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Reactive Oxygen Species and Human Spermatozoa

I. Effects on the Motility of Intact Spermatozoa and on Sperm Axonemes

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ABSTRACT: Mammalian spermatozoa are sensitive to oxygen-induced damages mediated by lipid peroxidation of the cell membrane. The aim of this study was to evaluate whether reactive oxygen species (ROS) could also induce axonemal damage. When Percoll-separated spermatozoa were treated with hydrogen peroxide, or the combination xanthine and xanthine oxidase (X + XO), there was a progressive decrease, leading to a complete arrest, in sperm flagellar beat frequency. Once demembranated in a medium containing magnesium adenosine triphosphate (Mg.ATP), ROS-immobilized spermatozoa still reactivated motility; however, the percentage and duration of motility obtained in these tests gradually decreased to zero in the next hour. In 50% of the cases, motility of intact spermatozoa spontaneously reinitiated after 6 to 24 hours of immobilization due to ROS treatment, although with percentages and beat frequencies lower than those of untreated spermatozoa. Studies using ROS scavengers (such as catalase, superoxide dismutase, and dimethylsulfoxide) indicated that hydrogen peroxide was the most toxic of the ROS involved, but that $\cdot O2^-$ and ·OH probably also played a role in immobilization of spermatozoa by ROS. The data suggest that ROS induce a chain of events leading to sperm immobilization, that axonemes are affected, and that limited endogenous repair mechanisms exist to reverse these damages.

Key words: Hydrogen peroxide, hydroxyl radical, demembranated spermatozoa, sperm axonemes, superoxide anion, sperm motility.

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The effects of reactive oxygen species (ROS), such as hydrogen peroxide, $\cdot O_2^-$, and ·OH, on sperm function and the toxicity of the fatty acid peroxides generated by their attack on the cell membrane phospholipids were recognized only a decade ago (Jones et al, 1979). Human spermatozoa are particularly sensitive to oxygen-induced damages mediated by lipid peroxidation because of their high content of polyunsaturated fatty acids and their lack of repair mechanisms (Aitken and Clarkson, 1987; Alvarez et al, 1987). Damaged or deficient spermatozoa form higher levels of ROS than normal spermatozoa, and could be a cause for idiopathic infertility (Aitken and Clarkson, 1988; Aitken and West, 1990). The production of ROS is also associated with loss of motility and decreased capacity for sperm–oocyte fusion (Aitken et al, 1989). In a recent study, Iwasaki and Gagnon (1992) showed that as many as 25% of semen samples from infertile men produced significant levels of ROS. They reported an inverse correlation between the percentage of motile spermatozoa and the amount of ROS detected. Semen contains scavenger systems to counteract the effects of ROS and prevent cellular damages. These systems include superoxide dismutase (SOD; Nissen and Kreysel, 1983), the glutathione peroxidase/reductase system (Alvarez et al, 1987; Alvarez and Storey, 1989), and catalase (Jeulin et al, 1989). Furthermore, a positive correlation between the level of SOD in spermatozoa and the duration of sperm motility has been reported (Alvarez et al, 1987).

Previously, it has been suggested that lipid peroxidation of the sperm membrane is responsible for the reduced sperm function observed after exposure to ROS (Jones et al, 1979; Alvarez et al, 1987). However, in these experiments, lipid peroxidation was observed after spermatozoa were incubated under conditions (vigorous shaking of a thin film of spermatozoa resuspended in a medium deficient in energy source) leading to a complete loss of motility as well as viability. In other systems, ROS have also been shown to produce extensive protein damage (Davies, 1987), cytoskeletal modifications (Hindshaw et al, 1986), and inhibition of a series of cellular mechanisms, such as mitochondrial respiration and protein DNA and RNA synthesis (Comporti, 1989).

The aim of the present study was to evaluate the effects of ROS on the motility of intact spermatozoa and on sperm

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axonemes under conditions in which sperm viability is not significantly altered and with levels of ROS compatible with those found in human semen and washed spermatozoa (Iwasaki and Gagnon, 1992). Demembranated sperm models (Gibbons and Gibbons, 1972; Mohri and Yanagimachi, 1980; de Lamirande et al, 1983) were used in this study to probe the integrity of the mechanics and energetics of flagellar beating. Our data show that sperm axonemes are affected by ROS, and that spermatozoa have some repair mechanisms to reverse the damage caused by ROS.

Materials and Methods

Materials

Catalase (from bovine liver), superoxide dismutase (SOD; from bovine erythrocytes), glutathione, mannitol, α-tocopherol, butylated hydroxytoluene (BHT), Triton X-100, nitroblue tetrazolium (NBT), horseradish peroxidase (HRPO), phenol, 4-aminoantipyrine, and phenol red were purchased from Sigma Chemical Company (St. Louis, MO). Xanthine and xanthine oxidase (from bovine milk) were obtained from Calbiochem (La Jolla, CA), and vanadium-free adenosine triphosphate (disodium salt) was obtained from Boehringer Mannheim (Montreal, Canada). Percoll was purchased from Pharmacia (Dorval, Canada) and collodion from JB EM Services (Dorval, Canada).

Preparation of Spermatozoa and Treatments

Whole semen samples were collected by masturbation after 3 days of sexual abstinence from healthy volunteers. The volunteer group consisted of 12 men, ranging in age from 22 years to 35 years, whose semen consistently had more than 300×10^6 spermatozoa with more than 60% progressive motility and no white blood cells. After liquefaction, samples were layered on discontinuous Percoll gradients. All Percoll solutions were buffered and made with isotonic with HEPES-balanced saline (HBS), which consisted of 130 mmol/L sodium chloride, 4 mmol/L potassium chloride, 0.5 mmol/L magnesium chloride, 14 mmol/L fructose, and 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) adjusted to pH 8.0 (Lessley and Garner, 1983; de Lamirande and Gagnon, 1991). The Percoll gradients were made of 0.2 ml of 95% Percoll and 2 ml of 65%, 40%, 20% Percoll. The gradients were centrifuged at room temperature at 1300 *g* for 30 minutes. Spermatozoa that were devoid of round cellular elements and debris and free of seminal plasma were recovered at the 65% to 95% interface and in the 95% Percoll layer. Percentages of motility ranged from 65% to 90%. When needed, sperm suspensions were diluted with the 95% Percoll solution (buffered and made isotonic with HBS), to a concentration of $100 \pm 20 \times 10^6$ sperm/ml. Spermatozoa were diluted with HBS, supplemented with the substances to be tested, or both. The final sperm concentration was $50 \pm 15 \times 10^6$ cells/ml and Percoll concentration was 45 ± 15%. Within these ranges of sperm and Percoll concentrations, ROS had similar effects on sperm motility.

