Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid

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ABSTRACT: Part of the reduction in boar sperm motility and fertility associated with hypothermic liquid storage and cryopreservation may be due to membrane lipid peroxidation. Lipid peroxidation was monitored by the shift from red to green fluorescence emission of the lipophilic probe 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, C11-BODIPY581/591 (BODIPY), as measured by fluorescence-activated flow cytometry in live sperm (negative for propidium iodide). Experiments were conducted with Percoll-washed sperm to determine the specificity of BODIPY oxidation in the presence of different reactive oxygen species generators and metal chelators. Compared with no FeSO4 and Na ascorbate, the combination of FeSO4 and Na ascorbate (FeAc) increased (P < 0.01) the percentage of sperm containing oxidized BODIPY from ≤1 to >70% and increased (P < 0.05) BODIPY fluorescence intensity/cell by 5- to 10-fold after a 30-min incubation. Motility was depressed (P < 0.05) after exposure to FeAc, but viability was not affected. Of the reactive oxygen species generators tested, BODIPY oxidation was specific for FeAc, because menadione and H2O2 had little or no effect. The oxidation of hydroethidine to ethidium was specific for menadione and H2O2; FeAc had no effect. The presence of the metal chelators EDTA or deferoxamine mesylate at 3 and 9 μM inhibited FeAc-induced BODIPY oxidation and maintained motility. Experiments were conducted to determine the effect of liquid storage at 17°C for 1 and 5 d and the effect of freeze-thawing on basal and FeAc-induced BODIPY oxidation. Basal BODIPY oxidation (no FeAc) was low in liquid stored and thawed viable sperm (1.3 and 3.4%, respectively). Although the incidence of basal or spontaneous membrane lipid peroxidation was low during liquid storage and after freeze-thawing, viable boar sperm were susceptible to FeAc-induced lipid peroxidation.

Key words: 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, lipid peroxidation, hydroethidine, flow cytometry, motility

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INTRODUCTION

The fertility of boar sperm after freeze-thawing or long-term hypothermic liquid storage is less than that of fresh liquid semen (Johnson, 1985; Waberski et al., 1994). Part of this reduction in sperm fertility may be due to oxidative damage from inappropriate formation of reactive oxygen species (ROS). Sperm isolated from fresh (Roca et al., 2004), hypothermic liquid stored (Cerolini et al., 2000), and thawed (Breininger et al., 2005) boar semen are susceptible to FeSO4 and ascorbate-catalyzed lipid peroxidation, as measured by malondialdehyde (MDA) formation. Measurement of MDA, a oxidation breakdown product derived from arachidonic acid (Spiteller, 2006), provides an estimate of lipid peroxidation in a population of cells (Storey, 1997) but has the disadvantages of not distinguishing between live and dead cells and not providing an estimate of the proportion of the live cell population that contains membrane lipid peroxidation.

A fluorescent fatty acid conjugate, 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, C11-BODIPY581/591 (BODIPY), is a membrane probe whose fluorescence changes irreversibly from red to green upon exposure to ROS and has been used to assess lipid peroxidation in living cells.
(Pap et al., 1999), including sperm of bovine (Brouwers and Gadella, 2003), equine (Ball and Vo, 2002), and ovine (Christova et al., 2004) species. Due to the high quantum yield of BODIPY-based probes and the high sensitivity of fluorescence techniques, the appearance of green fluorescence in BODIPY-labeled cells can be regarded as an indication of physiologically relevant exposure of phospholipids to ROS (Drummen et al., 2002; Haugland, 2005).

The objective of this study was to perform flow cytometric analysis of BODIPY oxidation in boar sperm as a marker for membrane lipid peroxidation to determine the effect of liquid storage for up to 5 d and freeze-thawing on membrane lipid peroxidation in viable boar sperm.

**MATERIALS AND METHODS**

The management of the boars (GPK5/EB5 boar line or derived from this line and female line GPK2/GPK1, Monsanto Choice Genetics, St. Louis, MO) used in this study was approved by the Beltsville Area Animal Care and Use Committee.

