimately washed with 10 ml BWW, centrifuged at $600 \times g$ for 15 min, and finally resuspended in BWW. All samples were cleared of contaminating leukocytes using magnetic Dynabeads coated with a monoclonal antibody directed against the common leukocyte antigen CD45 (Dynal, Oslo, Norway) and confirmed using a zymosan provocation assay (21).

Chemiluminescence

Luminol-peroxidase dependent assessment of extracellular H_2O_2 (21, 22) was recorded on a Berthold 953 luminometer at 37 C using 400-μl aliquots of spermatozoa at a concentration of 100×10^6 cells per ml. Cell-free media controls were also recorded during each experiment. Luminol was prepared as a 25-mM stock solution in dimethylsulfoxide (DMSO) and diluted in BWW to give a final concentration of 250 μM. Luminol was supplemented with horseradish peroxidase, freshly prepared as a 2 mg/ml stock solution in BWW, added to the sperm suspension to give a final peroxidase activity of 51.2 U/ml. The luminometer results were recorded as continuous traces and as integrated photon counts over the final 2 h after addition of stimulant after 15 min.

MitoSOX Red (MSR) and dihydroethidium (DHE)

Intracellular generation of $O_2^{-\bullet}$ was estimated using either DHE (8), a membrane permeant uncharged probe that reports overall cellular $O_2^{-\bullet}$ production, and MSR, a lipid soluble cation that is selectively targeted to the mitochondrial matrix. All fluorescent probes were purchased from Molecular Probes, Inc. (Eugene OR). For this assay, MSR stock solutions (5 mM in DMSO) were diluted in BWW and added to spermatozoa at 20×10^6 cells per ml to give a final concentration of 2 μM, and incubated for 15 min at 37 C. This was followed by centrifugation for 5 min at $600 \times g$ and resuspension in BWW. Spermatozoa loaded with the MSR dye were then divided into separate aliquots and treated with various mitochondrial inhibitors with the final cell concentration standardized at 10×10^6 cells per ml. SYTOX Green, a cell viability stain, was diluted in BWW and added to each treatment for the final 15-min incubation at a concentration of 0.05 μM. The MSR (red) and SYTOX Green (green) fluorescence was then measured on a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Argon laser excitation at 488 nm was coupled with emission measurements using 530/30 band pass (green) and 585/42 band pass (red) filters. Nonsperm-specific events were gated out, and 10,000 cells were examined. Before imaging the localization of these probes, live cells were fixed to glass slides using Cell-Tak (BD Biosciences, San Jose, CA). Images were then collected on a Zeiss LSM510 confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (488 nm) with emission collection at 500–530 nm (green) and helium neon laser excitation (543 nm) with emission collection at more than 560 nm (red).

Measurement of spontaneous ROS generation was performed by the addition of either MSR or DHE to spermatozoa at 10×10^6 cells per ml, giving a final concentration of 2 μM. SYTOX Green was also added to give a final concentration of 0.05 μM and incubated for 15 min at 37 C. This was followed by centrifugation for 5 min at $600 \times g$ and resuspension in BWW and analysis by flow cytometry as described previously.

Computer-assisted sperm analysis

Evaluation of sperm motility parameters was conducted using computer-assisted sperm analysis using a Hamilton Thorne Version 12 IVOS (Hamilton Thorne Biosciences, Beverly MA). For each measurement, a 2.5 μl aliquot of spermatozoa was loaded onto a standard four-chamber slide (Leja, NL, Nieuw-Vennep The Netherlands). A total of at least 200 or more spermatozoa were examined for each sample using standard settings (30 frames acquired at a frame rate of 60 Hz and a temperature of 37 C in 20-μm deep chambers). Samples were analyzed for percent motility as well as progressive motility (average path velocity of more than 25 μM/sec).

Lipid peroxidation assay

Lipid peroxidation was assessed using BODIPY (581/591) C_{11} as the probe (Molecular Probes). This probe incorporates into membranes where it undergoes a fluorescence emission shift upon peroxidation by lipid radicals (23). BWW/PVA was used because it was found that BSA binds the lipophilic BODIPY C_{11}. BODIPY C_{11} (5 μM) was added to 10×10^6 cells per ml, incubated for 30 min at 37 C, and washed twice ($650 \times g$ for 5 min) before the addition of mitochondrial inhibitors. At the end

of this incubation period, the viability indicator propidium iodide (10 μg /ml) was added 30 sec before the cells being analyzed on a FACSCalibur flow cytometer using an excitation wavelength of 488 nm. The FL-1 (530/30 nm band pass filter) was used to measure green fluorescence, and FL-3 (620 nm long pass) was used to measure the shift in red fluorescence upon staining with propidium iodide; 10,000 sperm specific events were collected per sample. Before imaging, live cells were fixed to glass slides using Cell-Tak. Images were then collected on a Zeiss LSM510 confocal microscope as described previously.

Statistical analysis

All experiments were repeated at least three times on independent samples and the results analyzed by ANOVA using Microsoft Excel (Microsoft Corp., Redmond, WA). Differences with a P value of less than 0.05 were regarded as significant.