Percoll-separated spermatozoa were treated at room temperature (22°C), unless stated otherwise, with hydrogen peroxide (0.1 to 5 mmol/L) or the combination of xanthine (0.1 to 2 mmol/L) and xanthine oxidase (0.025 to 0.1 U/ml). The ROS-scavenging capacities of various substances were also studied. Superoxide dismutase (SOD), catalase, dithiothreitol (DTT), glutathione (GSH), dimethylsulfoxide (DMSO), vitamin B_{12}, and mannitol were dissolved in HBS buffer. BHT and α-tocopherol were first dissolved in ethanol, then the ethanol was evaporated and the deposit was emulsified in HBS buffer by sonication. The table and figure legends provide information on the specific experimental conditions for each series of experiments.

Motility Measurements on Intact Spermatozoa

Motility of intact spermatozoa was evaluated at room temperature at various time intervals (15 to 30 minutes before motility arrest and hourly thereafter, except for the overnight period) after the beginning of ROS treatments. Measurements of beat frequency and percentage of motility were made from video recordings done on 10-μl portions of sperm suspension placed between Collodion-coated slides and coverslips (22 × 22 mm, thickness #1) using a high-speed camera (60 images/sec) as described by Chapeau and Gagnon (1987). The beat frequencies of at least 25 spermatozoa (randomly chosen, progressive, and without contact with other spermatozoa) were measured in each group for every time chosen. At least 200 spermatozoa were observed to determine the percentage of motility. In some experiments where the time constraint did not allow this type of motility assessment, a visual evaluation was performed. Rating systems were used to evaluate motility (+, + +, and + + + indicating 25%, 50%, and 75% motile spermatozoa, respectively) and beat frequencies (+, + +, and + + + indicating 1 to 2 Hz, 2 to 3 Hz, and 4 to 5 Hz, respectively).

Demembranation Reactivation Test

Axonemal integrity was assessed every 15 to 60 minutes after demembranation of spermatozoa in a medium containing 0.1% Triton X-100, 200 mmol/L sucrose, 25 mmol/L potassium glutamate (25 mmol/L glutamic acid brought to pH 8 with KOH), 1 mmol/L DTT, and 35 mmol/L Tris-HCl buffer pH 8 and reactivation with 0.5 mmol/L Mg.ATP (Mohri and Yanagimachi, 1980; de Lamirande et al, 1983). These tests were done with 0.25 ml medium in 15-mm petri dishes, and motility was again evaluated for the proportion of motile spermatozoa, quality of movement, and duration of reactivation (from the time Mg.ATP was added to the time when approximately 99% of all spermatozoa stopped moving). Beat frequencies were measured as described above for intact spermatozoa. The expression "positive test" means that a given intact sperm population could reactivate motility (at least 5% progressive spermatozoa) after demembranation in a medium containing Mg.ATP.

Detection of Reactive Oxygen Species

The generation of superoxide anion by XO + X was monitored by the change in absorbance (570 nm) of the NBT (pale yellow) to blue formazan (blue; Beauchamp and Fridovich, 1971). The presence of hydrogen peroxide in solution was assessed using the HRPO-dependent oxidation of phenol sulfophthalein (phenol red) to a blue derivative (Abs 630 nm; Pick and Mizel, 1981), or the HRPO-dependent oxidation of phenol in the presence of 4-aminoantipyrine to a red derivative (Abs 490 nm; Luck, 1963).

Viability Test

Equal volumes of sperm suspension, 5% eosin, and saturated nigrosin were mixed. The proportion of spermatozoa excluding the stain (alive) was determined. At least 200 cells were counted.

Results

Effect of Various Concentrations of Reactive Oxygen Species on Sperm Motility

The aim of the first series of experiments was to study the effects of ROS on sperm motility (intact and demembranated sperm models) and to determine concentrations that would stop motility without affecting cell viability. Preliminary results showed that hydrogen peroxide (2 and 5 mmol/L) or the superoxide-generating combination of xanthine oxidase (0.05 U/ml) plus xanthine (3 mmol/L) inhibited the motility of intact human spermatozoa in a short time (5 minutes to 30 minutes, depending on concentrations used). These treated spermatozoa were not reactivated with Mg.ATP after demembranation with Triton X-100 (Table 1). However, since these treatments also reduced sperm viability dramatically, the effects of lower concentrations of hydrogen peroxide and XO + X were investigated (Table 1). At very low doses of hydrogen peroxide (0.1 mmol/L) and of XO (0.05 U/ml) plus X (0.12 mmol/L), sperm motility (percentage, beat frequency) was unchanged for up to 8 hours. At intermediate concentrations (hydrogen peroxide = 0.25 or 0.5 mmol/L; XO = 0.05 U/ml; X = 0.3 or 0.6 mmol/L), sperm motility ceased 1 hour to 5 hours after treatment. At the time of complete arrest, the motility of demembranated spermatozoa could still be reactivated by the addition of Mg.ATP (positive test); however, in the next 30 minutes to 60 minutes, ROS-treated spermatozoa lost their capacity to reactivate motility after demembranation in the presence of Mg.ATP. Sperm viability was only slightly affected (by less than 20%). This observation was indirectly confirmed by spontaneous reinitiation of intact sperm motility (although usually with somewhat lower percentages than those observed in control samples), and positive demembranation–reactivation test results, several hours later. These concentrations of ROS-generating systems were used in most of the subsequent experiments, since they appeared to temporarily affect intact sperm motility and sperm axonemes. It should be noted that the ROS treatments used always had similar effects on sperm motility. The sequence of events leading to motility inhibition consistently followed the same pattern. In all sperm samples used, the original motility was high (65% to 90%), and there was always a progressive decrease in the flagellar beat frequency after ROS treatment, even if the percentage of motility remained at control levels. The duration of these

Table 1. *Effect of various concentration of hydrogen peroxide or xanthine and xanthine oxidase on the motility of intact and demembranated reactivated spermatozoa**

	Intact spermatozoa					Demembranation-reactivation test			
	Motility arrest		Motility reinitiation			Prevented		Reinitiated	
	Cases (%)	Time (hrs)	Cases (%)	Time (hrs)	Viability (% decrease)	Cases (%)	Time (hrs)	Cases (%)	Time (hrs)
Hydrogen peroxide (mmol/L)									
0.1	0	—	—	—	0	0			
0.25	50	2.3 ± 0.3 (1–4)	100	6–24	5–15	50	3.6 ± 0.6 (1.0–5.5)	100	6–24
0.5	100	1.6 ± 0.2 (0.5–4)	50	6–24	10–20	80	2.2 ± 0.2 (1–4)	50	6–24
1.0	100	0.5 ± 0.2 (0.3–1)	25	24	30–40	80	2.2 ± 0.3 (1–2)	50	24
2.0	100	0.4 ± 0.1 (0.2–0.5)	0	—	50–70	100	0.9 ± 0.1 (0.7–1)	0	—
5.0	100	0.3 ± 0.1 (0.1–0.5)	0	—	60–80	100	0.6 ± 0.2 (0.3–1)	0	—
Xanthine oxidase plus xanthine (mmol/L)									
0.12	0	—	—	—	0	0			
0.3	50	2.3 ± 0.3 (1–5)	100	5–24	5–10	30	3.5 ± 0.6 (3–5)	100	4–24
0.6	100	1.5 ± 0.1 (0.75–4)	50	5–24	10–20	90	2.2 ± 0.2 (1.5–4)	50	4–24
1.0	100	1.3 ± 0.2 (1–2)	0	—	30–50	100	2.1 ± 0.2 (1.5–3)	0	—
3.0	100	0.8 ± 0.2 (0.5–1)	0	—	50–70	100	1.1 ± 0.2 (0.8–1.5)	0	—

