Journal of Andrology, Vol. 23, No. 2, March/April 2002

Detection of Lipid Peroxidation in Equine Spermatozoa Based Upon the Lipophilic Fluorescent Dye C_{11}-BODIPY$^{581/591}$

BARRY A. BALL AND ANTHONY VO

From the Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California.

ABSTRACT: The lipophilic fluorescent probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$) was used to evaluate changes in lipid peroxidation in equine spermatozoa during both short-term exposure to ferrous sulfate and sodium ascorbate in the presence of cumene hydroperoxide as well as during storage of spermatozoa at 5°C for 48 hours. Peroxidation of C_{11}-BODIPY$^{581/591}$ was accompanied by a shift in fluorescence from red to green, and the relative amount of nonoxidized probe was determined as the ratio of red:(red + green) fluorescence as detected by either fluorescence microplate reader or by flow cytometry. The addition of Fe_2SO_4 (0 to 0.5 mM), low concentrations of sodium ascorbate, and the addition of cumene hydroperoxide increased peroxidation of C_{11}-BODIPY$^{581/591}$. The addition of high concentrations (10 or 20 mM) of sodium ascorbate or α-tocopherol reduced peroxidation of C_{11}-BODIPY$^{581/591}$ during short-term incubations. During storage at 5°C in a skim milk–based extender, equine spermatozoa demonstrated a progressive decline in motility and a small but significant increase in lipid peroxidation based upon ratiometric analysis of C_{11}-BODIPY$^{581/591}$. The addition of Fe_2SO_4 increased lipid peroxidation in cooled spermatozoa in a dose-dependent fashion and decreased sperm motility. The addition of α-tocopherol, however, did not reduce lipid peroxidation during cooled semen storage. These data demonstrate that the lipophilic fluorescent probe C_{11}-BODIPY$^{581/591}$ is a useful measurement of lipid peroxidation in equine spermatozoa and that there is an increase in lipid peroxidation during cooled storage of equine spermatozoa that is increased in the presence of ferrous promoters.

Key words: Stallion, semen, oxidative stress, α-tocopherol, fluorescent probe.

J Androl 2002;23:259–269

Equine spermatozoa, like those of other mammals, have a relatively high content of polyunsaturated fatty acids in their plasma membrane (Komarek et al, 1965; Parks and Lynch, 1992). The high polyunsaturated fatty acid content of spermatozoa is believed to be important in maintaining the fluidity and fusogenicity of the membrane, which are important in events such as acrosomal exocytosis (Flesch and Gadella, 2000). Unfortunately, the high polyunsaturated fatty acid content of sperm membrane also increases the susceptibility of spermatozoa to peroxidative damage (Jones and Mann, 1973; Aitken et al, 1993b; Storey, 1997; Halliwell and Gutteridge, 1999). Lipid peroxidation proceeds with the extraction of hydrogen and the formation of a number of reactive intermediates that can result in a chain reaction or propagation of peroxidation within the membrane (Aitken et al, 1993b; Storey, 1997). Extensive membrane lipid peroxidation results in a loss of membrane fluidity (Borst et al, 2000) and a concomitant loss of sperm function (Aitken et al, 1993b). Changes in sperm function as a consequence of lipid peroxidation include decreased sperm-oocyte fusion (Aitken et al, 1993a; Kodama et al, 1996), reduced sperm motility (Alvarez and Storey, 1982, 1983, 1984; Aitken et al, 1993a), alteration in membrane permeability and metabolism (Jones and Mann, 1977; Jones et al, 1979; Ohta et al, 1989), as well as damage to sperm chromatin (Hughes et al, 1996; Kodama et al, 1997; Donnelly et al, 1999).

Previously, detection of membrane lipid peroxidation in spermatozoa has been based primarily on the detection of end products of lipid peroxidation such as malondialdehyde or 4-hydroxyalkenals (Jones and Mann, 1973; Aitken et al, 1993b; Gomez et al, 1998; Baumber et al, 2000) or by the evaluation of extracted membrane lipids using thin-layer chromatography (Jones and Mann, 1977; Alvarez and Storey, 1992). Although these methods are sensitive and can detect the end-point reaction products of lipid peroxidation, they are relatively elaborate and provide only an indirect measure of lipid peroxidation without subcellular resolution of the membrane changes (Pap et al, 2000).

As an alternative to the detection of end products such as malondialdehyde, a variety of lipophilic fluorescent probes have been used to assess lipid peroxidation in so-

Supported by the John P. Hughes Endowment and by the Center for Equine Health with funds provided by the Oak Tree Racing Association, the State of California pari-mutuel fund, and contributions by private donors.

Correspondence to: Dr Barry A Ball, Room 1133 Tupper Hall, Department of Population Health and Reproduction, University of California at Davis, Davis, CA 95616 (e-mail: baball@ucdavis.edu).

Received for publication August 16, 2001; accepted for publication October 31, 2001.

matic cells. These include *cis*-paranaric acid (van den Berg et al, 1992) and various derivative of fluoroscein (Makrigiorgos et al, 1997; Chung and Benzie 2000). Recently, the application of a fluorescent fatty acid probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$) has been described in a number of studies with somatic cells as a means to monitor lipid peroxidation in living cells (Naguib, 1998; Pap et al, 1999, 2000; Borst et al, 2000). The probe has the advantage of being used in a ratiometric method to determine membrane lipid peroxidation, which reduces problems with artifacts due to differences in uptake and distribution of the probe (Pap et al, 2000). The probe shifts from red (em = 581; ex = 591) to green (ex = 500; em = 510) upon oxidation, and the ratio of red and green fluorescence has been used as a measurement of lipid peroxidation in living cells (Pap et al, 2000). The dye is relatively nonfluorescent in solution and has been evaluated by fluorescence microplate reader, by epifluorescence microscopy, by confocal microscopy, and by flow cytometry (Pap et al, 1999, 2000). The fluorescence changes in C_{11}-BODIPY$^{581/591}$ reflect indirectly the oxidation of unsaturated fatty acids (Borst et al, 2000), and the peroxidation rate of C_{11}-BODIPY$^{581/591}$ is comparable to that of endogenous fatty acids (approximately twofold greater than arachadonic acid; Pap et al, 1999). The probe possesses 2 conjugated double bonds that are susceptible to oxidation, and oxidation of these diene bonds results in a shift in the wave length for both excitation and emission of the probe (Naguib, 1998; Borst et al, 2000).

The objective of the study reported here was to evaluate lipid peroxidation of equine spermatozoa based upon evaluation of the ratiometric fatty acid probe, C_{11}-BODIPY$^{581/591}$, and to evaluate lipid peroxidation in spermatozoa stored for 48 hours at 5°C.

Materials and Methods

Reagents

Unless otherwise noted, all chemicals and reagents were obtained from Sigma Chemical Company, St Louis, Mo. C_{11}-BODIPY$^{581/591}$ was obtained from Molecular Probes (Eugene, Ore). Because bovine serum albumin (BSA) acts as an antilipoperoxidative agent (Alvarez and Storey, 1995), a modified Tyrodes medium (TALP) in which BSA was replaced by polyvinyl alcohol (1.0 mg/mL) was used in all experiments (Bavister, 1989).