Results

Mitochondrial ROS generation by human spermatozoa

Evidence that human sperm mitochondria have the potential to generate ROS was obtained using mitochondrial electron transport inhibitors to disrupt the flow of electrons through the ETC and a peroxidase-based, chemiluminescence system to detect the release of H_2O_2 into the extracellular space. Although normal human spermatozoa exhibited extremely low rates of spontaneous ROS generation, addition of antimycin A and myxothiazol (10 μM), both of which act at complex III of the ETC, resulted in a significant increase in redox activity (Fig. 1A; $P < 0.01$). Myxothiazol binds close to the b_L heme of this complex, allowing ubiquinol (QH_2) to access the Rieske iron sulfur to undergo a one electron oxidation to create the semiquinone radical, $Q^{-\bullet}$ (Fig. 2), while preventing the latter from passing its electrons on to cytochrome b_L. Antimycin A treatment also leads to the generation of $Q^{-\bullet}$ by inhibiting the reoxidation of heme b_L through its capacity to disrupt electron transfer from heme b_H to $Q^{-\bullet}$ (Fig. 2) (24). As a consequence of these interactions, both myxothiazol and antimycin A stimulate the generation of $Q^{-\bullet}$, which then stabilizes by shedding its electrons to oxygen to create $O_2^{-\bullet}$ in the intramembranous space. This $O_2^{-\bullet}$ then dismutates to H_2O_2 under the influence of superoxide dismutase and escapes to the outside of the cell, where it can be detected by the luminol-peroxidase monitoring system. Rotenone (an inhibitor of electron transfer from FeSN-2 cluster to ubiquinone; Fig. 2) had a minor stimulatory effect on H_2O_2 release at 10 μM (Fig. 1A), possibly because the free radicals generated with this compound are directed into the mitochondrial matrix where they can be neutralized by the antioxidant enzymes (superoxide dismutase and glutathione peroxidase) that abound at this site (Fig. 2). Stigmatellin (10 μM), which inhibits the transfer of electrons from QH_2 to the Rieske iron sulfur cluster and prevents semiquinone ($Q^{-\bullet}$) formation (Fig. 2), failed to induce mitochondrial ROS generation.

Human sperm mitochondria spontaneously consumed approximately 30 nmol $O_2/10^6$ cells per hour (Fig. 1B), but this rate was more than doubled by the addition of the uncoupling agent, carbonyl cyanide-m-chlorophenylhydrazone (CCCP). The importance of this effect is illustrated in Fig. 1C, which reveals that the addition of CCCP significantly increased mitochondrial ROS production by both antimycin A and myxothiazol ($P < 0.05$). This highlights the fact that $\Delta\psi$ is not essential for mitochondrial ROS production by human spermatozoa. On the contrary, the collapse of $\Delta\psi$ with reagents such as CCCP can significantly enhance free radical generation, if electron flow through the ETC is impeded.

Further confirmation of the ability of human spermatozoa to generate ROS was secured using a fluorescent indicator of mitochondrial superoxide production, MSR. This probe is a membrane permeant derivative of the DNA-sensitive fluorochrome, DHE, which allows for the highly selective detection of $O_2^{-\bullet}$ in the mitochondria of live cells (8). Once in these organelles, MSR is oxidized by $O_2^{-\bullet}$ and exhibits bright red fluorescence upon binding to nucleic acids. Use of this reagent revealed a very low level of mitochondrial ROS generation in untreated normal human spermatozoa from the high-density region of discontinuous Percoll gradients (Fig. 3A). However, treatment with rotenone (10 μM), which diverts mitochondrial electron flow to generate ROS in the mitochondrial matrix, stimulated a significant time-dependent increase in the percentage of MSR positive cells, measured by flow cytometry.

Confocal imaging revealed that cells treated with rotenone exhibited MSR localization to the midpiece and base of the sperm head as the activated probe reacted with DNA, initially in the mitochondrial matrix and then in the sperm nucleus (Fig. 3B). Treatment of human spermatozoa with antimycin A also stimulated a significant time-dependent increase in mitochondrial superoxide generation, however, this time the probe only bound to nuclear DNA because the ROS were generated in the intermembranous space (Fig. 2) and never gained access to the DNA located in the mitochondrial matrix (Fig. 3C). Myxothiazol and stigmatellin were omitted from this experiment due to the former's ability to auto-oxidize the probe and the latter's intrinsic autofluorescence.

Mitochondrial ROS and defective sperm function

Although the ability to induce mitochondrial ROS in healthy donor samples is an important finding, it was of interest to determine the relevance of mitochondrial ROS generation in the etiology of defective sperm function. For this purpose, spontaneous mitochondrial ROS generation was compared between the defective spermatozoa recovered from the low-density region of Percoll gradients and functional gametes pelleting in high-density Percoll. This analysis revealed that a significantly greater proportion of the compromised, low-density, human spermatozoa produced mitochondrial ROS compared with the high-density cells ($P < 0.001$; Fig. 4A). In addition, a strong exponential negative correlation ($R^2 = 0.8048$) was also recorded between MSR positivity and sperm motility in these cell populations (Fig. 4B). The MSR signal was also highly correlated ($R^2 = 0.6821$) with the total level of spontaneous ROS generation by human spermatozoa measured by DHE (Fig. 4C).