**Sperm Processing**

Semen collection and determination of sperm concentration were conducted as previously described (Guthrie and Welch, 2005). Sperm were isolated from semen constituents and extenders using a Percoll (P-1644, Aldrich, Milwaukee, WI) 2-step discontinuous gradient centrifugation, consisting of 35 and 70% isotonic Percoll (Guthrie and Welch, 2005), as described in detail by Harrison et al. (1992). Freshly collected and thawed semen were Percoll-washed at a sperm concentration of 150 × 10^6/mL in Beltsville thawing solution (BTS, Minitube of America, Verona, WI; Guthrie and Welch, 2005), and semen that was stored in liquid form was Percoll-washed at the storage concentration of 30 × 10^6 sperm/mL in a modified noncapacitating Tyrode’s medium (TYR, prepared as described earlier; Guthrie and Welch, 2005) containing no CaCl$_2$ and in which BSA was replaced with 0.1% polyvinyl alcohol.

**Cryopreservation**

The boar semen cryopreservation protocol (Guthrie and Welch, 2005) was based on one published previously (Almlid and Johnson, 1988). Sperm (1 × 10^9 sperm/mL) were frozen in 0.5-mL French straws (13408/0010, Minitube of America, Verona, WI) in a freezing extender composed of 11% lactose (wt/vol; L2643, Sigma-Aldrich, St. Louis, MO) and 20% chicken egg yolk (vol/vol, isolated from whole eggs) with final concentrations of 3% glycerol (vol/vol; G6279, Sigma-Aldrich) and 0.5% Equex STM paste (vol/vol; Nova Chemical Sales, Scituate, MA). The contents of the straws were frozen in liquid N$_2$ vapor in a programmable freezer (IceCube 1810, Minitube of America), and the contents of the straws were thawed as previously described (Guthrie and Welch, 2005).

**Sperm Motion Analysis**

The percentage of motile sperm was determined in 20-micron 4-chamber glass counting slides (SC 20-01 FA, Leja Products, Nieuw-Vennep, The Netherlands) using a Hamilton Thorne IVOS version 12, motion analysis system (Hamilton Thorne Biosciences, Beverly, MA) set for boar sperm analysis (Vyt et al., 2004; Douglas-Hamilton et al., 2005). Sperm were incubated at a concentration of 30 × 10^6/mL at 37°C in a temperature-controlled water bath, and aliquots were removed at specific times for motion analysis.

**C$_{11}$-BODIPY$_{581/681}$ Oxidation**

Loading of sperm with BODIPY was similar to the procedure described by Brouwers and Gadella (2003). Before conduct of the incubation treatments, BODIPY (3861, Molecular Probes Inc., Eugene, OR) was added to Percoll-washed sperm to a final concentration of 2 μM from a working solution of 2 mM in ethanol at a sperm density of 60 million/mL. After aerobic incubation at 37°C for 30 min, excess probe was removed by washing the cells once with TYR (centrifugation at 300 × g for 7 min). To determine viability, after incubating the sperm with ROS generators, inhibitors, or both, for varying lengths of time, sperm were loaded with propidium iodide (PI) at 9.6 μM (P-4170, Sigma-Aldrich) from a working solution of 2.4 mM in deionized distilled water and incubated a further 10 min at 25°C before flow cytometry.

A Beckman-Coulter Epics XL-MCL analyzer (Beckman-Coulter, Hialeah, FL) equipped with a single 488-nm excitation source was used for all flow cytometric analyses. A forward- and side-scatter gate was used to select single sperm from clumps and debris. Green fluorescence from oxidation of BODIPY in individual sperm was collected in fluorescence detector 1, with a 525-nm band pass filter, and red fluorescence from PI was collected in fluorescence detector 4, with a 675-nm band pass filter. Statistical regions (System II software, Beckman-Coulter) were drawn to delineate the viable sperm population (low intensity PI fluorescence) and were used to determine the percentage of viable sperm, the percentage of viable sperm with lipid peroxidation (green BODIPY fluorescence), and the mean BODIPY fluorescence intensity/cell (mean channel fluorescence number). The variables recorded for each sample were percentage of viable sperm containing oxidized BODIPY, BODIPY fluorescence intensity/cell, and viability (percentage of sperm with low intensity PI fluorescence).
ROS Formation

Detection of ROS formation in the viable sperm incubation treatments was determined by flow cytometric analysis of the oxidation of hydroethidine (HE) to ethidium in cells that excluded the impermeant nuclear dye Yo Pro1 (Molecular Probes, Eugene, OR), as previously described (Guthrie and Welch, 2006). The variables recorded for each sample were percentage of viable sperm containing oxidized HE, ethidium fluorescence intensity/cell (mean channel fluorescence number, fluorescent detector 3), and viability (percentage of sperm with low intensity Yo Pro1 fluorescence).