* Percoll washed human spermatozoa were treated with different concentrations of hydrogen peroxide (H_2O_2) or xanthine and xanthine oxidase (0.05 U/ml) at time zero. The percentage of sperm samples in which motility inhibition occurred (percent of cases) and the time at which motility was completely arrested (mean ± SEM, range between parenthesis) were noted. The percentage of sperm preparations in which motility spontaneously reinitiated and the time at which this reinitiation occurred (range) were noted, as well as the frequency of these events. Demembranation reactivation tests were performed on the same sperm samples, and the time at which reactivation of demembranated spermatozoa was not possible (prevented, mean ± SEM, range between parenthesis), as well as the time at which this reactivation was again possible (reinitiated, range), were noted. Cell viability was measured at 24 hours, using the eosin–nigrosin stain procedure, and compared to that of the control sperm population (no treatment). Individually, xanthine (up to 1 mmol/L), xanthine oxidase (up to 0.5 U/ml), and uric acid (up to 1 mmol/L, neutralized; end product of xanthine and xanthine oxidase reaction) had no significant effect on sperm motility. Percoll (10% to 80%) did not change the effects observed with reactive oxygen species treatments. Values given are for the range of the 3 to 20 different sperm preparations tested.

events varied and was a function of the sperm specimen used, but they were always followed by a dramatic fall in motility to zero in a short interval of time (30 minutes). As reported in Table 1, however, this loss of motility occurred at various times between 0.5 hours and 4.0 hours, depending on the sperm preparation. For this reason, representative results are presented in the following sections.

Presence of Superoxide and of Hydrogen Peroxide in Incubation Mixtures

Because of variations in the time at which sperm motility was completely arrested (see above), it seemed important to test for the presence of ROS in solution. In incubation mixtures containing XO (0.05 U/ml) and X (0.6 mmol/L), measurements of NBT reduction indicated that superoxide was generated over a 25-minute period (Fig. 1A). In the presence of spermatozoa (50 to 100 × 10^6 cells/ml) the same kinetics were observed, but the maximal absorbance obtained was lower (by 10% to 20%), possibly reflecting a scavenging effect of spermatozoa (data not shown). When X + XO was incubated 25 minutes before the addition of NBT, no reduction of this compound was observed. This result can be explained by the short half life (1 millisecond) of the superoxide anion. The combination of X (0.6 mmol/L) and XO (0.05 U/ml) also generated hydrogen peroxide (from the spontaneous dismutation of O_2^-), which reached a concentration of 0.25 mmol/L 15 minutes to 30 minutes after mixing (Fig. 1B). In the presence of spermatozoa (50 or 100 × 10^6 cells/ml), the amount of hydrogen peroxide in solution rapidly decreased, and was less than 20% of the original concentration after 60 minutes of incubation.

Hydrogen peroxide (0.5 mmol/L) was present in HBS buffer much longer, remaining at 75% of the original concentration 90 minutes after preparation (Fig. 1C). However, in the presence of spermatozoa (50 to 100 × 10^6 cells/ml), the amount of hydrogen peroxide in solution decreased rapidly, and there was only 10% to 20% of the original concentration after 60 minutes of incubation. The concentrations of hydrogen peroxide observed after 60 minutes of incubation were insufficient to affect sperm motility.

Effects of Hydrogen Peroxide and Xanthine Oxidase plus Xanthine on Sperm Motility

The effects of hydrogen peroxide and XO + X treatments (at concentrations found to consistently produce a complete inhibition of motility without affecting viability; see above and Table 1) on the motility of intact spermatozoa and on the motility obtained in demembranation–reactivation tests run in parallel are presented in Figure 2. Intact sperm motility (percentage and beat frequency), and demembranated–reactivated sperm motility (percentage, beat frequency, and duration of movement) were determined. In intact spermatozoa, the percentage of motility remained at the control