Sample Preparation

Semen samples were collected from light-horse stallions with an artificial vagina and filtered to remove gel. Raw semen was layered on a discontinuous Percoll gradient (40%/80%) and centrifuged (300 × *g* for 20 minutes) to isolate motile spermatozoa from seminal plasma (Drobnis et al, 1991). Spermatozoa were then washed in a modified TALP (325 mOsm) and resuspended to a concentration of 200 × 10^6 cells/mL. Washed spermatozoa were loaded with 10 μM C_{11}-BODIPY$^{581/591}$ in dimethyl sulfoxide (DMSO; final concentration <1%) for 30 minutes at 38°C. Unbound probe was removed by centrifugation, and sperm were resuspended to a concentration of 400 × 10^6 cells/mL in modified TALP.

Measurement of C_{11}-BODIPY$^{581/591}$ Fluorescence

After loading with C_{11}-BODIPY$^{581/591}$ and application of appropriate treatments, the samples were aliquoted in duplicate or triplicate wells of a 96-well microplate (100 × 10^6 cells/mL; 200 μL final volume), and fluorescence was determined with a microplate reader (Model HTS 7000, Perkin-Elmer; Norwalk, Conn). Depending upon the experiment, plates were evaluated at 5- to 15-minute intervals for red and green fluorescence for a period of 30 to 120 minutes. Between times, plates were maintained at 38°C in 5% CO_2. In order to determine both red and green fluorescence of C_{11}-BODIPY$^{581/591}$ each microplate was evaluated at the following excitation and emission wavelengths: red, $\lambda_{excitation}$ = 590 and $\lambda_{emission}$ = 635 nm; green, $\lambda_{excitation}$ = 485 and $\lambda_{emission}$ = 535 nm.

In addition to red and green fluorescence, the ratio of red:(red + green) fluorescence was determined as a measure of oxidation of C_{11}-BODIPY$^{581/591}$ (Pap et al, 1999, 2000). Blank wells evaluated included sperm in modified TALP alone as well as C_{11}-BODIPY$^{581/591}$ without sperm and modified TALP alone.

Experiment 1—Effect of Cumene Hydroperoxide and Promoters

After loading with C_{11}-BODIPY$^{581/591}$ sperm were incubated with cumene hydroperoxide (CuOOH: 0, 25, 50, 75, or 100 μM) in the presence of 0.64 mM Fe_2SO_4 and 20 mM sodium ascorbate (Gomez et al, 1998; Baumber et al, 2000; experiment a). In a subsequent experiment, sperm loaded with C_{11}-BODIPY$^{581/591}$ were incubated with CuOOH (0 or 50 μM), Fe_2SO_4 (0, 0.01, 0.05, 0.10, or 0.50 mM) and sodium ascorbate (0, 0.1, 0.5, or 10 mM) (experiment b). In both experiments, red and green fluorescence were determined at 15-minute intervals for 120 minutes.

Experiment 2a—Effect of α-Tocopherol

After loading with C_{11}-BODIPY$^{581/591}$, spermatozoa were incubated with α-tocopherol (0, 100, or 500 μM) for 15 minutes. Samples were then incubated with 50 μM CuOOH, Fe_2SO_4 (0.01 or 0.05 mM), and sodium ascorbate (0, 0.1, 10, or 20 mM). Red and green fluorescence were determined at 15-minute intervals for 75 minutes.

Experiment 2b—Effect of α-Tocopherol With Varying CuOOH Concentrations

In an additional experiment, spermatozoa loaded with C_{11}-BODIPY$^{581/591}$ were incubated with α-tocopherol (0, 50, or 500 μM) for 15 minutes. Samples were then incubated with CuOOH (0, 25, 50, or 75 μM), 0.05 mM Fe_2SO_4, and sodium ascorbate (0 or 0.1 mM). Red and green fluorescence were determined at 10-minute intervals for 30 minutes.

At the termination of each of these experiments, a separate

aliquot of each sample was subjected to flow cytometry for the simultaneous determination of red and green fluorescence. Samples were diluted to a concentration of 10×10^6 cells/mL in modified TALP. A total of approximately 10 000-gated events were analyzed per sample via flow cytometry (FACScan; Becton-Dickinson, Franklin Lakes, NJ). The green fluorescence (FL1) was collected through a 525-nm bandpass filter and the red fluorescence (FL2) was collected through a 635-nm bandpass filter. The mean channel fluorescence was determined for both red and green and a ratio of red:(red + green) was determined as for the microplate reader.

Experiment 3—Lipid Peroxidation in Chilled Spermatozoa

Immediately after collection, raw semen was diluted (1:1 v/v) with a nonfat, dried skim milk extender (NFDSM extender, EZ-mixin; Animal Reproduction Resources, Chino, Calif). Spermatozoa were separated from seminal plasma by centrifugation ($300 \times g$ for 10 minutes at 20°C), and resuspended to 200×10^6 cell/mL in extender. Washed spermatozoa were loaded with 10 μM C_{11}-BODIPY$^{581/591}$ in DMSO (final concentration <1%) for 30 minutes at 38°C. Unbound dye was removed by centrifugation, and sperm were resuspended to a concentration of 50×10^6 cells/mL in NFDSM extender. Treatments were as follows: control (0 mM Fe_2SO_4 and 0 mM sodium ascorbate), promoters (Fe_2SO_4, 0, 0.05, 0.1, 0.5 mM), and sodium ascorbate (0 or 0.1 mM), as well as α-tocopherol (1.0 or 10 mM). Triplicate samples were prepared for each treatment (1.0 mL final volume in a 1.5-mL microcentrifuge tube) for subsequent analysis at 0, 24, and 48 hours. Samples were transferred to a 600-mL water bath (30°C) in a polystyrene box and stored at 5°C. At 0, 24, and 48 hours, sperm motility was determined via computer-assisted semen analysis (CASA; HTM CEROS, Hamilton-Thorne Research, Beverly, Mass) after samples were warmed to 37°C for 10 minutes (except for the sample at 0 hours, which was analyzed immediately).

Motility analysis was conducted by CASA, and fluorescence of C_{11}-BODIPY$^{581/591}$ was determined at 0, 24, and 48 hours after spermatozoa were washed by centrifugation ($300 \times g$ for 10 minutes) and resuspended in modified TALP before determining red and green fluorescence intensity.

Statistical Analysis

Data were analyzed by repeated measures analysis of variance, and differences between individual means were compared with the Fisher protected least-significant difference. In some experiments, data were analyzed by regression analysis or by the Pearson correlation coefficient (Statview, SAS Institute, Cary, NC). Data are presented as least-squares means ± SEM.

Results

Experiment 1—Effect of CuOOH and Promoters

After initial loading of the probe, green fluorescence of spermatozoa loaded with C11-BODIPY$^{581/591}$ was similar to that of spermatozoa without the probe, whereas red fluorescence was significantly increased in spermatozoa loaded with probe compared with spermatozoa alone (Figure 1). Spermatozoa loaded with the probe had a higher red fluorescence but similar green fluorescence intensity compared to 10 μM C_{11}-BODIPY$^{581/591}$ in TALP alone (Figure 1).

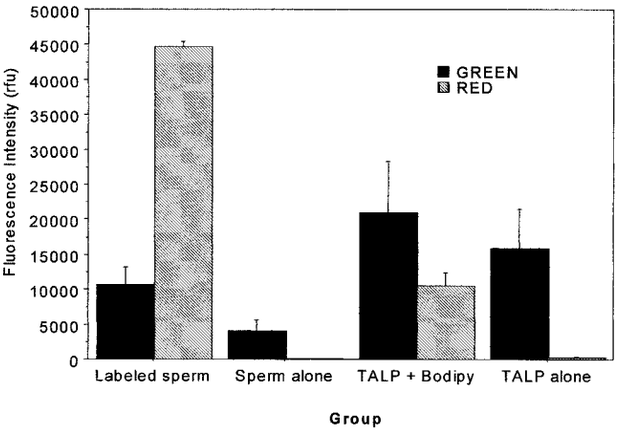

Figure 1. Red (hatched bar) and green (solid bar) fluorescence intensity (expressed as relative fluorescence units, rfu) for spermatozoa before (sperm alone) and after (labeled sperm) loading with 10 μM C_{11}-BODIPY$^{581/591}$ and for TALP with or without 10 μM C_{11}-BODIPY$^{581/591}$.