To determine whether a direct causal relationship existed between poor motility and mitochondrial ROS generation, the impact of the latter on membrane lipid peroxidation, a known inhibitor of sperm movement (2, 25), was determined using the fluorescent probe BODIPY C_{11}. After 4-h incubation, it was

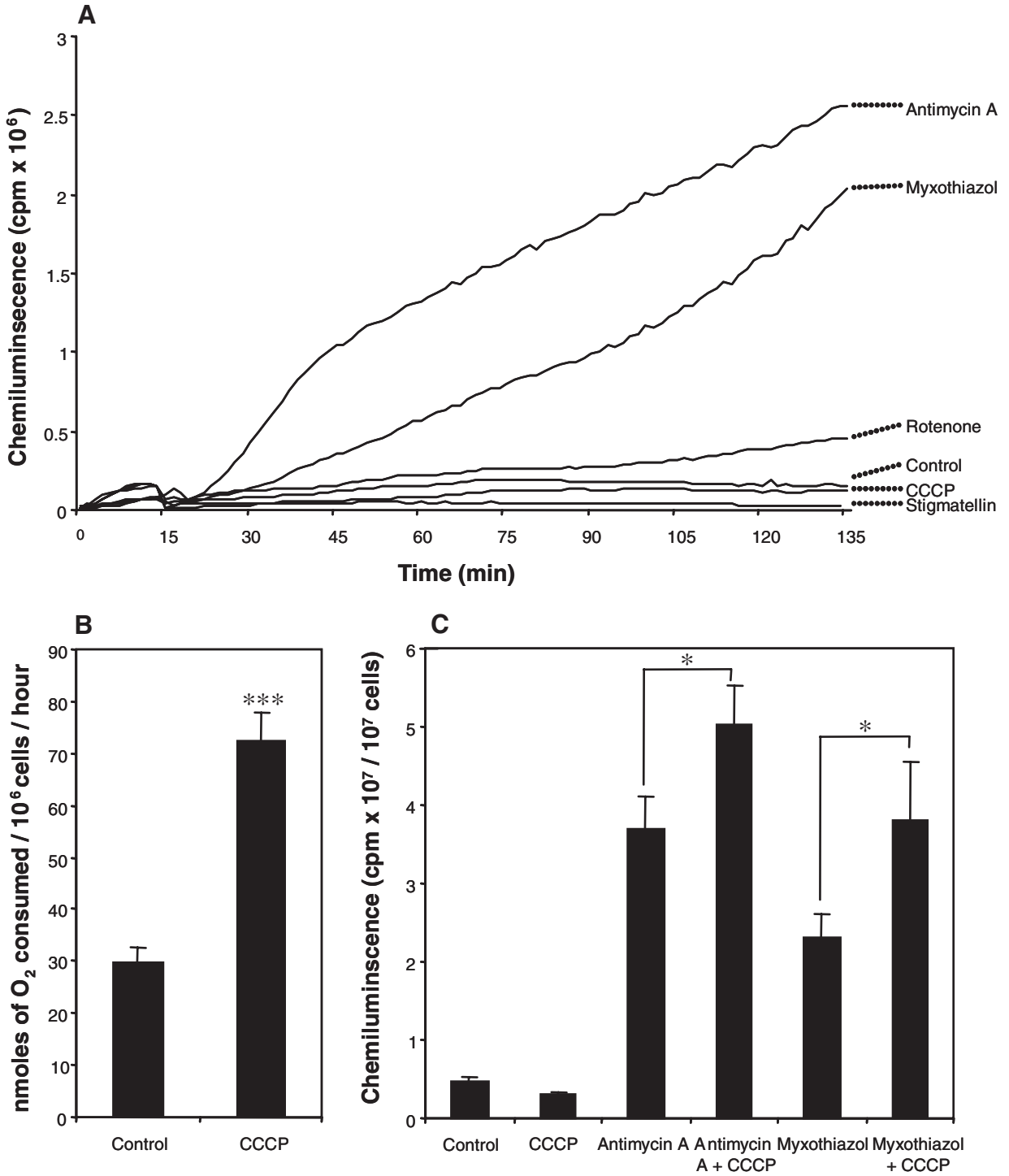

FIG. 1. Analysis of the impact of mitochondrial inhibitors (10 μM) on mitochondrial ROS generation in human spermatozoa measured by luminol-peroxidase dependent chemiluminescence. A, Representative trace of the luminol-peroxidase dependent chemiluminescence exhibited by human spermatozoa in the presence of mitochondrial inhibitors (10 μM). B, The impact of CCCP involved induction of a significant increase in oxygen consumption upon dissipation of the $\Delta\psi$ ($P < 0.001$). C, Integrated photon counts reveal a significant increase in chemiluminescence exhibited by both antimycin A and myxothiazol in the presence of CCCP ($P < 0.05$).

revealed that only when mitochondrial ROS formation was induced in the matrix by rotenone was a significant increase in lipid peroxidation observed ($P < 0.001$; Fig. 5A). The lipid peroxidation stimulated by rotenone was induced in a time-dependent manner (Fig. 5B) and was targeted to the mitochondria in the sperm midpiece (Fig. 5, C–F). The induction of ROS generation in the intermembranous space with antimycin A had no such effect (Fig. 5A).