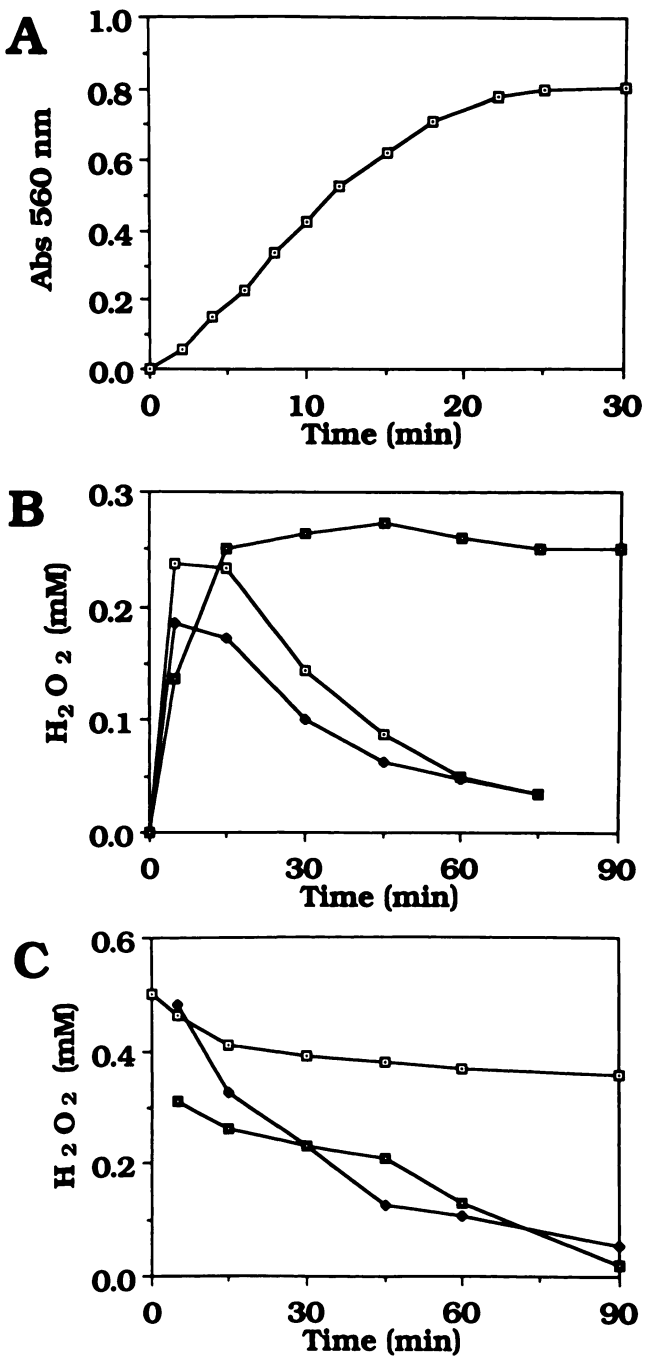

FIG. 1. Detection of reactive oxygen species in incubation mixtures containing xanthine and xanthine oxidase or H_2O_2. (A) The generation of superoxide anion by the combination XO (0.05 U/ml) + X (0.6 mmol/L) was monitored by the reduction of nitroblue tetrazolium (pale yellow) to blue formazan (blue). (B) The generation of H_2O_2 by the combination xanthine oxidase (0.05 U/ml) and xanthine (0.6 mmol/L). H_2O_2 was assessed using the horseradish peroxidase-dependent oxidation of phenol, in the presence of 4-aminoantipyrine, to a red derivative. X + XO alone = –■–; or in the presence of spermatozoa 50 × 10^6 cells/ml = –□–; or 100 × 10^6 cells/ml = –◆–. (C) Presence of H_2O_2 in solution. H_2O_2 was assessed using the horseradish peroxidase-dependent oxidation of phenol red to a blue derivative. H_2O_2 alone = –□–; with spermatozoa 50 × 10^6 cells/ml = –◆–; or 100 × 10^6 cells/ml = –■–.

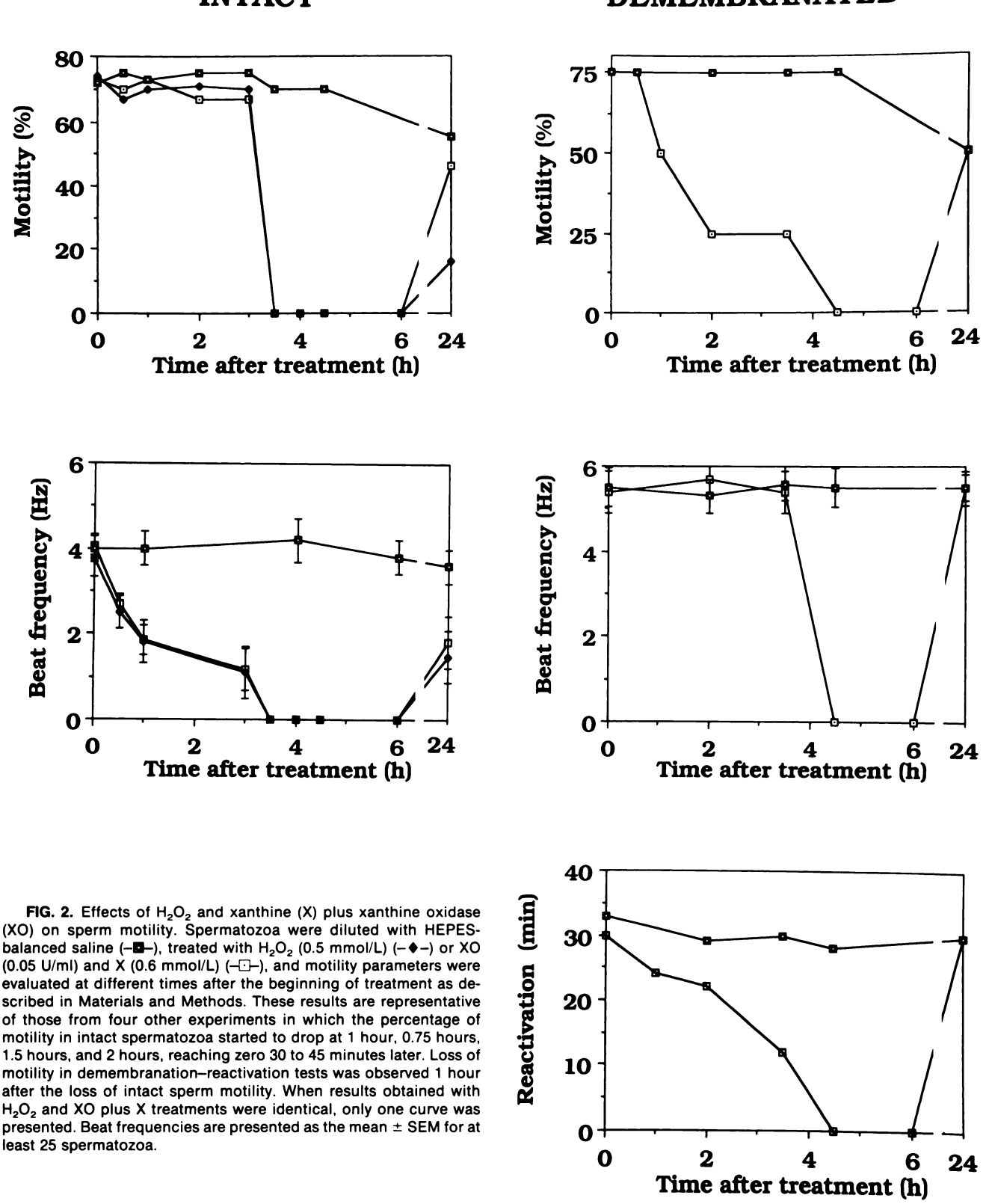

FIG. 2. Effects of H_2O_2 and xanthine (X) plus xanthine oxidase (XO) on sperm motility. Spermatozoa were diluted with HEPES-balanced saline (–■–), treated with H_2O_2 (0.5 mmol/L) (–◆–) or XO (0.05 U/ml) and X (0.6 mmol/L) (–□–), and motility parameters were evaluated at different times after the beginning of treatment as described in Materials and Methods. These results are representative of those from four other experiments in which the percentage of motility in intact spermatozoa started to drop at 1 hour, 0.75 hours, 1.5 hours, and 2 hours, reaching zero 30 to 45 minutes later. Loss of motility in demembranation–reactivation tests was observed 1 hour after the loss of intact sperm motility. When results obtained with H_2O_2 and XO plus X treatments were identical, only one curve was presented. Beat frequencies are presented as the mean ± SEM for at least 25 spermatozoa.

level for the first 3 hours after ROS treatment and then dropped to zero in the next 30 minutes. On the other hand, there was an immediate and progressive decrease in the beat frequency. At various times after treatment, these spermatozoa were demembranated and reactivation was attempted. The percentage of motility and the duration of reactivation obtained decreased progressively, whereas the beat frequency of reactivated spermatozoa did not change during this period. It should also be noted that, when intact spermatozoa were immobilized, the result of the demembranation–reactivation test was still positive for the next 60 minutes to 75 minutes. Intact sperm could spontaneously reinitiate motility 6 hours to 24 hours after treatment, although with reduced percentage and beat frequency. Their demembranated–reactivated counterparts had the same percentage of motility and beat frequency as the untreated spermatozoa tested at the same time points.