There was a significant ($P < .001$) effect of CuOOH concentration and time on peroxidation of C_{11}-BODIPY$^{581/591}$ in the presence of promoters as determined by changes in green and red fluorescence as well as the ratio of red:(red + green) during the 120-minute incubation period (Figure 2). Cumene hydroperoxide concentrations of >75 μM resulted in a decreased green and subsequently an increased red:(red + green) ratio. Regression analysis indicated a significant ($P < .0001$) concentration dependent effect of CuOOH on green and red fluorescence as well as the ratio after 120 minutes of incubation based upon a second-order polynomial regression (Figure 3). Oxidation of the probe was greatest ($P < .01$) during the first 30 minutes of incubation; however, red, green, and ratio fluorescence differed at 0 hours, indicating that peroxidation of the probe was well underway before the first analysis.

In experiment 1b, there were significant ($P < .05$) effects of Fe_2SO_4, CuOOH, sodium ascorbate, and time on peroxidation of C_{11}-BODIPY$^{581/591}$ as determined by changes in ratio fluorescence (Figure 4). There was a significant ($P < .05$) time by treatment interaction for Fe_2SO_4 and ascorbate. Across all concentrations of Fe_2SO_4 and sodium ascorbate, peroxidation of C_{11}-BODIPY$^{581/591}$ was increased ($P < .01$) in the presence of CuOOH. In the absence of CuOOH and ascorbate, there was a significant ($P < .05$) concentration-dependent effect of Fe_2SO_4. The addition of CuOOH or low (0.1 or 0.5

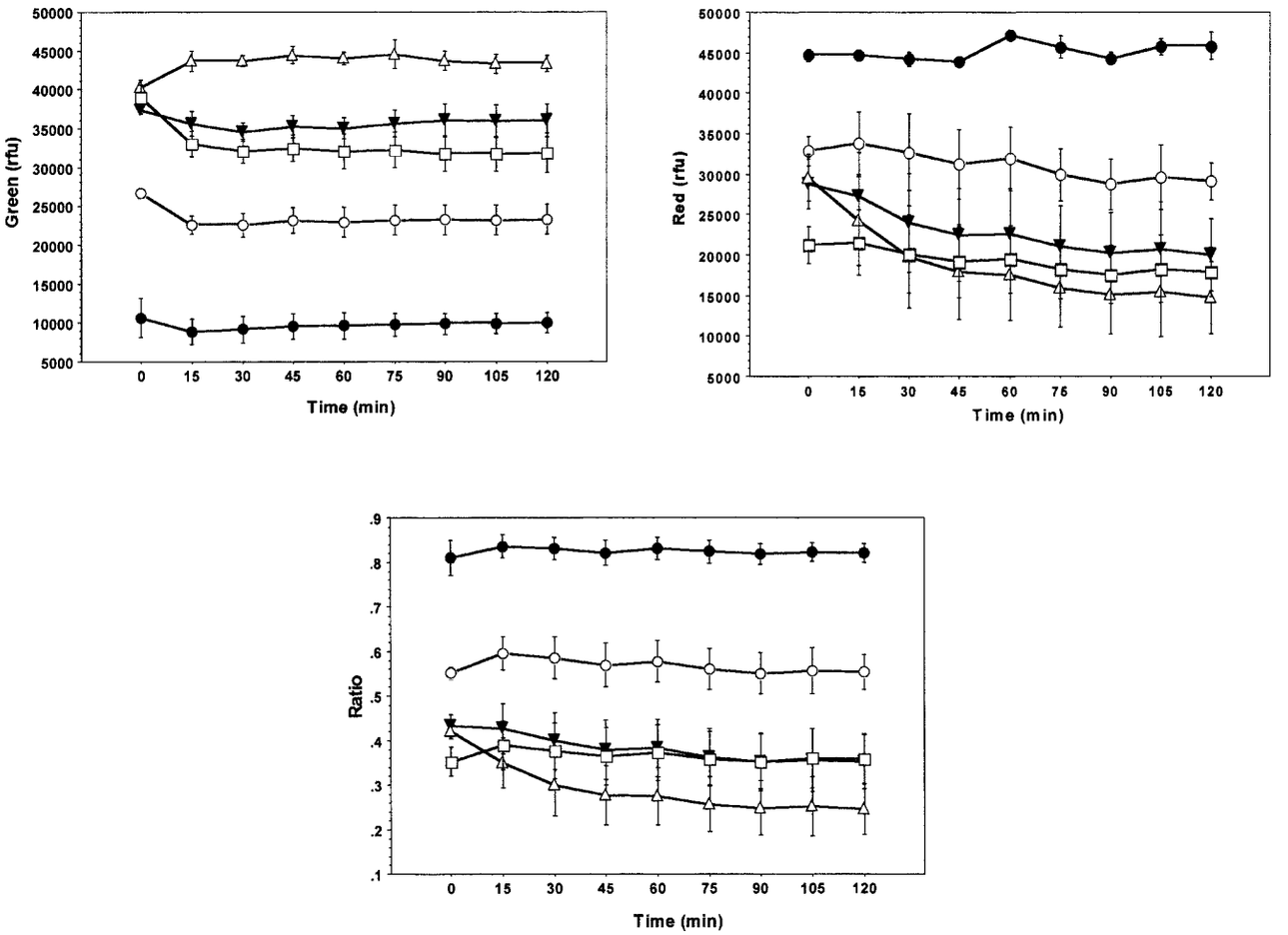

Figure 2. Effect of CuOOH (0 μM, ●; 25 μM, ○; 50 μM, ▼; 75 μM, △; or 100 μM, □) in the presence of 0.64 mM Fe_2SO_4 and 20 mM sodium ascorbate on relative fluorescence intensity (rfu: green and red) and ratiometric fluorescence intensity (red:(red + green)) of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ during a 120-minute incubation. Data are mean ± SEM.

mM) concentrations of ascorbate resulted in an increased lipid peroxidation at all concentrations of Fe_2SO_4 of more than zero; however, in the absence of ferrous promoter, CuOOH or ascorbate did not increase lipid peroxidation. The addition of 10 mM sodium ascorbate reduced the peroxidation of C_{11}-BODIPY$^{581/591}$ compared with lower concentrations of sodium ascorbate (Figure 4).

Experiment 2a—Effect of α-Tocopherol

There were significant ($P < .05$) effects of Fe_2SO_4, sodium ascorbate, α-tocopherol, and time on peroxidation of C_{11}-BODIPY$^{581/591}$ as determined by ratiometric analysis of fluorescence intensity (Figure 5). There was a significant ($P < .0001$) time by treatment interaction for all treatments. The addition of α-tocopherol reduced ($P < .01$) peroxidation of C_{11}-BODIPY$^{581/591}$ in a dose-dependent manner. The addition of 0.05 mM Fe_2SO_4 increased peroxidation of the probe compared with 0.01 mM Fe_2SO_4. The addition of 0.1 mM sodium ascorbate increased ($P < .05$) peroxidation of C_{11}-BODIPY$^{581/591}$, whereas the addition of higher concentrations (10 or 20 mM) of sodium ascorbate decreased ($P < .05$) peroxidation of the probe (Figure 5).