Preliminary Experiments

The flow cytometric 2-dimensional dot plots of BODIPY- and PI-stained sperm from fresh semen in the absence of ROS generators revealed a relatively homogeneous population, with little or no oxidized BODIPY (low basal BODIPY oxidation, data not shown). Fluorescence intensity/cell of this population changed little during incubation at 37°C for 120 min. When a BODIPY response marker was set just above this sperm population on each dot plot, the percentage of sperm containing oxidized BODIPY was low, usually 1% or less.

Experiments were conducted in freshly collected sperm to establish the concentrations of FeSO₄ [iron (II) heptahydrate, 215422, Sigma-Aldrich] and Na ascorbate (Na L-ascorbate, A7631, Sigma-Aldrich) as well as the incubation time required for BODIPY oxidation to develop in a large proportion of the sperm population without total loss of motility or substantial loss of viability. The combinations of FeSO₄ and Na ascorbate concentrations tested ranged from 0 to 25 and 0 to 250 μM, respectively, and the incubation time ranged from 5 to 120 min at 37°C. The percentage of sperm containing oxidized BODIPY increased to at least 88% after a 30-min incubation with 1 μM FeSO₄ and Na ascorbate at 10 and 30 μM, whereas longer exposure to lower concentrations of FeSO₄ and Na ascorbate or exposure to concentrations greater than 10 and 30 μM, respectively, for only 5 min did not increase the percentage of sperm containing oxidized BODIPY above 18% (data not shown). The percentage of motile sperm decreased by approximately one-third (P < 0.05) during incubation with FeSO₄ at concentrations of 1 or 3 μM and Na ascorbate at concentrations of 1 to 30 or 3 to 30 μM, for 120 min, and viability did not change (P = 0.10) during the same period (data not shown). For subsequent experiments, a combination of FeSO₄ and Na ascorbate (FeAc) of 1 and 30 μM, respectively (designated 1/30) was used.

Experiments 1A and 1B: Specificity of BODIPY and HE Oxidation

Experiments 1A and 1B were conducted to determine the specificity of FeAc, H₂O₂ (216763, Sigma-Aldrich) and menadione (MEN; M5625, Sigma-Aldrich) for BODIPY and HE oxidation, respectively. Sperm in both experiments underwent computer-assisted analysis of motility. For each experiment, 1 ejaculate was collected from each of 4 boars, and each sperm sample was divided into 4 portions for aerobic incubation for 30 and 120 min at 37°C with 4 treatments: control, 30 μM MEN, 300 μM H₂O₂, and 1/30 μM FeAc. The concentrations of MEN and H₂O₂ selected were based on previous studies (Guthrie et al., 2006; Guthrie and Welch, 2006). The experimental design for each experiment was a randomized complete block with boar as the blocking variable, the 4 treatments as fixed effects, and incubation time as a repeated measure.

Experiments 2A, 2B, and 2C: Effects of Metal Chelators on C₁₁-BODIPY⁵₈₁⁶₆₈₁ Oxidation

Experiments 2A, 2B, and 2C were conducted to determine if the metal chelator EDTA and the Fe chelator deferoxamine mesylate (DFM) attenuated FeAc-induced BODIPY or HE oxidation. In experiment 2A, 1 ejaculate was collected from each of 3 boars, and each sperm sample was divided into 6 portions for aerobic incubation for 30 and 120 min at 37°C with 6 treatment combinations of FeAc and DFM (D9533, Sigma-Aldrich; μM): 0/0 and 0, 0/0 and 9, 1/30 and 0, 1/30 and 1, 1/30 and 3, and 1/30 and 9. In experiment 2B, 1 ejaculate was collected from each of 2 boars, and each sperm sample was divided into 6 portions for aerobic incubation for 30 and 120 min at 37°C with 6 treatment combinations of FeAc and EDTA (μM): 0/0 and 0, 0/0 and 9, 1/30 and 0, 1/30 and 1, 1/30 and 3, and 1/30 and 9. For experiments 2A and 2B, BODIPY oxidation was measured at 30 min, and motility was measured at 30 and 120 min. The experimental design was a randomized complete block, with the 6 treatments as fixed effects and incubation time as a repeated measure for motility.