Effects of Reactive Oxygen Species on Demembranated Spermatozoa

Reactivation of demembranated spermatozoa, when it did occur, was always accompanied by normal beat frequencies (5.5 to 6 Hz; Fig. 2). Furthermore, addition of hydrogen peroxide (up to 5 mmol/L), XO + X (up to 0.6 mmol/L), or the products of the reaction of X + XO (30 minutes incubation) to control demembranated–reactivated spermatozoa did not affect motility parameters (percentage, type, and duration of movement; data not shown). These data suggest that the axonemal force-generating dynein ATPase was not directly affected by ROS. The possibility that a cytosolic dynein inhibitor, present in ROS-treated spermatozoa, could be responsible for the lowered beat frequency observed in these spermatozoa was examined. Cytosolic extracts (de Lamirande et al, 1986) made by freezing and thawing concentrated untreated and ROS-immobilized spermatozoa were prepared and used as media for the demembranation–reactivation test for untreated spermatozoa. Cytosolic extracts from ROS-treated spermatozoa allowed as high a level of reactivated motility (percentage, beat frequency, and duration) as did extracts from untreated spermatozoa (data not shown).

Effect of the Concentration of Xanthine Oxidase

To determine whether the period of time during which ROS are produced had any influence on the chain of events leading to sperm immobilization, a set of experiments was performed in which the amount of xanthine was fixed at 0.6 mmol/L but the amount of xanthine oxidase varied (Fig. 3). As the concentration of enzyme was increased (ROS generated were produced over shorter time periods; data not shown), the effects on sperm motility became more pronounced. With the lowest concentration of XO (0.025 U/ml), sperm motility disappeared completely at 3 hours, but reappeared at 6 hours. However, demembranation–reactivation test results were always positive, although lower levels of motility (% and duration) were observed. With the highest concentration of enzyme (0.1 U/ml), sperm motility was permanently lost 1 hour after the begin-

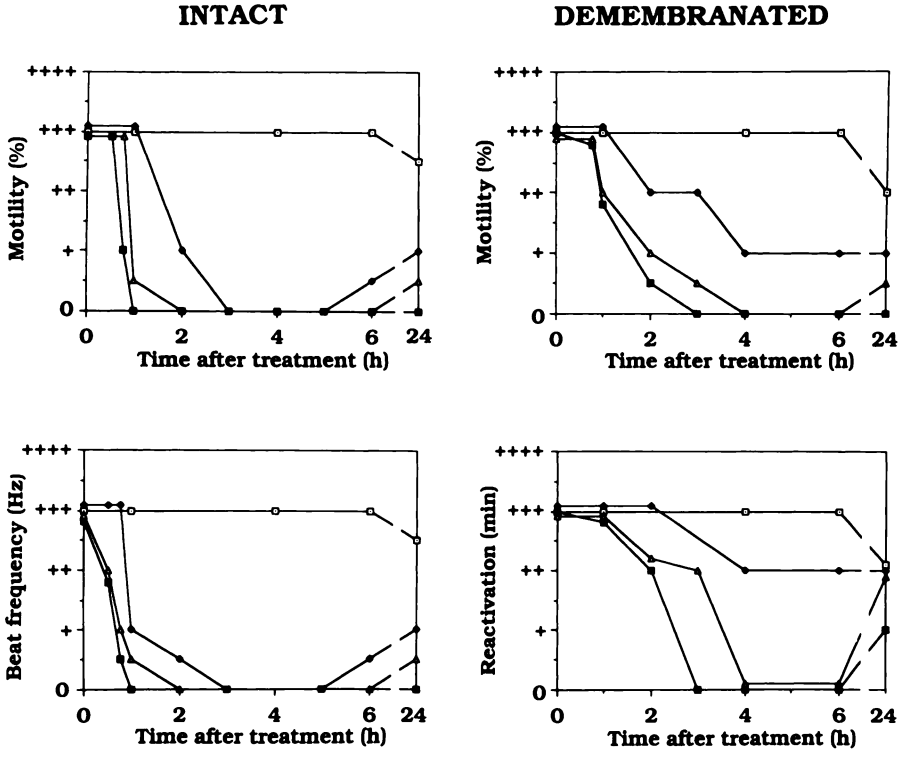

FIG. 3. Effects of various concentrations of xanthine oxidase on the motility of spermatozoa treated with xanthine (X) plus xanthine oxidase (XO). Spermatozoa were treated with xanthine (0.6 mmol/L) and xanthine oxidase at time zero. Untreated spermatozoa (–□–); XO, 0.025 U/ml (–◆–); XO, 0.05 U/ml (–△–); XO, 0.10 U/ml (–■–). Motility of intact spermatozoa was evaluated visually. For percentages, + + + = ≥ 75%; + + = ≥ 50%; + = ≥ 25%. For beat frequencies, + + + = 4–5 Hz; + + = 2–3 Hz; + = 1–2 Hz. Demembranation–reactivation tests were performed in parallel and percentages and duration of motility were evaluated. For percentages, see above. For duration, + + + = 15–20 minutes; + + = 10–15 minutes; + = 5–10 minutes. When spermatozoa in a sample showed intermediate degrees of motility, intermediate values were plotted on the graphs. These results are representative of three other experiments in which the motility of intact spermatozoa was arrested at 0.75 hours, 1 hour, and 1.5 hours with the highest concentration of XO. Loss of motility in demembranation–reactivation tests was observed 1 hour after the loss of intact sperm motility.

ning of treatment. The axonemes were also more affected, since 3 hours after treatment the motility of spermatozoa in the demembranation–reactivation test was decreased to zero. The total amount of ROS produced, as well as the period of time over which they were produced, appeared important in the overall effects of ROS on spermatozoa.

Effect of the Duration of Reactive Oxygen Species Treatment

Time course experiments were performed to examine the relationship between loss of motility and the time of contact between spermatozoa and ROS. Spermatozoa treated with XO + X (0.05 U/ml and 0.6 mmol/L, respectively) were washed on small Percoll gradients (0.4 ml of 20% Percoll layered over 0.05 ml of 95% Percoll in a microfuge tube) at various times after treatment (Fig. 4). Even 1 minute of contact between spermatozoa and ROS was sufficient to induce a significant decrease in the beat frequency (to 1 to 2 beats/sec) of intact spermatozoa and a decrease of motility in the corresponding demembranation–reactivation test results (Fig. 4). With longer periods of exposure (5 minutes and 15 minutes), the effects of ROS became more pronounced, lasted longer, and were less reversible. When the experiment was repeated with hydrogen peroxide (0.5 mmol/L), similar data were obtained, although 5 to 10 minutes of contact with ROS before washing was needed to produce a significant decrease in sperm motility (equivalent to the effect the ROS produced in 1 minute by 0.05 U/ml XO + 0.6 mmol/L X). These data suggest that the initial burst of ROS was the most important in the production of deleterious effects on spermatozoa

Effect of Temperature on the Inhibition of Motility Caused by Hydrogen Peroxide or Xanthine Oxidase plus Xanthine

Spermatozoa treated at 37°C instead of 22°C were much more affected by hydrogen peroxide and XO + X. The concentrations of these substances had to be reduced by a factor of 3 to 5 (hydrogen peroxide = 0.1 mmol/L; XO = 0.05 U/ml; xanthine = 0.2 mmol/L) to obtain comparable losses of motility without significant losses of viability. Except for the difference in the concentration of ROS used, similar effects on intact sperm motility and in the demembranation–reactivation test results were obtained at 37°C and 22°C (data not shown).