There was a strong positive correlation between mean channel fluorescence determined by flow cytometry and relative fluorescence intensity measured via the microplate reader for ratiometric fluorescence values ($R = 0.80$; $P < .0001$). Within individual replicates, correlation coefficients ranged from 0.85 to 0.99 (data not presented).

Experiment 2b—Effect of α-Tocopherol With Varying CuOOH Concentrations

There were significant ($P < .001$) main effects of CuOOH, sodium ascorbate, α-tocopherol, and time on peroxidation of C_{11}-BODIPY$^{581/591}$ as determined by ratiometric analysis of fluorescence intensity (Figure 6). There was a significant ($P < .0001$) time-by-treatment interaction for all treatments as well as a significant interaction

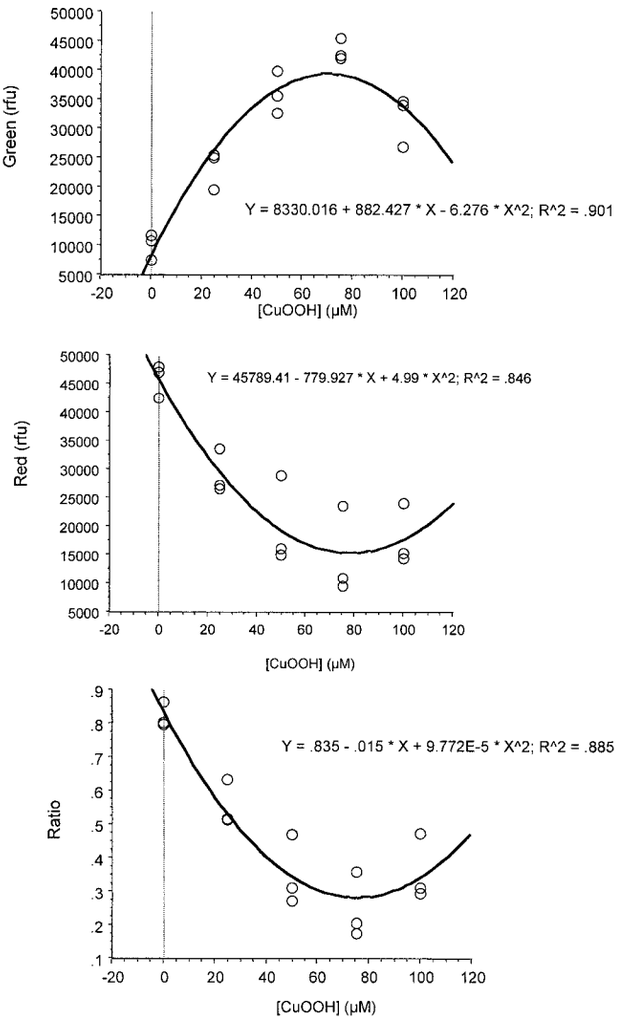

Figure 3. Regression plots for CuOOH concentration versus green, red, and ratio fluorescence of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ after 120-minute incubation in the presence of 0.64 mM Fe_2SO_4 and 20 mM sodium ascorbate. Data are expressed as second-order polynomial regressions, and the regression equations are presented within individual graphs.

between ascorbate and α-tocopherol. The addition of 0.1 mM ascorbate increased ($P < .0001$) peroxidation of C_{11}-BODIPY$^{581/591}$. In the absence of ascorbate, CuOOH did not affect peroxidation of the probe, and the addition of 500 μM α-tocopherol reduced ($P < .05$) peroxidation of the probe (data not shown). In the presence of 0.1 mM ascorbate, the addition of all concentrations of CuOOH increased ($P < .05$) peroxidation of the probe; however, there was no dose response detected across the range of concentrations used. In the presence of 0.1 mM ascorbate, α-tocopherol reduced ($P < .05$) peroxidation of the probe in a dose-dependent manner.

Evaluation of spermatozoa loaded with C_{11}-BODIPY$^{581/591}$ by flow cytometry after a 30-minute incubation revealed significant ($P < .001$) effects of CuOOH, sodium ascorbate, and α-tocopherol on the ratio of mean channel fluorescence (Figure 7). The addition of α-tocopherol reduced the peroxidation of C_{11}-BODIPY$^{581/591}$ ($P < .0001$) based upon the ratio of mean channel fluorescence in a dose-dependent manner. The addition of 0.1 mM ascorbate increased ($P < .0001$) peroxidation of C_{11}-BODIPY$^{581/591}$, and the addition of all concentrations of CuOOH increased ($P < .0001$) peroxidation of the probe; however, there was no dose response detected across the range of CuOOH concentrations used.

There was a strong positive correlation between the ratio of mean channel fluorescence determined by flow cytometry and the ratio of relative fluorescence intensity measured via the microplate reader ($R = 0.80$; $P < .0001$; Figure 8). For individual replicates, correlation coefficients ranged from 0.71 to 0.92.

Experiment 3—Lipid Peroxidation in Chilled Spermatozoa

There were significant ($P < .0001$) effects of Fe_2SO_4 and time, but not sodium ascorbate on peroxidation of C11-BODIPY$^{581/591}$ as determined by the ratio of red:(red + green) fluorescence (Figure 9a) as well as on sperm motility (Figure 9b). The addition of increasing concentrations of Fe_2SO_4 increased ($P < .01$) peroxidation in a dose-dependent manner (Figure 9a). However, only the highest concentration of Fe_2SO_4 (0.5 mM) significantly reduced sperm motility (Figure 9b). The addition of either 1 or 10 mM α-tocopherol did not affect peroxidation of C_{11}-BODIPY$^{581/591}$ or progressive motility during sperm storage at 5°C for 48 hours (data not shown). In the absence of either promoters or α-tocopherol, there was a slight but significant ($P < .01$) increase in peroxidation during the 48-hour storage at 5°C (Figure 9a).

Discussion

Application of C_{11}-BODIPY$^{581/591}$ to Evaluate Oxidative Stress in Equine Spermatozoa

In the present study, the lipophilic probe C_{11}-BODIPY$^{581/591}$ was used to determine the oxidation status of equine spermatozoa. Based upon previous reports, this probe appears to have several advantages over other oxidation-sensitive fluorescent probes (Pap et al, 1999, 2000), and our present findings support the usefulness of this probe to evaluate oxidative stress in equine spermatozoa. Because the C_{11}-BODIPY$^{581/591}$ probe is lipophilic, it is readily loaded into cell membranes. Although not critically tested in our studies, the probe did not appear to adversely affect sperm viability during prolonged exposure (up to 48 hours for cooled spermatozoa; data not presented), and as such, is compatible with evaluation of lipid oxidation status of