In experiment 2C, 1 ejaculate was collected from each of 2 boars, and each sperm sample was divided into 6 portions for aerobic incubation for 30 min at 37°C with 6 H₂O₂ and EDTA treatment combinations (μM): 0 and 0, 0 and 1,000, 300 and 0, 30 and 10, 300 and 100, and 300 and 1,000. The oxidation of HE was measured at 30 min. The experimental design was a randomized complete block, with the 6 treatments as fixed effects.

Experiment 3: Effects of Hypothermic Liquid Storage on C₁₁-BODIPY⁵₈₁⁶₆₈₁ Oxidation

This experiment was conducted to compare the effects of hypothermic liquid storage in normal BTS (3.35 mM EDTA) and modified BTS (without EDTA) on basal and FeAc-induced BODIPY oxidation in boar sperm. One ejaculate each from 2 boars and 2 ejaculates each from 2 additional boars were extended separately to 30 million sperm/mL in normal BTS or in modified BTS and divided into 5 portions for storage: at 25°C for 30 min in normal BTS (d 0); at 17°C for 24 h in normal or modified...
BTS; and at 17°C for 120 h (d 5) in normal or modified BTS. After storage and Percoll washing, each sperm sample was divided into 2 portions for aerobic incubation at 37°C for 30 and 120 min with or without FeAc (1/30 μM). The oxidation of BODIPY was measured at 30 min, and motility was measured at 30 and 120 min. Two statistical models were used. Model 1 excluded d 0 data, and the experimental design was a randomized complete block with duration of storage (d 0, 1/30, and 5) as the whole plot variable, FeAc treatment as a split-plot variable, and incubation time as a repeated measure for motility. Model 2 excluded the modified BTS data, and the experimental design was a randomized complete block with duration of storage (d 0, 1, and 5) as the whole plot variable, FeAc treatment as a split-plot variable, and incubation time as a repeated measure for motility.

**Experiment 4: Effects of Freeze-Thawing on C11-BODIPY581/681 Oxidation**

This experiment was conducted to determine the changes in basal and FeAc-inducible membrane lipid peroxidation in thawed sperm in the absence and presence of varying concentrations of EDTA. After Percoll washing, a thawed sperm sample from each of 6 boars was divided into 6 portions for aerobic incubation for 30 and 120 min at 37°C, with 6 treatment combinations of FeAc and EDTA (μM): 0/0 and 0, 0/0 and 9, 1/30 and 0, 1/30 and 1, 1/30 and 3, and 1/30 and 9. The oxidation of BODIPY was measured at 30 min, and motility was measured at 30 and 120 min. The experimental design was a randomized complete block, with the 6 treatment combinations as fixed effects and incubation time as a repeated measure for motility.

**Statistical Analysis**

The data were analyzed using the MIXED procedure (Little et al., 1996; SAS Inst. Inc., Cary, NC), as described for each experiment. The model diagnostics included testing for a normal distribution of the error residuals and for homogeneity of treatment variance (Little et al., 1996). Comparison of least squares means was made by the LSMEANS statement using the DIFF option.

**RESULTS**

**Experiment 1 (A, B)**

The specificity of BODIPY and HE oxidation, in response to treatment with 3 different ROS generators, is shown in Tables 1 (experiment 1A) and 2 (experiment 1B), respectively. In experiment 1A, basal BODIPY oxidation in the absence of ROS generators was very low, with less than 1% of viable sperm containing oxidized BODIPY. The percentage of sperm containing oxidized BODIPY did not change (P = 0.41) from 30 to 120 min of incubation at 37°C. After FeAc treatment, the mean percentage of sperm containing oxidized BODIPY at 30 and 120 min was over 90%, greater (P < 0.01) than the means in the absence of FeAc. In the presence of FeAc, BODIPY fluorescence intensity/cell increased (P < 0.05) 9-fold at 30 min compared with its absence and continued to increase (P < 0.05) from 30 to 120 min. Compared with the absence of ROS generators, incubation with MEN and H2O2 had no effect on the percentage of sperm with BODIPY oxidation or on BODIPY fluorescence intensity (LSMEANS test, Table 1).