Finally, we examined whether the induction of free radical generation and lipid peroxidation in the mitochondrial matrix could have a major impact on the movement characteristics of these cells. In the presence of glucose, no short-term (4 h) changes

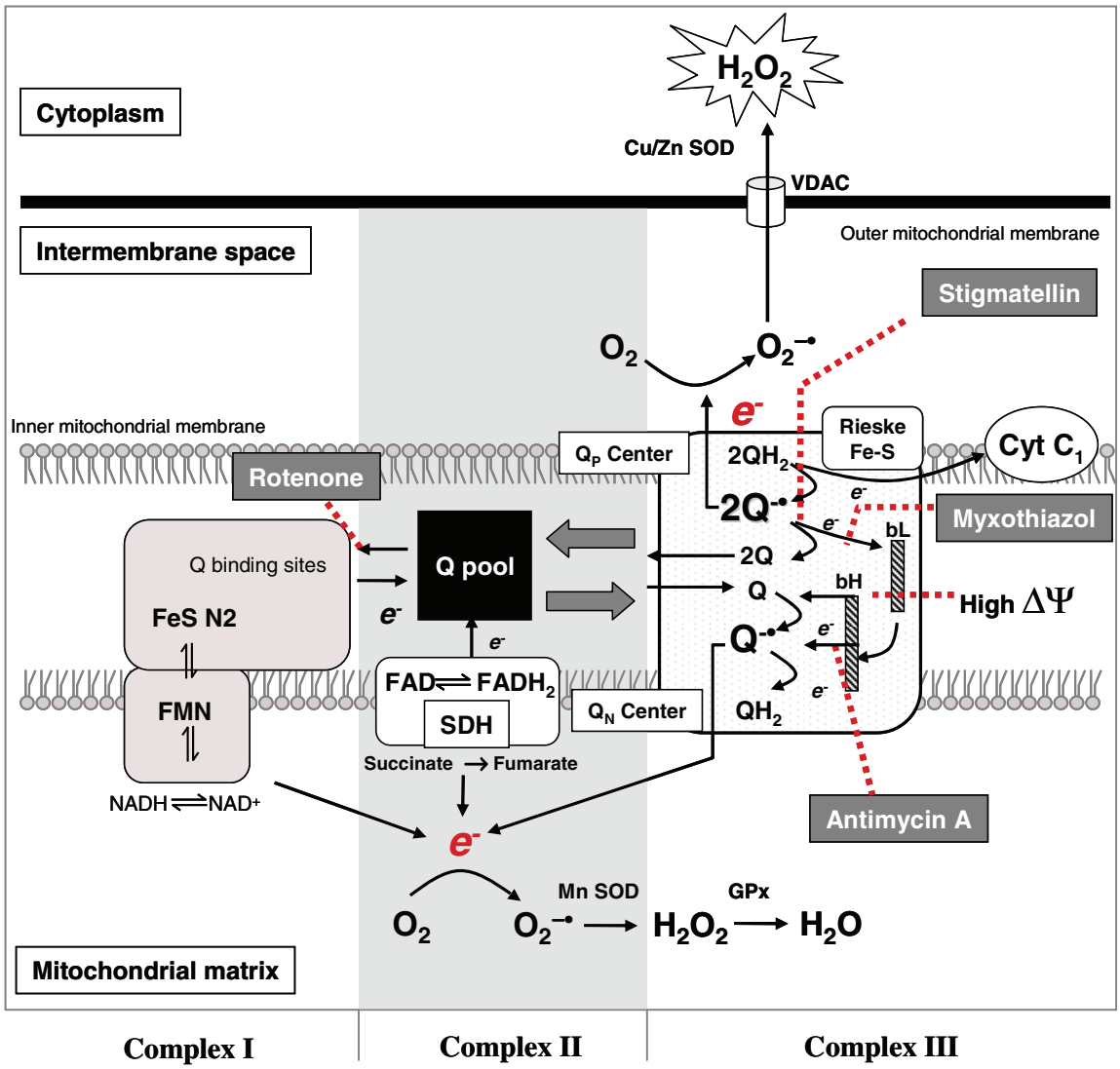

FIG. 2. Schematic diagram showing the main pathways of electron flux through the mitochondrial ETC. Sites of action for the mitochondrial inhibitors used in this study are also indicated. Cu, Copper; Cyt C, cytochrome C; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GPx, glutathione peroxidase; Mn, manganese; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Q, ubiquinone; SDH, succinate dehydrogenase; SOD, superoxide dismutase; VDAC, voltage dependent anion channel; Zn, zinc.

in sperm movement were seen in the face of mitochondrial ROS generation stimulated by antimycin A or rotenone (data not shown), presumably because glycolysis compensated for any lack of ATP production on the part of the mitochondria (26, 27). On the other hand, when the incubation period was extended to 24 h, then rotenone, but not antimycin, induced a significant loss of both total and progressive motility (Fig. 6, A and B), in keeping with the lipid peroxidation results presented in Fig. 5. Causative relationships between the induction of mitochondrial ROS generation, peroxidative damage, and impaired movement were indicated by the preservation of sperm motility observed by concomitant exposure of rotenone-treated cells to the chain-breaking antioxidant, α-tocopherol (Fig. 6, A and B).