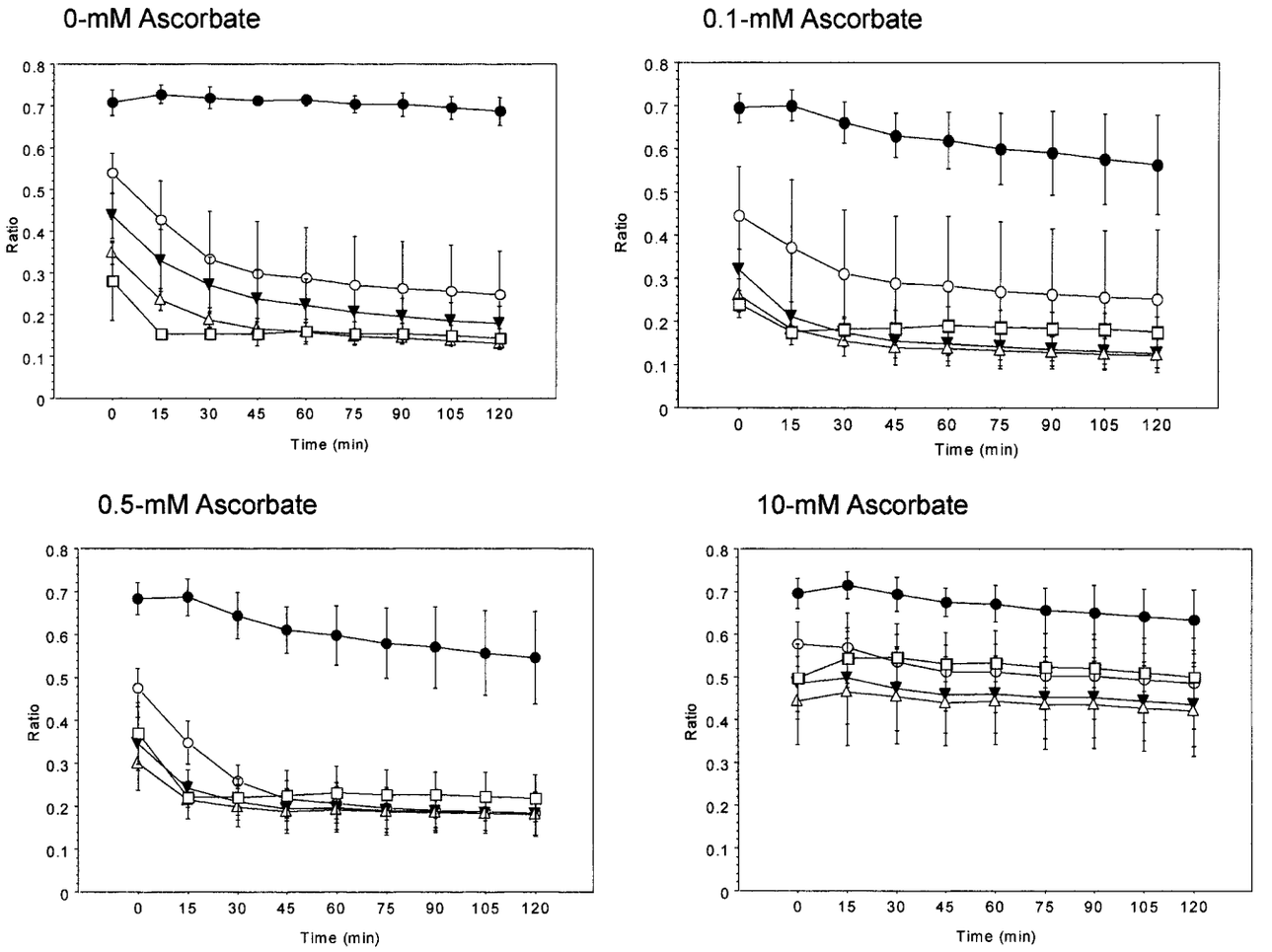

Figure 4. Effect of Fe_2SO_4 (0.0 mM, ●; 0.01 mM, ○; 0.05 mM, ▼; 0.1 mM, △; and 0.5 mM, □), sodium ascorbate (0, 0.1, 0.5, or 10 mM) in the presence of 50 μM CuOOH on ratiometric fluorescence of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$. Data are mean ± SEM.

live spermatozoa. In the present study, results obtained with the fluorescence microplate reader agreed well with those obtained via flow cytometry based upon the ratiometric evaluation of the red (nonoxidized) and green (oxidized) form of the probe when compared at either 30 or 75 minutes after the application of an oxidative stress. The multi-well approach is particularly well suited for the evaluation of the effects of multiple promoters and antioxidants in dose-response studies as presented here.

Effect of CuOOH, Promoters, and Antioxidants on Oxidation of C_{11}-BODIPY$^{581/591}$

Organic hydroperoxides such as CuOOH have been used frequently in the study of lipid peroxidation (van den Berg et al, 1992; Borst et al, 2000). Cumene hydroperoxide appears to act via the addition of a hydroperoxide to unsaturated double bonds, and the reaction is significantly increased in the presence of metal ions (van den Berg et al, 1992; Borst et al, 2000). In our study, CuOOH induced a dose-dependent increase in oxidation of C_{11}-BODIPY$^{581/591}$ in the presence of 0.64 mM Fe_2SO_4 and 20 mM ascorbate. In contrast, in the absence of a ferrous promoter and ascorbate, CuOOH did not induce an increase in peroxidation of C_{11}-BODIPY$^{581/591}$. In experiment 1a, C_{11}-BODIPY$^{581/591}$ was oxidized in a dose-dependent manner in the presence of CuOOH plus promoters; however, concentrations of CuOOH greater than 75 μM appeared to result in a decline in green fluorescence and a slight increase in red fluorescence. This change resulted in a higher ratio of red:(red + green) fluorescence. The reason for the reduced green fluorescence with CuOOH concentrations higher than 75 μM is not clear, although self-quenching or resonance energy transfer has been suggested to alter fluorescence intensity with this probe (Pap et al, 2000).