In experiment 1B, the dot-plot marker was set just above the viable sperm population in the absence of ROS generators (Guthrie and Welch, 2006). Less than 1% of this cell population contained oxidized HE, and this number did not change from 30 to 120 min of incubation at 37°C (LSMEANS test, Table 2). Compared with the absence of ROS generators, incubation with 300 μM H2O2 or 30 μM MEN increased (P < 0.05) the percentage of sperm with HE oxidation to a maximum, greater than 80%, by 30 min and increased (P < 0.05) ethidium fluorescence intensity/cell 3- to 4-fold. By con-

<p>| Table 1. Comparison of the effects of H2O2, menadione (MEN), and FeSO4-Na ascorbate (FeAc) on C11-BODIPY581/591 (BODIPY) oxidation in viable sperm of boars (Exp. 1A) |
|-----------------|----------------|----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>H2O2, μM</th>
<th>MEN, μM</th>
<th>FeAc, μM</th>
<th>Incubation time, min</th>
<th>Sperm with oxidized BODIPY, %</th>
<th>BODIPY fluorescence intensity/sperm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0/0</td>
<td>30</td>
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<td>0.30 ± 0.06²</td>
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<td>120</td>
<td>99.0 ± 0.4²⁴</td>
<td>3.66 ± 0.80²⁴</td>
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²,³Means within a column with no superscript letter in common differ (P < 0.05).

⁴Values are means ± SEM for ejaculates from 4 boars.

²FeAc = a combination of FeSO4 and Na ascorbate, expressed as μM/μM.

³Mean channel fluorescence number, fluorescence detector 1 output.
Table 2. Comparison of the effects of \( \text{H}_2\text{O}_2 \), menadione (MEN), and \( \text{FeSO}_4\text{-Na ascorbate} \) (FeAc) on hydroethidium (HE) oxidation in viable sperm of boars (Exp. 1B)

<table>
<thead>
<tr>
<th>( \text{H}_2\text{O}_2 ), ( \mu\text{M} )</th>
<th>MEN, ( \mu\text{M} )</th>
<th>FeAc, ( \mu\text{M} )</th>
<th>Incubation time, min</th>
<th>Sperm with oxidized HE, %</th>
<th>Ethidium fluorescence intensity/sperm ( ^{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0/0</td>
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<td>0.5 ± 0.2 ( ^{a} )</td>
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<td>1.51 ± 0.15 ( ^{a} )</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0/0</td>
<td>30</td>
<td>98.4 ± 0.8 ( ^{b} )</td>
<td>6.44 ± 0.27 ( ^{b} )</td>
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<tr>
<td>0</td>
<td>30</td>
<td>1/30</td>
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<td>99.5 ± 0.1 ( ^{b} )</td>
<td>6.69 ± 0.13 ( ^{b} )</td>
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<tr>
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<td>0/0</td>
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<td>6.44 ± 0.27 ( ^{b} )</td>
</tr>
<tr>
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<td>30</td>
<td>1/30</td>
<td>120</td>
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\( ^{a,b} \)Means within a column with no superscript letter in common differ (\( P < 0.05 \)).

1Values are means ± SEM for ejaculates from 3 boars.

2FeAc = a combination of \( \text{FeSO}_4 \) and \( \text{Na ascorbate} \), expressed as \( \mu\text{M} \).

3Mean channel fluorescence number, fluorescence detector 3 output.

Table 3. Effects of 30 min of incubation with Fe chelator deferoxamine (DFM) on basal and \( \text{FeSO}_4\text{-Na ascorbate} \) (FeAc) induced \( \text{C}_{11} \)-BODIPY \( ^{581/591} \) (BODIPY) peroxidation in viable sperm of boars (Exp. 2A)

<table>
<thead>
<tr>
<th>FeAc, ( \mu\text{M} )</th>
<th>DFM, ( \mu\text{M} )</th>
<th>Sperm with oxidized BODIPY, %</th>
<th>BODIPY fluorescence intensity/sperm ( ^{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/0</td>
<td>0</td>
<td>0.1 ± 0.1 ( ^{a} )</td>
<td>0.30 ± 0.03 ( ^{a} )</td>
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<tr>
<td>0/30</td>
<td>0</td>
<td>98.5 ± 0.3 ( ^{b} )</td>
<td>3.05 ± 0.36 ( ^{b} )</td>
</tr>
<tr>
<td>1/30</td>
<td>1</td>
<td>87.9 ± 2.7 ( ^{c} )</td>
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<td>9</td>
<td>0.2 ± 0.1 ( ^{a} )</td>
<td>0.30 ± 0.03 ( ^{a} )</td>
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\( ^{a,b} \)Means within a column with no superscript letter in common differ (\( P < 0.05 \)).