Discussion

The results obtained in this study demonstrate for the first time that human spermatozoa are capable of mitochondrial ROS production. Treatments that stimulated production of the unstable semiquinone radical, $Q^{-\bullet}$, increased ROS release into the extracellular space, as observed with antimycin, myxothiazol, and, to a lesser extent, rotenone. Rotenone is distinguished by the fact that the ROS are generated at complex I rather than complex III and, as a result, are located in the mitochondrial matrix rather than the intermembranous space (Fig. 2). Superoxide generated in the latter is rapidly dismutated to H_2O_2 in the cytoplasm and escapes to the extracellular space (Fig. 1A), causing minimal peroxidative damage to the cell (Fig. 5, A and B). Rotenone, on the other hand, stimulates ROS production in the mitochondrial matrix. This accounts for the ability of activated MSR to stain mitochondrial DNA in the presence of rotenone but not with antimycin (Fig. 3B). The scavenging of ROS in the mitochondrial matrix by mitochondrial superoxide dismutase and glutathione peroxidase would have accounted for the minimal release of H_2O_2 into the extracellular space after rotenone treatment (Fig. 1A), despite the high levels of mitochondrial ROS generation

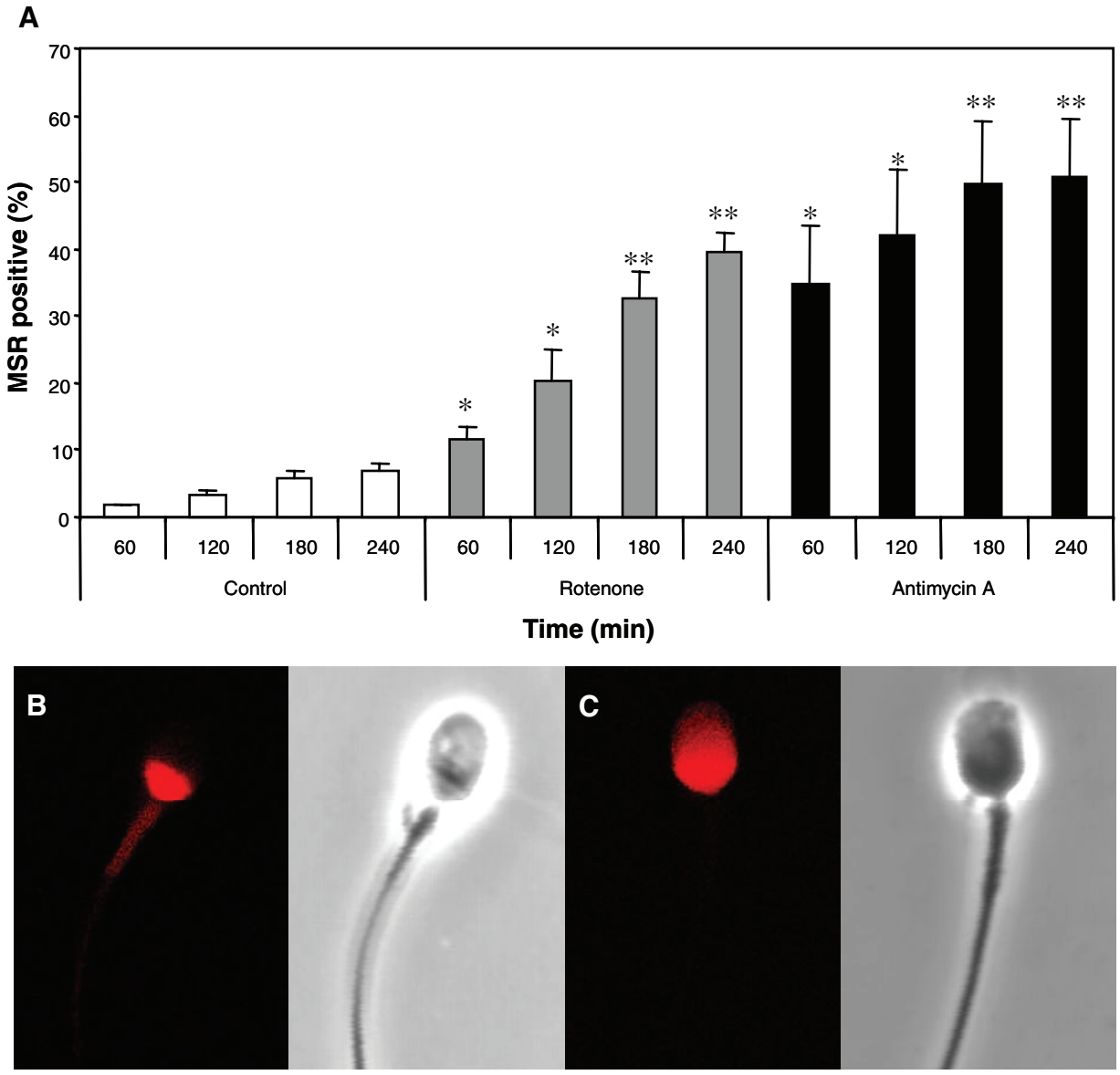

FIG. 3. Analysis of the impact of mitochondrial inhibitors (10 μM) on mitochondrial ROS generation in human spermatozoa measured by MSR. A, ANOVA analysis revealed highly significant increases in mitochondrial ROS in the presence of mitochondrial inhibitors rotenone and antimycin A (*, $P < 0.05$; **, $P < 0.01$). Confocal imaging of the cells loaded with MSR and treated with rotenone (B) revealed red fluorescence localized to the midpiece and posterior head regions of the spermatozoa; however, in the presence of antimycin A (C), the fluorescence was confined to the base of the sperm head. Magnification, ×3,000.