Effect of Reactive Oxygen Scavengers on the Motility of Spermatozoa Treated with Hydrogen Peroxide or Xanthine Oxidase plus Xanthine

To determine which of the ROS (O_2^-, H_2O_2, and OH) was responsible for decreased motility, the effects of a series of ROS scavengers were tested on spermatozoa incubated with hydrogen peroxide or X+XO. Catalase (8 μg/ml) totally counteracted the toxic effects of ROS treatment (Fig. 5). Spermatozoa treated with ROS and catalase could not be differentiated from untreated spermatozoa. With a lower concentration of catalase (3 μg/ml), there was a protection against the loss of motility (percentage), but not against a

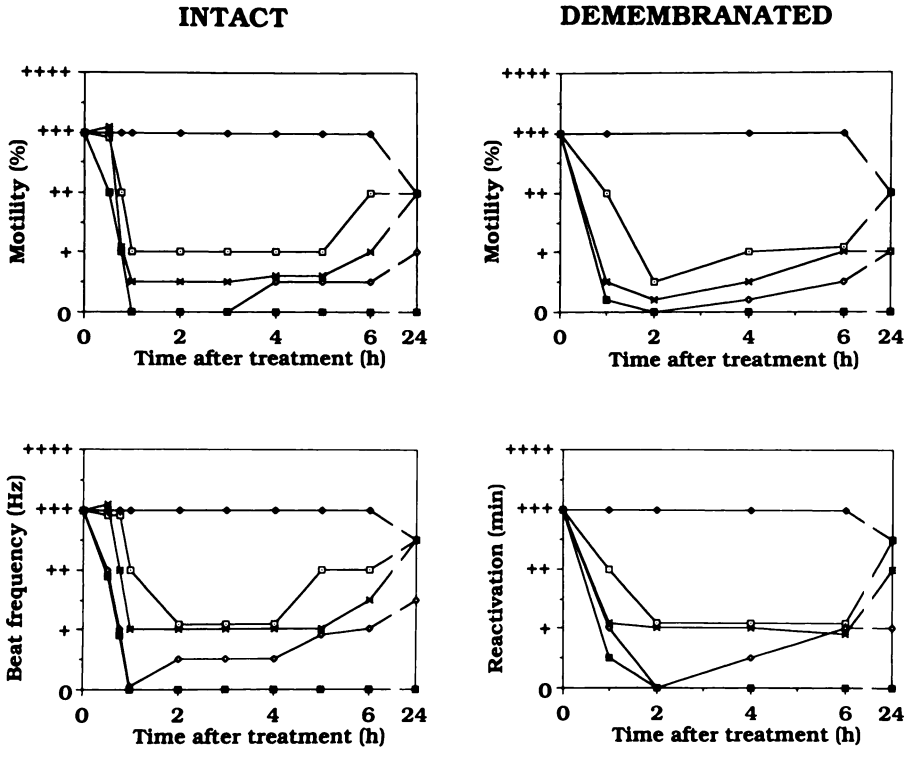

FIG. 4. Effects of various incubation periods between spermatozoa and reactive oxygen species (ROS) on the motility of spermatozoa treated with xanthine plus xanthine oxidase. Spermatozoa were treated with xanthine (0.6 mmol/L) and xanthine oxidase (0.05 U/ml) at time zero. They were washed on a small Percoll gradient at various times after the beginning of treatment. Untreated spermatozoa (–◆–); 1-minute contact with ROS (–□–); 5-minute contact with ROS (*); 15-minute contact with ROS (–◇–); total contact, no wash (–■–). These results are representative of three experiments in which the extent of motility loss was similar but happened at 1 hour, 1.5 hours, and 2 hours (intact spermatozoa).

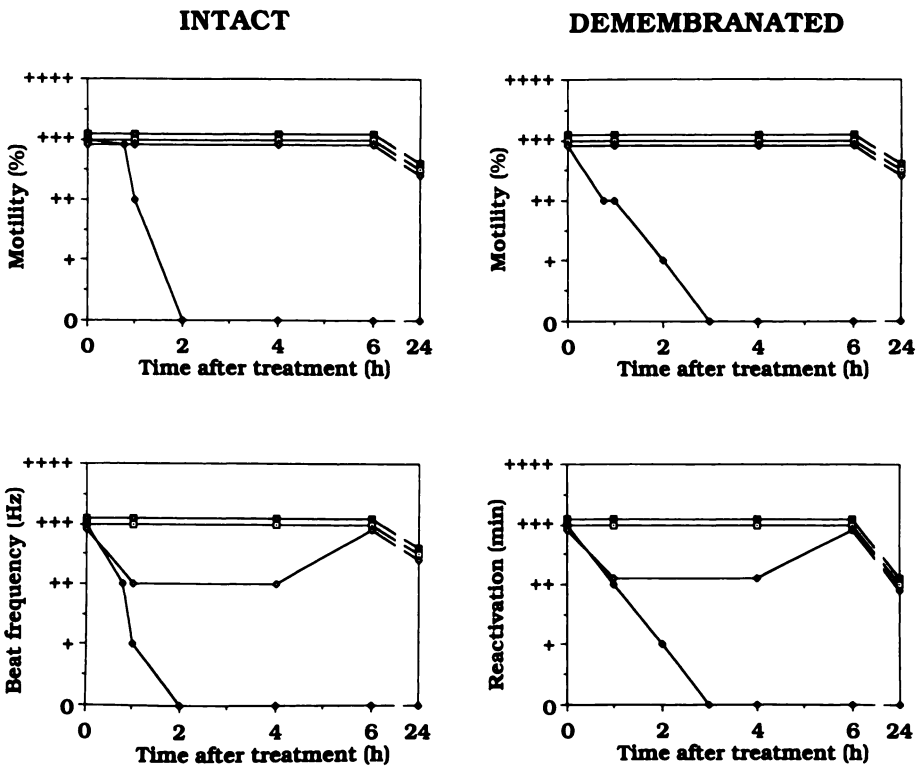

FIG. 5. Effect of catalase on motility inhibition due to reactive oxygen species (ROS) treatments. Spermatozoa were treated with H_2O_2 (0.5 mmol/L) or xanthine oxidase (0.1 U/ml) and xanthine (0.6 mmol/L) in the absence or presence of catalase. Untreated spermatozoa, (–□–); ROS-treated spermatozoa, (–◆–); ROS treatment with catalase 8 μg/ml, (–■–); ROS treatment with catalase 3 μg/ml (–◇–). Motility was evaluated as described in the legend for Figure 3. These results are representative of three other experiments in which similar effects were observed but motility of intact spermatozoa was lost at 1 hour, 1.5 hours, and 2 hours. Loss of motility in demembranation–reactivation tests was observed 1 hour after the loss of intact sperm motility.

temporary decrease in beat frequency (to 2 to 3 beats/sec). Furthermore, the duration of motility observed with demembranated–reactivated spermatozoa was less (by 20% to 30%) than that observed with untreated spermatozoa.