1Values are means ± SEM for an ejaculate from each of 3 boars.

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Table 2: Comparison of the effects of \( \text{H}_2\text{O}_2 \), menadione (MEN), and \( \text{FeSO}_4\text{-Na ascorbate} \) (FeAc) on hydroethidium (HE) oxidation in viable sperm of boars (Exp. 1B).

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<th>Incubation time, min</th>
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2FeAc = a combination of \( \text{FeSO}_4 \) and \( \text{Na ascorbate} \), expressed as \( \mu\text{M} \).

3Mean channel fluorescence number, fluorescence detector 3 output.

Figure 1: Effects of menadione (MEN), \( \text{H}_2\text{O}_2 \) (HP), and \( \text{FeSO}_4\text{-Na ascorbate} \) (FeAc) on the percentage of motile boar sperm after incubation at 37°C (30 min, ■; 120 min, □). Values are means ± SEM for an ejaculate from each of 7 boars (Exp. 1A and 1B). \( ^{a,b} \)Means with no letter in common differ (\( P < 0.05 \)).

Experiment 2 (A, B, C)

The effect of the Fe chelator DFM on FeAc-induced BODIPY oxidation is shown in Table 3 (experiment 2A). Compared with the absence of FeAc, the presence of FeAc alone increased (\( P < 0.05 \)) the percentage of sperm with oxidized BODIPY from less than 1 to 98.5% and increased BODIPY fluorescence intensity/cell 10-fold. The addition of DFM to FeAc decreased (\( P < 0.05 \)) the percentage of sperm with oxidized BODIPY and BODIPY fluorescence intensity/cell; the responses to 3 and 9 \( \mu\text{M} \) DFM were not different than the responses in the absence of FeAc (LSMEANS test, Table 3). Sperm viability as measured by the exclusion of PI from the sperm averaged 91% in experiment 2A and was not affected (\( P = 0.99 \)) by treatment (data not shown).

Motility for the treatment groups having no FeAc treatment was maintained at 70 to 75% during the 30 and 120 min of incubations (Figure 2). Compared with the absence of FeAc, motility in the presence of FeAc
Lipid peroxidation in boar sperm

Figure 2. Effects of the Fe chelator deferoxamine mesylate (DFM) on basal and FeSO$_4$-Na ascorbate- (FeAc) induced inhibition of the percentage of motile boar sperm after incubation at 37°C (30 min, □; 120 min, △). Values are means ± SEM for an ejaculate from each of 4 boars (Exp. 2A). a,bMeans with no letter in common differ ($P < 0.05$).

did not change after 30 min (LSMEANS test, Figure 2) but decreased ($P < 0.05$) to 30% after 120 min. Motility at 120 min in the presence of FeAc with 3 and 9 $\mu M$ DFM was maintained at a level that was not different from motility in the absence of FeAc (LSMEANS test, Figure 2).

In experiment 2B, EDTA was used at the same concentrations as DFM in experiment 2A. The pattern of response to EDTA in the presence of FeAc with respect to inhibition of BODIPY peroxidation and maintenance of motility was similar to that found in response to DFM; 3 and 9 $\mu M$ EDTA attenuated or eliminated ($P < 0.05$) the FeAc-induced BODIPY oxidation and inhibition of motility (data not shown).

Because EDTA was found to be effective in antagonizing FeAc induction of BODIPY oxidation, EDTA at greater dosages was administered to fresh boar sperm in experiment 2C to investigate its effect on H$_2$O$_2$-induced HE oxidation. Amounts of EDTA up to 1,000 $\mu M$ did not inhibit H$_2$O$_2$-induced HE oxidation (data and LSMEANS tests not shown).

Experiment 3

The results of hypothermic liquid semen storage in normal and modified BTS (model 1) showed that compared with its presence, the absence of EDTA in the BTS storage extender had no effect on BODIPY oxidation fluorescence intensity ($P = 0.85$), percentage of cells with oxidized BODIPY ($P = 0.22$), viability ($P = 0.60$), or motility ($P = 0.49$) during storage (data not shown). For model 2 (data summarized in Table 4), only FeAc treatment ($P < 0.0001$) was a source of variation for BODIPY oxidation. Fluorescence intensity and percentage of cells with oxidized BODIPY were not affected by storage time ($P = 0.20$ and 0.23, respectively) or the interaction of FeAc treatment with storage time ($P = 0.20$ and 0.07, respectively). Basal BODIPY oxidation in the absence of FeAc on d 0 through 5 averaged 1.0%, and BODIPY fluorescence intensity/cell averaged 0.3. Compared with no FeAc, the presence of FeAc increased the percentage of viable sperm containing oxidized BODIPY to an overall mean of 82% and BODIPY fluorescence intensity/cell to an overall mean of 1.3. The viability on day zero, 91%, was greater ($P < 0.05$) than the viability on d 1 and 5 (85.5 and 82%, respectively); means in the presence or absence of FeAc did not differ ($P = 0.83$).