In subsequent experiments there was not a significant

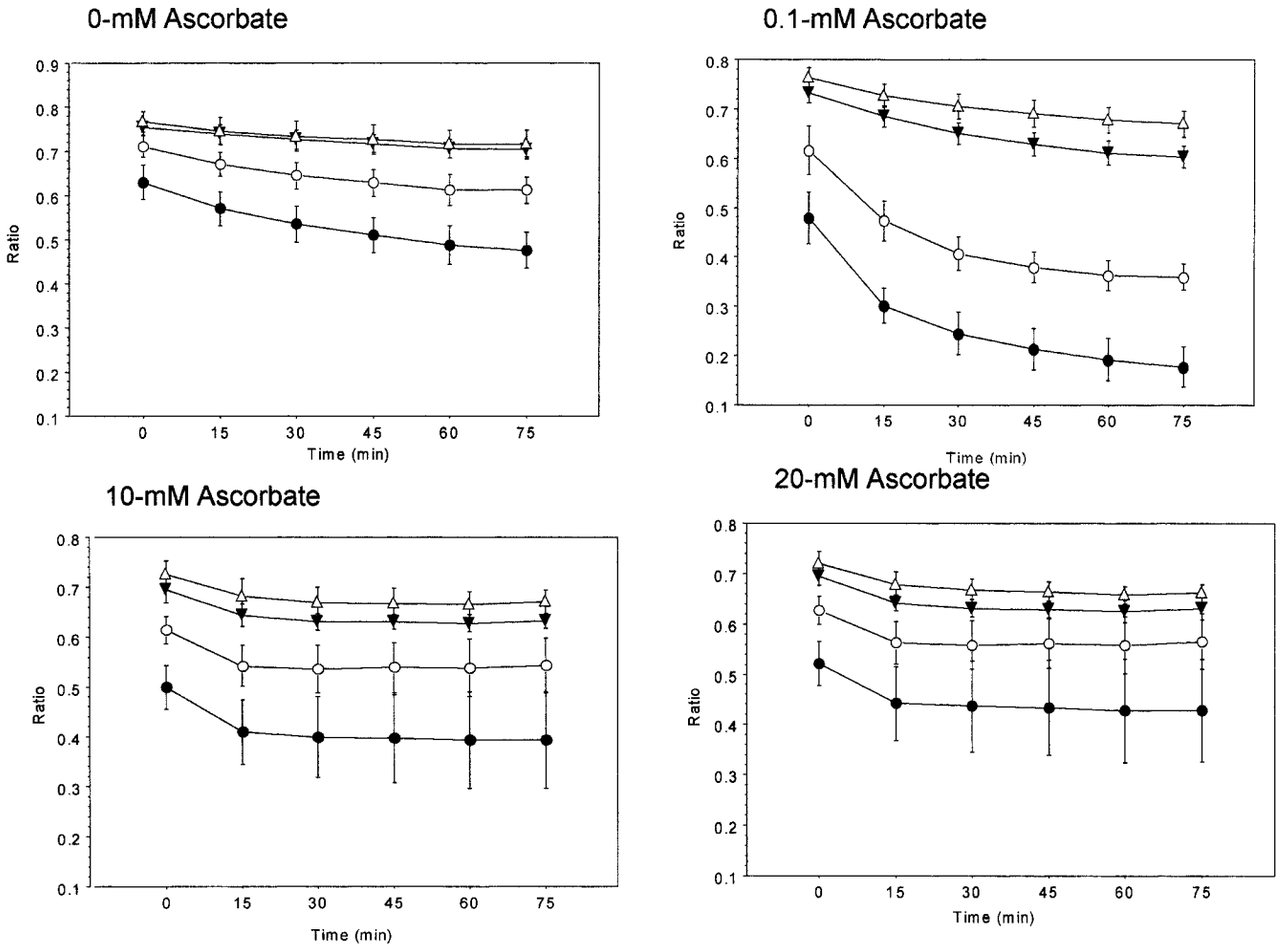

Figure 5. Effect of α-tocopherol on peroxidation of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ in the presence of 50 μM CuOOH, 0.01 mM Fe_2SO_4, and sodium ascorbate (0, 0.1, 10, or 20 mM). Spermatozoa were incubated with α-tocopherol (0 μM, ●; 100 μM, ○; 500 μM, ▼; or 1.0 mM, △). Data are mean ± SEM.

dose-response effect for the addition of CuOOH in the presence of lower concentrations of promoters. In these experiments, it appears that the promoter concentration may have limited the rate of oxidation of C_{11}-BODIPY$^{581/591}$. Similar observations were made previously in experiments in which hemin concentrations limited the rate of oxidation of C_{11}-BODIPY$^{581/591}$ with CuOOH (Pap et al, 1999).

As previously demonstrated, the addition of low concentrations of ferrous promoter and ascorbate significantly increased lipid peroxidation in the absence of CuOOH. Ascorbate plus Fe^{2+} promoters have been used extensively to induce rapid lipid peroxidation in a variety of cell types including spermatozoa (Jones and Mann 1977; Aitken et al, 1993b; Kodama et al, 1996; Storey, 1997; Gomez et al, 1998). The ferrous ion promotes the catalysis of lipid peroxides to alkoxyl and peroxyl radicals, which appear to be important in the propagation of the chain reaction of lipid peroxidation in the sperm membrane (Aitken et al, 1993b). Ferrous sulfate increased oxidation of C_{11}-BODIPY$^{581/591}$ in a dose-dependent fashion in the absence of CuOOH, and low concentrations of sodium ascorbate appeared to enhance the oxidation of C_{11}-BODIPY$^{581/591}$. This effect appears to be due to the pro-oxidant effect of low concentrations of ascorbate, which act to promote oxidation by redox cycling of Fe^{3+} to Fe^{2+} (Buettner and Jurkiewicz 1996). At higher concentrations ascorbate acted as an antioxidant. This concentration-dependent crossover effect of ascorbate has been well characterized (Buettner and Jurkiewicz 1996).

Higher concentrations of the water-soluble antioxidant, ascorbate, and α-tocopherol decreased oxidation of C_{11}-BODIPY$^{581/591}$ in a dose-dependent manner. Although α-tocopherol reduced the rate of lipid peroxidation, some oxidation of C_{11}-BODIPY$^{581/591}$ continued in the presence of CuOOH and promoters during incubation. The antioxidant is eventually consumed, and the resulting toco-

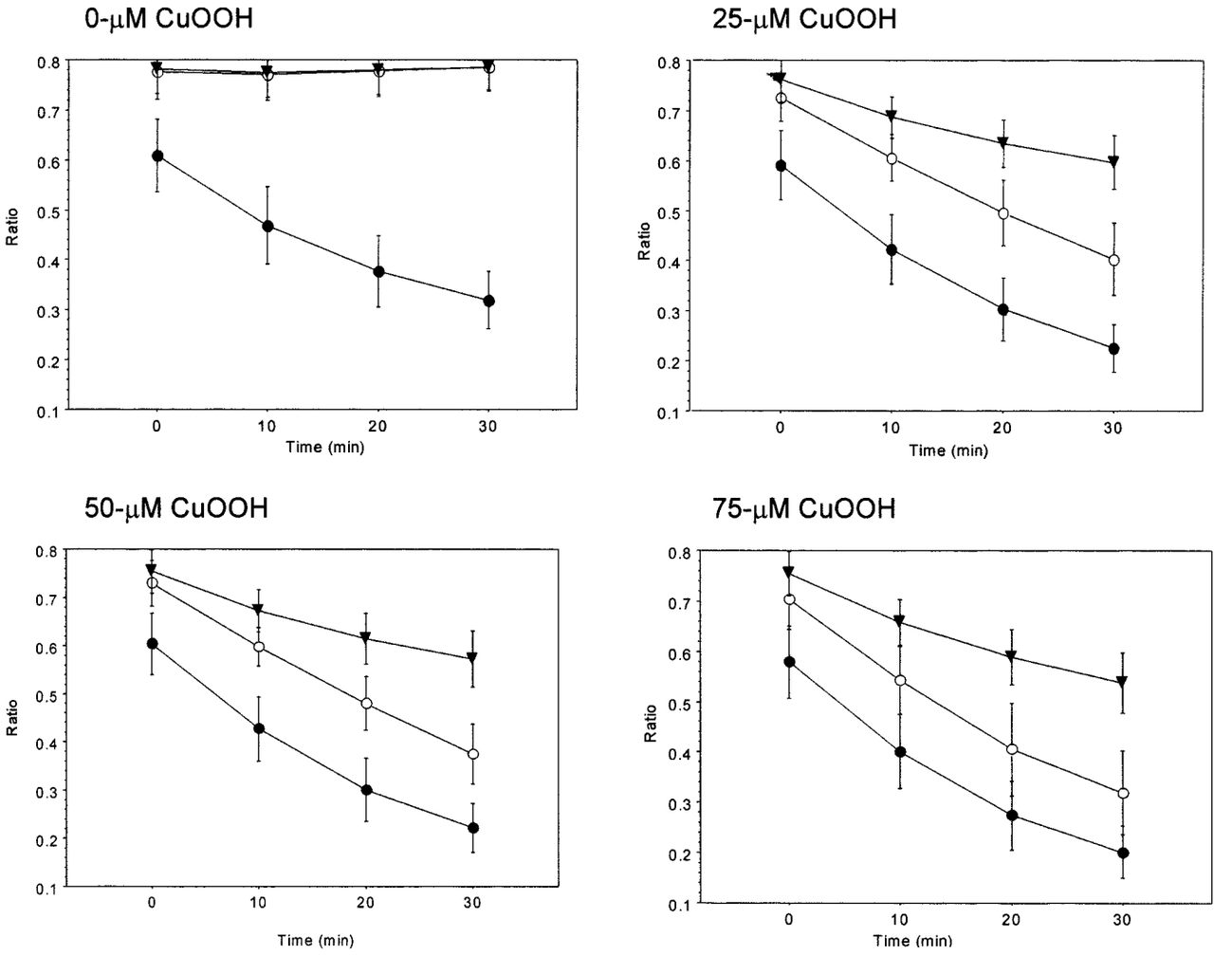

Figure 6. Effect of α-tocopherol (0 μM, ●; 50 μM, ○; or 500 μM, ▼) in the presence of 0.1 mM ascorbate, and CuOOH (0 μM, 25 μM, 50 μM, or 75 μM) on peroxidation of spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$. Data are mean ± SEM.

pheroxyl radical must be reduced to regenerate α-tocopherol (Niki, 1996). Ascorbate appears to act synergistically with α-tocopherol via the regeneration of α-tocopherol from its oxidized form (Buettner and Jurkiewicz 1996; Niki, 1996). In our study, there were significant interactions between ascorbate and α-tocopherol in the extent of lipid peroxidation induced by CuOOH and promoters.