indicated by MSR (Fig. 3, A and B). This interpretation would also account for the ability of rotenone, but not antimycin, to induce peroxidative damage in the midpiece of the spermatozoa (Fig. 5C). The fact that such damage took more than 24 h to manifest itself suggests that peroxidative damage could only be induced once the production of ROS in the mitochondrial matrix had overwhelmed the intramitochondrial antioxidant defense enzymes. This peroxidative damage, in turn, induced a progressive loss of motility in terms of both the percentage of spermatozoa that were motile and the progressiveness of this motility via mechanisms that could be reversed by the concomitant presence of the antioxidant, α-tocopherol (Fig. 6, A and B). The notion that rotenone stimulates complex I to generate ROS that can only be detected once matrix antioxidant protection has been overwhelmed also accords with earlier studies of cardiac subsarcolemmal mitochondria (28).

The fact that this inhibition of sperm motility was observed in the presence of a glycolytic substrate, glucose, suggests that the oxidative stress created by rotenone had induced permanent damage to the motility apparatus. This reflects the *in vivo* situation where a powerful inverse relationship was observed between mitochondrial ROS generation and sperm motility, despite the presence of glucose in the incubation medium to compensate for any defect in the ability of the mitochondria to generate ATP (Fig. 4, A and B). These results echo previous studies that clearly demonstrated an inverse relationship between the peroxidation status of human spermatozoa and their competence for movement (2). However, this is the first study to identify aberrant mitochondrial activity as a probable source of the free radicals responsible for this damage.

We have previously reported a negative correlation between sperm motility and the measurement of ROS generation

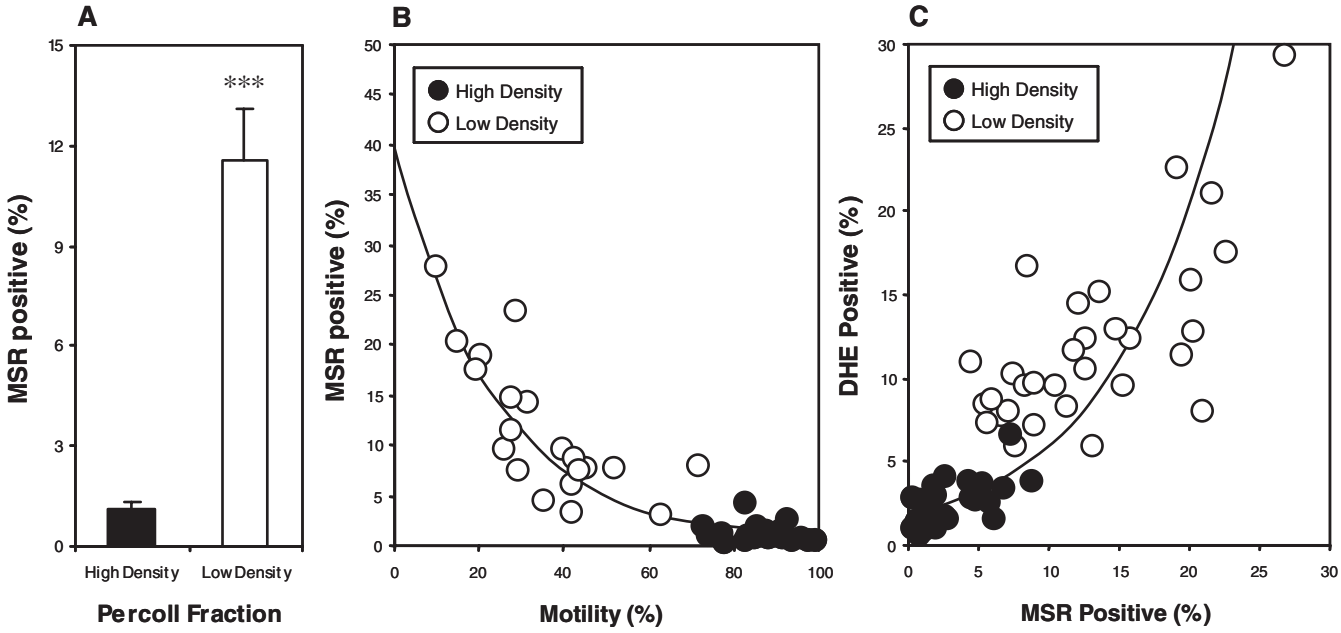

FIG. 4. Generation of mitochondrial ROS by human spermatozoa. A, A significant increase ($P < 0.001$) in spontaneous MSR activity generated by leukocyte free low-density Percoll fractions in comparison with their high-density Percoll counterparts. B, A highly significant exponential negative correlation ($R^2 = 0.8048$) was observed between the spontaneous MSR signal generated by live human spermatozoa and cell motility, using Percoll fractionated spermatozoa. C, A significant correlation ($R^2 = 0.6821$) was also observed between free radical generation by the mitochondria (MSR fluorescence) and total ROS generation by the sperm population (DHE fluorescence).