Analysis of motility showed that storage time ($P = 0.001$), FeAc treatment ($P < 0.0001$), incubation time ($P = 0.004$), and the interaction of FeAc and incubation time ($P = 0.02$) were the only sources of variation (Figure 3). Motility in the absence of FeAc did not differ and did not decrease from 30 to 120 min on any day (LSMEANS test, Figure 3). Compared with no FeAc, the presence of FeAc on d 0 decreased ($P < 0.05$) motility after 120 min of incubation but not at 30 min. By contrast, on d 1 and 5, compared with no FeAc, the presence

Table 4. Effects of hypothermic liquid storage in Beltsville thawing solution on basal and FeSO$_4$-Na ascorbate- (FeAc) induced changes in C$_{11}$-BODIPY$_{581/591}$ (BODIPY) oxidation in viable boar sperm (Exp. 3)$^1$

<table>
<thead>
<tr>
<th>Storage time, d</th>
<th>FeAc, $^2$ $\mu M$</th>
<th>Sperm with oxidized BODIPY, %</th>
<th>BODIPY fluorescence intensity/cell $^3$</th>
<th>Cell viability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/0</td>
<td>1.3 ± 0.3$^a$</td>
<td>0.36 ± 0.04$^a$</td>
<td>90.1 ± 2.6$^a$</td>
</tr>
<tr>
<td></td>
<td>1/30</td>
<td>75.0 ± 0.2$^b$</td>
<td>1.17 ± 0.12$^b$</td>
<td>92.6 ± 0.8$^b$</td>
</tr>
<tr>
<td>1</td>
<td>0/0</td>
<td>0.8 ± 0.2$^b$</td>
<td>0.22 ± 0.02$^b$</td>
<td>85.7 ± 2.0$^b$</td>
</tr>
<tr>
<td></td>
<td>1/30</td>
<td>81.9 ± 5.5$^b$</td>
<td>1.25 ± 0.14$^b$</td>
<td>85.2 ± 1.8$^b$</td>
</tr>
<tr>
<td>5</td>
<td>0/0</td>
<td>1.0 ± 0.3$^a$</td>
<td>0.25 ± 0.03$^b$</td>
<td>83.4 ± 1.6$^b$</td>
</tr>
<tr>
<td></td>
<td>1/30</td>
<td>88.6 ± 4.5$^b$</td>
<td>1.58 ± 0.17$^b$</td>
<td>81.0 ± 2.8$^b$</td>
</tr>
</tbody>
</table>

$^a,b$Means within a column with no superscript letter in common differ ($P < 0.05$).

$^1$Values are means ± SEM for 6 ejaculates.

$^2$FeAc  = a combination of FeSO$_4$ and Na ascorbate, expressed as $\mu M/\mu M$.

$^3$Mean channel fluorescence number, fluorescence detector 1 output.
Effects of hypothermic liquid storage in Beltsville thawing solution on basal and FeSO$_4$-Na ascorbate-(FeAc) induced inhibition of the percentage of motile boar sperm after incubation at 37°C (30 min, □; 120 min, □□). Values are means ± SEM for ejaculates from 6 boars (Exp. 3). a–f Means with no letter in common differ (P < 0.05). of FeAc decreased (P < 0.05) motility at both 30 and 120 min.