Evaluation of Lipid Peroxidation in Equine Spermatozoa Stored at 5°C

Although a decline in sperm motility and viability is noted during cooled storage, there has been little research to examine changes in membrane lipid peroxidation in equine spermatozoa during storage at 5°C. Our present results indicate that during storage for 48 hours, there was an increase in peroxidation of C_{11}-BODIPY$^{581/591}$ accompanied by a decline in motility. Further, the addition of a ferrous promoter increased lipid peroxidation and decreased motility of equine spermatozoa stored at 5°C. Although there is little information available concerning levels of either free or bound iron in stallion semen, a recent report indicated that the iron-binding protein, lactoferrin, was a major secretory protein in the equine epididymis (Fouchecourt et al, 2000). It appears that lactoferrin may associate with spermatozoa (Jin et al, 1997) and may act to decrease the amount of free iron available to initiate lipid peroxidation. Together, these studies suggest that metal ion contamination of semen during processing could result in adverse effects on semen storage due to increased lipid peroxidation.

The addition of micromolar amounts of α-tocopherol did not decrease oxidative stress in equine spermatozoa stored at 5°C. Likewise, we previously reported that the addition of similar concentrations of α-tocopherol did not

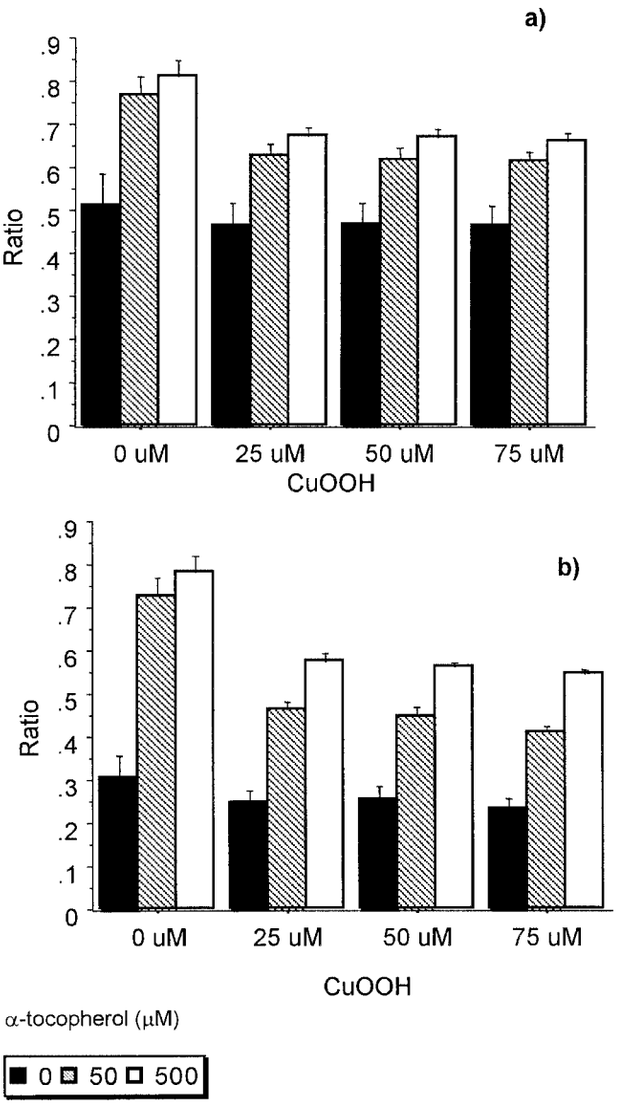

Figure 7. Effect of CuOOH (0, 25, 50, or 75 μM) and α-tocopherol (0 μM, solid bar; 50 μM, hatched bar; 500 μM, open bar) on the ratio of mean channel fluorescence (red:(red + green)) of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ after 30-minute incubation in the presence of 0.05 mM Fe_2SO_4 in the absence of ascorbate **(a)** or in the presence **(b)** of 0.1 mM sodium ascorbate.

improve the maintenance of motility of equine spermatozoa during storage at 5°C (Ball et al, 2001). These observations for equine semen differ from a recent study with boar semen in which the addition of α-tocopherol improved both the maintenance of motility as well as decreased levels of malondialdehyde present during storage at 19°C (Cerolini et al, 2000). These differences suggest that considerable species or technical differences exist in the effect of vitamin E on maintenance of sperm motility and viability. For example, positive effects have been reported in stored turkey spermatozoa (Donoghue and Don-

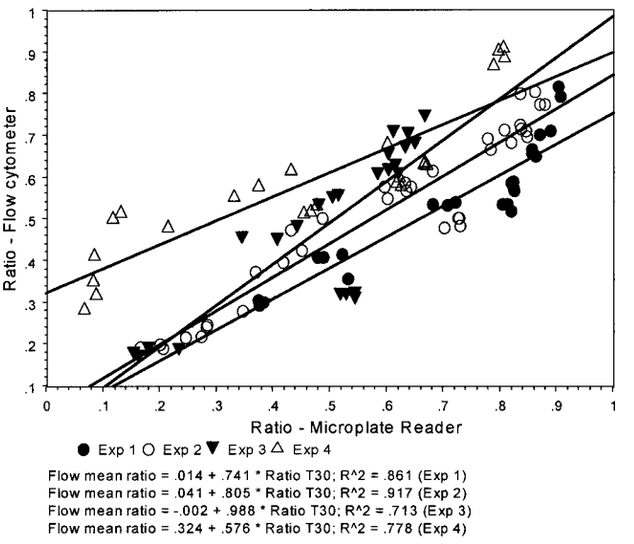

Figure 8. Relationship between the ratio of mean channel fluorescence (red:(red + green)) determined by flow cytometry and the ratio of fluorescence intensity determined by the microplate reader for equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ after 30-minute incubation in the presence of 0.05 mM Fe_2SO_4 as determined by linear regression analysis. Regression equations and corresponding coefficients of determination are shown for individual experiments.