in human spermatozoa using DHE as the probe (8). Using a combination of mass spectrometry, spectrofluorimetry, and nuclear magnetic resonance spectroscopy, we confirmed that one of the primary products being measured with this probe was $O_2^{-\bullet}$. Furthermore, we concluded that this ROS signal was not mitochondrial because it could not be significantly affected by either rotenone or CCCP. However, it is clear from the results presented in this study that mitochondrial ROS generation does not depend on the maintenance of mitochondrial membrane potential. Indeed, oxygen consumption was increased when $\Delta\psi$ was collapsed with CCCP and, in the presence of reagents that disrupt electron flow through the ETC, this loss of membrane potential was associated with a significant increase in ROS generation (Fig. 1C). In a similar fash-

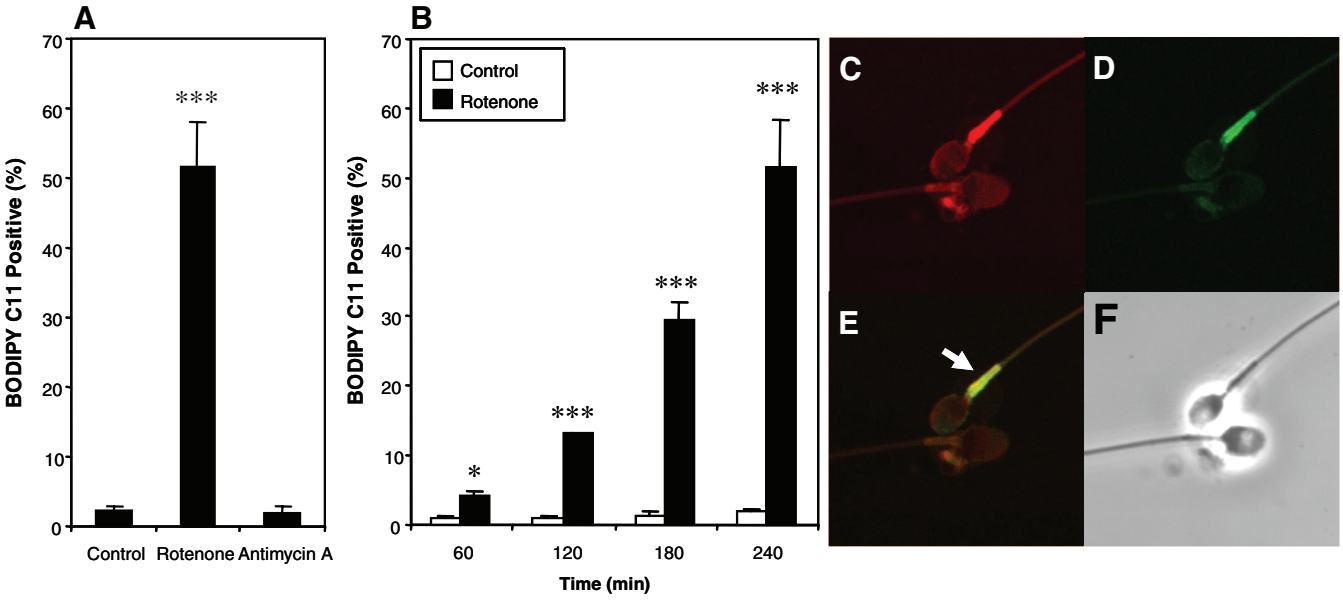

FIG. 5. Lipid peroxidation in human spermatozoa measured by BODIPY C_{11} fluorescence. A, The ability of mitochondrial inhibitors (10 μM) to stimulate lipid peroxidation in human spermatozoa. Spermatozoa were loaded with BODIPY C_{11} and then exposed to mitochondrial inhibitors over 240 min. Only rotenone induced a significant increase in lipid peroxidation ($P < 0.001$). B, This rotenone response was time dependent (*, $P < 0.05$; ***, $P < 0.001$). Confocal imaging of rotenone treated spermatozoa indicating red fluorescence associated with integration of the nonoxidized probe into the membrane of the spermatozoa (C), the green fluorescence indicating probe peroxidation (D), the overlayed images revealing the localization of lipid peroxidation in the midpiece, which appears yellow (E) (*arrow*), and the phase contrast image (F). Magnification, ×1,500.