Superoxide dismutase (SOD) was much less effective than catalase as scavenger for ROS (Fig. 6). Even at a very high concentration (1 mg/ml), SOD could not prevent a temporary decrease in the percentage of motility (to +/++) and in the beat frequency (to 2 to 3 beats/sec) of intact spermatozoa treated with ROS. When these spermatozoa were demembranated and reactivated, however, their motility was similar (in percentage and duration of movement) to that of untreated spermatozoa (Fig. 6). With lower doses (0.1 to 0.25 mg/ml) of SOD, the effects of ROS on sperm motility were more pronounced and lasted longer than with the higher concentration of SOD.

The protection against ROS effects allowed by DMSO (140 mmol/L) was limited (Fig. 7). The motility of intact spermatozoa was completely inhibited, but could reinitiate 2 hours to 4 hours later (Fig. 6), whereas 6 hours to 24 hours were necessary in the absence of DMSO (Table 1, Fig. 2). Lower concentrations of DMSO (70 and 35 mmol/L) did not afford any protection.

Other ROS scavengers or antioxidants had various effects (data not shown). In the presence of DTT (1 mmol/L) or GSH (1 mmol/L), motility arrest due to ROS was delayed but not prevented. Mannitol (100 mmol/L), sodium benzoate (100 mmol/L), vitamin B_{12} (0.1 mmol/L), and BHT (1 mmol/L) did not modify the inhibitory effects of ROS on sperm motility. α-tocopherol (10 mmol/L) provided partial protection against ROS effects: motility was lost but reappeared within 2 to 3 hours.

Discussion

The present data indicate that ROS affect sperm axonemes. We first evaluated the effects of various concentrations of ROS on sperm axonemes. Percoll-separated spermatozoa, immobilized after contact with high concentrations of hydrogen peroxide (2 or 5 mmol/L, as used by Alvarez and Storey, 1989), could not reactivate motility after demembranation in a medium containing Triton X-100 and Mg.ATP. These data suggest that ROS affected the sperm axoneme. However, considering the high doses of ROS used and the resulting loss of viability, this axonemal damage may have been due to protein damage after cell death rather than to ROS treatment itself. On the other hand, when concentrations of hydrogen peroxide (0.5 mmol/L) and XO (0.05 U/ml) plus X (0.6 mmol/L) were adjusted to allow inhibition of motility (in 100% of the cases) but with marginal effects on sperm viability (Table 1), the integrity of sperm axonemes, as measured by the demembranation–reactivation test, was still affected. Using these moderate ROS concentrations, the motility of ROS-treated spermatozoa could spontaneously reinitiate several hours after immobilization, although with somewhat lower percentages

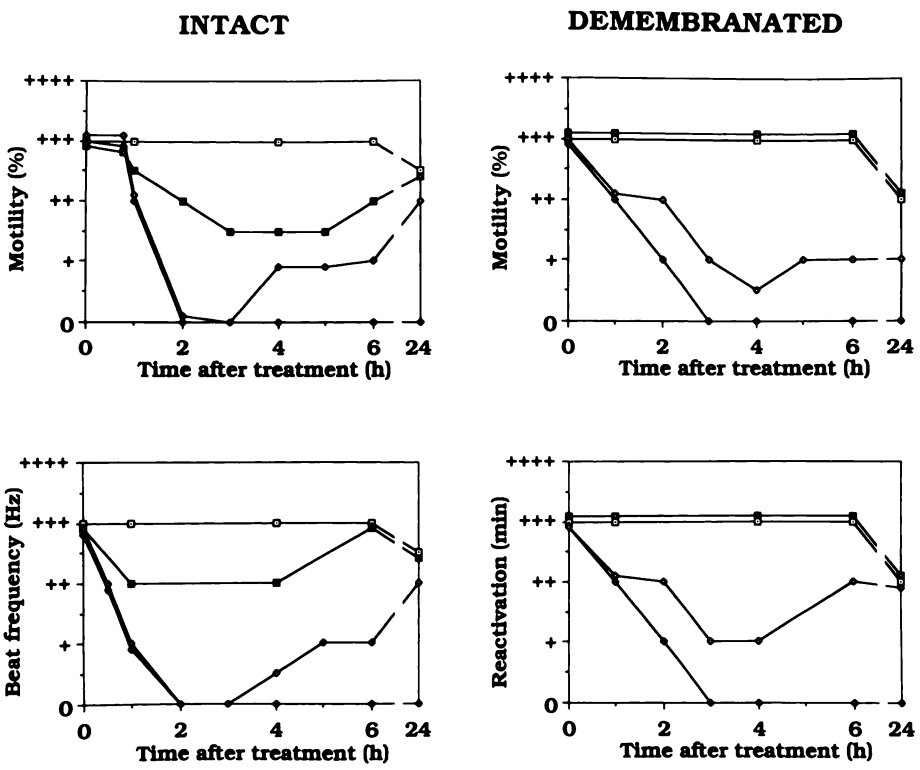

FIG. 6. Effect of superoxide dismutase (SOD) on motility inhibition due to reactive oxygen species (ROS) treatments. Spermatozoa were treated with H_2O_2 (0.5 mmol/L) or xanthine oxidase (0.1 U/ml) and xanthine (0.6 mmol/L) in the absence or presence of SOD. Untreated spermatozoa, (–□–); ROS-treated spermatozoa, (–♦–); ROS treatment and SOD 1 mg/ml, (–■–); ROS treatment with SOD 200 μg/ml, (–◇–). Evaluation of motility parameters is described in the legend for Figure 3. These results are representative of two experiments in which similar effects were observed but motility of intact spermatozoa was lost at 1 hour and 1.5 hours.

than untreated spermatozoa (Table 1, Fig. 2). Simultaneously, axonemes recovered their functional state, showing a motility in the demembranation–reactivation test that was often similar to that observed with untreated spermatozoa. It is also noteworthy that the amount of ROS generated by XO + X or hydrogen peroxide in the present study was of the same order of magnitude as that found in the semen of some infertile patients (Iwasaki and Gagnon, 1992). These authors reported an inverse correlation between the percentage of motile spermatozoa and the amount of ROS detected.

The possibility of a direct action of ROS on the axoneme is unlikely, since hydrogen peroxide (up to 4 mmol/L) and XO (0.05 U/ml) + X (0.6 mmol/L) had no significant effect on the motility parameters (percentage of motility, beat frequency, and duration) when added to control demembranated–reactivated spermatozoa. The data from two other series of experiments are consistent with the hypothesis that the changes observed at the axonemal level were secondary to other changes induced by ROS. The presence of ROS lasted for 30 minutes to 120 minutes, depending on the ROS studied (Fig. 1), yet spermatozoa were immobilized 1 hour to 4 hours after the beginning of incubation, a time at which ROS were absent or present at very low concentrations. Spermatozoa subjected to XO + X for 1 minute and then washed on small Percoll gradient had decreased motility, even if these substances and the ROS were removed (Fig. 4). These data suggest that the inhibitory action of ROS on sperm motility is not direct, but is mediated by other substances. It is known that lipid hydroperoxides are formed by the action of ROS on membrane lipids and that they are themselves toxic (Jones et al, 1979). Lipid hydroperoxides can also be degraded to 4-hydroxyalkenals (Comporti, 1989). One of these products, (E)-4-hydroxy-2-nonenal, was found to be spermicidal at a concentration of 50 μmol/L (Selley et al, 1991). These compounds are very reactive and highly toxic for most cellular biochemical reactions (such as mitochondrial respiration and protein, DNA and RNA synthesis; Comporti, 1989), and could be the intermediate factors responsible for the effects observed in the experiments reported here.