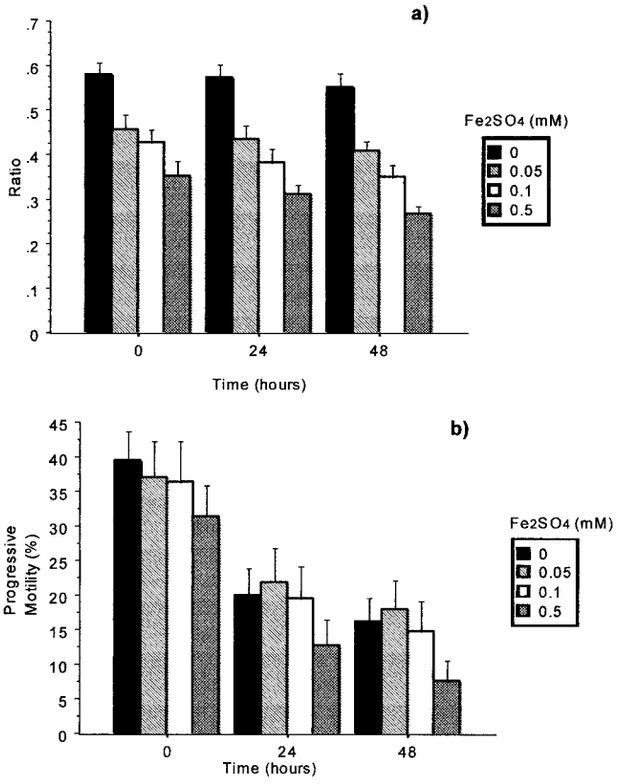

Figure 9. Effect of Fe_2SO_4 on peroxidation of equine spermatozoa loaded with 10 μM C_{11}-BODIPY$^{581/591}$ as determined by the ratio of red:(red + green) fluorescence (top panel) as well as progressive motility (bottom panel) of spermatozoa (determined by CASA) during 48-hour storage at 5°C.

oghue 1997), but not in ram spermatozoa (Upreti et al, 1997). In addition, a number of factors may alter the rate of lipid peroxidation in spermatozoa. The rate of lipid peroxidation increases with temperature, dissolved O_2 content, decreased pH, and the relative unsaturation of membrane lipids (Storey, 1997). There also appear to be wide differences in the type and relative antioxidant capacity between semen samples from different species (Ball et al, 2000) that may also influence susceptibility to lipid peroxidation. The composition of media or extender used may further affect antioxidant capacity of a sample (Storey, 1997). Therefore, differences in the relative effect of Vitamin E may be due to storage conditions (5°C vs 19°C), membrane lipid composition, or relative levels of antioxidants and enzyme scavengers between semen from different species.

Acknowledgments

We thank B. Stewart, M.A. Scott, M. Wessel, G. Pereira, and D.A. Carver for assisting with semen collection.

References

Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil.* 1993a;98:257–265.

Aitken RJ, Harkiss D, Buckingham DW. Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev.* 1993b;35:302–315.

Alvarez JG, Storey BT. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa: its effect on sperm motility. *Biol Reprod.* 1982; 27:1102–1108.

Alvarez JG, Storey BT. Role of superoxide dismutase in protecting rabbit spermatozoa from O2 toxicity due to lipid peroxidation. *Biol Reprod.* 1983;28:1129–1136.

Alvarez JG, Storey BT. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. *Biol Reprod.* 1984;30: 323–331.

Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J Androl.* 1992; 13:232–241.

Alvarez JG, Storey BT. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev.* 1995;42:334–346.

Ball BA, Gravance CG, Medina V, Baumber J, Liu IKM. Catalase activity in equine semen. *Am J Vet Res.* 2000;61:1026–1030.

Ball BA, Medina V, Gravance CG, Baumber J. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. *Theriogenology.* 2001;56:577–589.

Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MCG. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl.* 2000;21:895–902.

Bavister BD. A consistently successful procedure for in vitro fertilization of Golden hamster eggs. *Gamete Res.* 1989;23:139–158.

Borst JW, Visser NV, Kouptsova O, Visser AJ. Oxidation of unsaturated phospholipids in membrane bilayer mixtures is accompanied by membrane fluidity changes. *Biochim Biophys Acta.* 2000;1487:61–73.

Buettner GR, Jurkiewicz BA. Chemistry and Biochemistry of Ascorbic Acid. In: Cadenas E, Packer L, eds. *Handbook of Antioxidants.* New York, NY: Marcel Dekker; 1996:91–115.

Cerolini S, Maldjian A, Surai P, Noble R. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci.* 2000;58:99–111.

Chung WY, Benzie IF. Probe-assisted flow cytometric analysis of erythrocyte membrane response to site-specific oxidant stress. *Cytometry.* 2000;40:182–188.

Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis.* 1999;14:505–512.

Donoghue AM, Donoghue DJ. Effects of water- and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poultry Sci.* 1997;76:1440–1445.

Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using Sephadex columns, washing, or Percoll gradients. *J Androl.* 1991;12:201–208.

Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta.* 2000; 1469:197–235.

Fouchecourt S, Metayer S, Locatelli A, Dacheux F, Dacheux JL. Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. *Biol Reprod.* 2000;62:1790–1803.

Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Androl.* 1998;21:81–94.

Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine.* Oxford, United Kingdom: Clarendon Press; New York, NY: Oxford University Press; 1999.

Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men using a modified comet assay. *Mol Hum Reprod.* 1996;2:613–619.

Jin YZ, Bannai S, Dacheux F, Dacheux JL, Okamura N. Direct evidence for the secretion of lactoferrin and its binding to sperm in the porcine epididymis. *Mol Reprod Dev.* 1997;47:490–496.

Jones R, Mann T. Lipid peroxidation in spermatozoa. *Proc R Soc Lond B Biol Sci.* 1973;184:103–107.

Jones R, Mann T. Damage to ram spermatozoa by peroxidation of endogenous phospholipids. *J Reprod Fertil.* 1977;50:261–268.

Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril.* 1979;31:531–537.

Kodama H, Kuribayashi Y, Gagnon C. Effect of sperm lipid peroxidation on fertilization. *J Androl.* 1996;17:151–157.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril.* 1997;68:519–524.

Komarek RJ, Pickett BW, Gibson EW, Lanz RN. Composition of lipids in stallion semen. *J Reprod Fertil.* 1965;10:337–342.

Makrigiorgos GM, Kassis AI, Mahmood A, Bump EA, Savvides P. Novel fluorescein-based flow-cytometric method for detection of lipid peroxidation. *Free Radic Biol Med.* 1997;22:93–100.

Naguib YMA. A fluorometric method for measurement of peroxyl radical scavenging activities of lipophilic antioxidants. *Anal Biochem.* 1998; 265:290–298.

Niki E. a-Tocopherol. In: Cadenas E, Packer L, eds. *Handbook of Antioxidants.* New York, NY: Marcel-Dekker; 1996:3–26.

Ohta A, Mohri T, Ohyashiki T. Effect of lipid peroxidation on membrane-bound Ca2+-ATPase activity of the intestinal brush-border membranes. *Biochim Biophys Acta.* 1989;984:151–157.

Pap EH, Drummen GP, Post JA, Rijken PJ, Wirtz KW. Fluorescent fatty acid to monitor reactive oxygen in single cells. *Methods Enzymol.* 2000;319:603–12.

Pap EH, Drummen GP, Winter VJ, et al. Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY(581/591). *FEBS Lett.* 1999;453:278–282.

Parks JE, Lynch DV. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology.* 1992;29:255–266.

Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod.* 1997;3:203–313.

Upreti G, Jensen K, Oliver J, Duganzich D, Munday R, Smith J. Motility of ram spermatozoa during storage in a chemically-defined diluent containing antioxidants. *Anim Reprod Sci.* 1997;48:269–278.

van den Berg JJ, Op den Kamp JA, Lubin BH, Roelofsen B, Kuypers FA. Kinetics and site specificity of hydroperoxide-induced oxidative damage in red blood cells. *Free Radic Biol Med.* 1992;12:487–498.