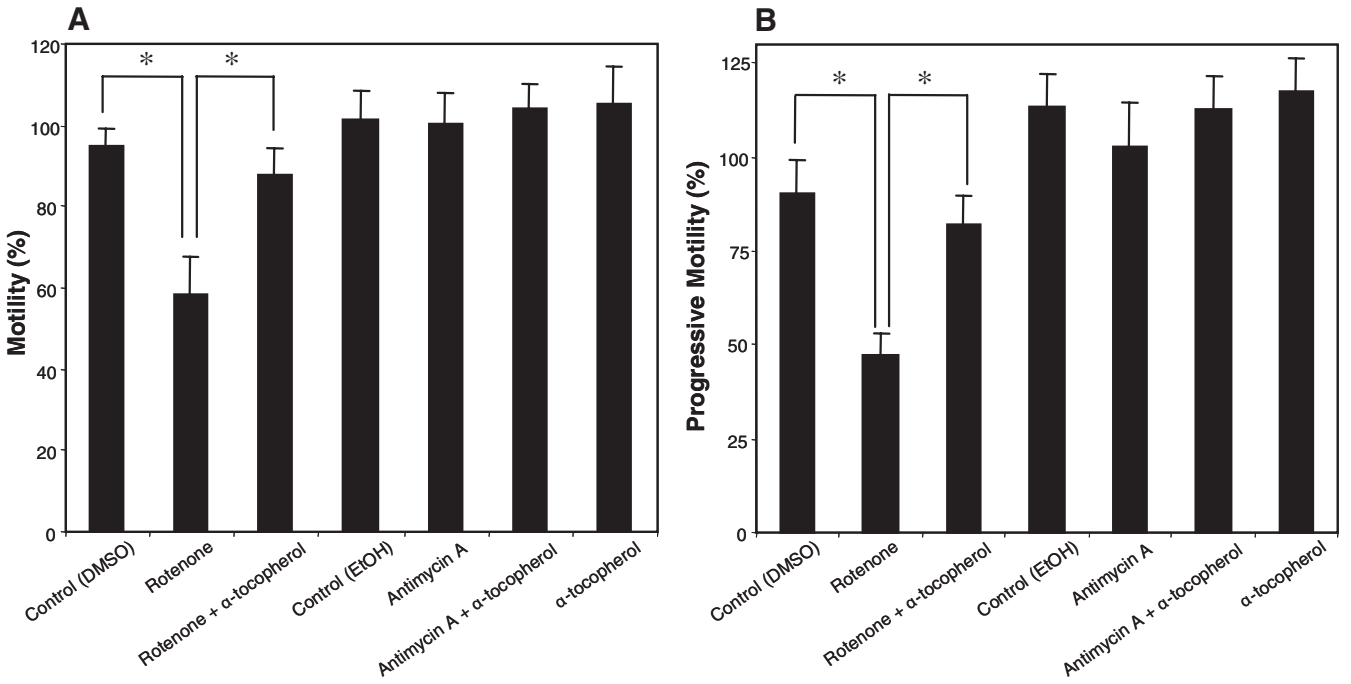

FIG. 6. Effect of mitochondrial ROS generation on sperm motility. All values expressed as a percentage of control (untreated) samples. Sperm total motility (A) and progressive motility (B) after pretreatment with α-tocopherol (0.5 mM) for 1 h, followed by exposure to mitochondrial inhibitors over 24 h; only treatment with rotenone resulted in a significant reduction in both total and progressive sperm motility (*, $P < 0.05$). However, this decline was prevented by the prior addition of α-tocopherol (*, $P < 0.05$). EtOH, Ethyl alcohol.

ion, CCCP has enhanced ROS production in carcinoma cell lines (29).

It is also clear from the data presented in Fig. 4 that the overall generation of ROS detected by DHE is highly correlated with the mitochondrial ROS generation. This mitochondrial ROS generation probably constitutes one of the more important sources of ROS in these highly susceptible cells. Because MSR is a cation, it is selectively targeted to the mitochondrial matrix and, thus, more able to respond to $O_2^{-\bullet}$ generated in this location than the uncharged DHE. This may explain why MSR stains more cells than DHE (Fig. 4C) when less than 20% of the cells are positive. The R^2 value associated with the DHE:MSR correlation (Fig. 4C; $R^2 = 0.6821$) suggests that 32% of ROS generation detected by DHE cannot be explained by variation in mitochondrial activity alone. Other sources of ROS are possible (21, 30) Where mitochondrial ROS are involved, the present data suggest that optimal activity depends on two factors: a disruption of $\Delta\psi$ and the impeded flow of electrons through the ETC. Previous studies have already reported that there is an inverse relationship between sperm motility and mitochondria membrane potential (31–33). Because mitochondrial ATP production is not required for the maintenance of motility in the presence of glucose (26), this association must be indirect and potentially mediated by oxidative stress. The second condition that must be met for optimal mitochondria ROS generation is perturbation of electron flow through the ETC. We have recently demonstrated that the presence of unesterified PUFAs such as arachidonic or docosahexaenoic acid stimulates ROS generation by human spermatozoa (34). Intriguingly, PUFAs have also been shown to collaps $\Delta\psi$ and trigger mitochondrial ROS generation by interfering with electron flow at complexes I and III (35). Furthermore, the unsaturated fatty acid content of human spermatozoa is positively correlated with ROS generation by these cells and negatively correlated with their motility (36, 37). In light of these data, we hypothesize that the presence of high levels of unesterified PUFAs in human spermatozoa triggers mitochondrial ROS generation from complexes I and III that overwhelm the limited antioxidant defenses offered by these cells. This results in a state of oxidative stress that induces peroxidative damage in the sperm tail, disrupting motility and plausibly accounting for the high levels of oxidative DNA damage seen in human spermatozoa (38).

In summary, these findings highlight the potential importance of aberrant mitochondrial activity in the etiology of defective sperm function, one of the most significant causes of human infertility (39).

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