One of the striking effects observed after ROS treatment was the rapid decrease in the beat frequency of intact spermatozoa. The possibility of a lowered dynein ATPase activity after ROS treatment could explain this phenomenon, but it appears unlikely, since the beat frequencies obtained in demembranation–reactivation tests were always similar to those of control spermatozoa (Fig. 2). The possibility that ROS-treated spermatozoa contain an intracellular inhibitor of dynein ATPase also appears unlikely, since cytosolic extracts from control and ROS-treated spermatozoa allowed similar levels of reactivated motility of control spermatozoa. However, this possibility can not be rejected, because these cytosolic inhibitors could be unstable, too diluted, or tested in suboptimal conditions. Another explanation for the low beat frequency observed in ROS-treated spermatozoa is

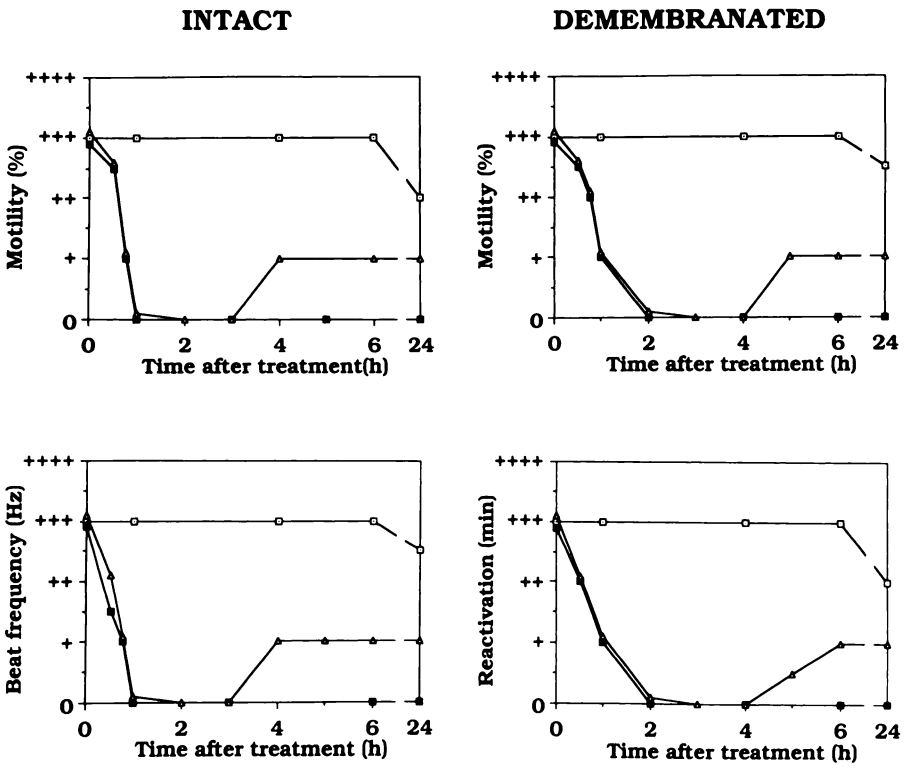

FIG. 7. Effect of dimethylsulfoxide (DMSO) on motility inhibition due to reactive oxygen species (ROS) treatments. Spermatozoa were treated with H_2O_2 (0.5 mmol/L) or xanthine oxidase (0.1 U/ml) and xanthine (0.6 mmol/L) in the absence or the presence of DMSO. Untreated spermatozoa, (–⊡–); ROS-treated spermatozoa, (–■–); ROS treatment with DMSO 140 mmol/L, (–△–). Motility evaluation is described in the legend for Figure 3. These results are representative of three experiments in which similar effects were observed but motility of intact spermatozoa was lost at 1 hour, 1.5 hours, and 2 hours. Loss of motility in demembranation–reactivation tests was observed 1 hour after the loss of intact sperm motility.

a decrease in the intracellular ATP concentration. Furthermore, since ATP is a substrate for several essential enzymes in spermatozoa (such as ATPases and protein kinases), it is conceivable that a decrease in its concentration is responsible for the arrest of motility (de Lamirande and Gagnon, 1992). Catalase (Jeulin et al, 1989), SOD (Alvarez and Storey, 1989), and the glutathione peroxidase/reductase system (Alvarez et al, 1987; Alvarez and Storey, 1989) are known scavenger systems for sperm ROS. The wide variations in levels of scavenging systems reported in spermatozoa (Alvarez et al, 1987) and the fact that the effects of ROS treatment varied in time course from one sample to another suggest the importance of these scavengers in guarding against loss of motility. Of the agents tested, only catalase could totally prevent the effects of XO + X or hydrogen peroxide (Fig. 5). Since this enzyme is specific for hydrogen peroxide, this result supports the suggestion made by Alvarez and Storey (1989) that hydrogen peroxide is the most toxic ROS for spermatozoa. However, the fact that SOD and DMSO also afforded partial protection against ROS treatment (Figs. 6, 7) suggests that $\cdot O_2^-$ and $\cdot OH$ play a minor role in ROS immobilization of spermatozoa.

It has been suggested that several enzymatic activities other than dynein ATPase play a role in the regulation of axonemal movement, including cAMP-dependent protein kinase (Lindemann, 1978; Tash et al, 1987), protein methylase (Gagnon et al, 1986), axonemal protease (de Lamirande and Gagnon, 1986; Cosson and Gagnon, 1988), and axonemal transglutaminase (de Lamirande and Gagnon, 1989). Temporary inhibition of one or more of these enzymes, or of the synthesis of their substrates due to ROS treatment, could explain the results reported here. Major structural damage, irreversible modifications of proteins, or permeabilization of cell membranes are less likely explanations for the results obtained, since spermatozoa can reinitiate motility after a few hours of immobilization. The following paper will address the role played by cAMP-dependent protein phosphorylation in spermatozoa treated with ROS (de Lamirande and Gagnon, 1992).

The data presented here provide evidence that ROS treatment can affect sperm axoneme function. The effects observed on sperm motility (of intact and demembranated–reactivated cells) are probably due to a chain of reactions induced by ROS rather than to a direct reaction of ROS with axonemal proteins. Under these conditions, limited endogenous repair mechanisms exist to reverse the damages caused by ROS, since motility can spontaneously reinitiate 6 hours to 24 hours after exposure to ROS. The fact that ROS production in semen was found to be inversely correlated with motility stresses the need for further study of the mechanisms of ROS actions on spermatozoa and of repair mechanisms.

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