**Experiment 4**

In the absence of FeAc and EDTA, the basal level of BODIPY peroxidation was low; only 3.4% of thawed sperm contained oxidized BODIPY (Table 5). Examination of dot plots for thawed sperm (Figure 4, panel A, quadrant 1) and fresh sperm (data not shown) revealed a small subpopulation of thawed viable sperm containing oxidized BODIPY that was absent or reduced in fresh sperm. Compared with no FeAc, the inclusion of 9 μM EDTA alone caused a slight decrease (P < 0.05) in the percentage of sperm containing oxidized BODIPY to 1.2%. Compared with no FeAc, the presence of FeAc increased the percentage of sperm containing oxidized BODIPY to 75.4%. The presence of 3 or 9 μM EDTA with FeAc maintained the percentage of sperm containing oxidized BODIPY and BODIPY fluorescence intensity/cell at low levels that were not different from the levels in sperm in the absence of FeAc and EDTA (LSMEANS test, Table 5). The dot plots in Figure 4 show the increase in BODIPY green fluorescence intensity caused by FeAc (panel B, quadrant 1) compared with the absence of FeAc (panel A, quadrant 1) and shows the inhibitory effect of EDTA on FeAc-induced BODIPY peroxidation (panel C, quadrant 1).

Mean viability of thawed sperm in the absence of FeAc was approximately 64% (Table 5). The presence of FeAc alone decreased (P < 0.05) viability to 48.9%. The presence of EDTA (all concentrations) with FeAc maintained viability at levels that did not differ from the absence of FeAc (LSMEANS test, Table 5). The fluorescence of BODIPY in nonviable sperm was not statistically analyzed in this experiment, but the percentage of nonviable sperm with high intensity BODIPY fluorescence was approximately 80% with a mean fluorescence intensity that was equal to or greater than the mean for viable sperm treated with FeAc (see Figure 4, panel B).

Motility of thawed sperm in the absence of FeAc (with or without EDTA) was stable at about 23% from 30 to 120 min, but FeAc alone decreased (P < 0.05) motility at 30 and 120 min compared with no FeAc (Figure 5). The inclusion of 3 or 9 μM EDTA with FeAc completely blocked the effect of FeAc by maintaining motility at levels similar to sperm incubated in the absence of FeAc.

**DISCUSSION**

The assay of BODIPY oxidation is accepted as superior to MDA analysis as a method of measuring lipid peroxidation in cultured cells (Pap et al., 1999) or mammalian sperm (Ball and Vo, 2002; Brouwers and Gadeilla, 2003). Our objective was to establish the assay un-

![Figure 3](image-url)

**Figure 3.** Effects of hypothermic liquid storage in Beltsville thawing solution on basal and FeSO$_4$-Na ascorbate-(FeAc) induced inhibition of the percentage of motile boar sperm after incubation at 37°C (30 min, □; 120 min, □□). Values are means ± SEM for ejaculates from 6 boars (Exp. 3). a–f Means with no letter in common differ (P < 0.05).
Figure 4. Flow cytometric 2-dimensional dot plots of fluorescence intensities of 10,000 individual boar sperm from thawed semen stained with C11-BODIPY591/691 (BODIPY) and propidium iodide (PI). Sperm were incubated for 30 min at 37°C in the absence (panel A) or in the presence of FeSO₄-Na ascorbate (FeAc, 1 and 30 μM, respectively; panel B) and with FeAc and 9 μM EDTA (panel C). Propidium iodide was added to distinguish between membrane intact and permeant cells. Data in quadrants 1 and 3 represent viable sperm with low intensity PI fluorescence. In the absence of FeAc (panel A), only 4% of viable thawed sperm contained oxidized BODIPY. The presence FeAc (panel B) increased the percentage of sperm containing oxidized BODIPY to 78% of the viable sperm. Treatment with EDTA blocked the effect of FeAc (panel C), with only 4% of the viable population containing oxidized BODIPY.

Figure 5. Effects of metal chelator EDTA on FeSO₄-Na ascorbate- (FeAc) induced inhibition of the percentage of motile boar sperm after incubation at 37°C (30 min, ■; 120 min, □). Values are means ± SEM for an ejaculate from each of 6 boars (Exp. 4). a,bMeans with no letter in common differ (P < 0.05).

Lipid peroxidation in boar sperm

Under conditions that would reveal early changes in lipid peroxidation and motility before spontaneous lipid peroxidation or the effects of ROS generators became lethal. We then determined the incidence and level of lipid peroxidation during hypothermic liquid storage and after thawing of frozen sperm.

Mammalian sperm contain abundant mitochondrial and cytoplasmic superoxide dismutase (Mennella and Jones, 1980), so it is likely that H₂O₂ or ROS derived from H₂O₂ is responsible for damage to cell structure and function. That H₂O₂ readily passes through membranes makes it a major threat to lipids through the Fenton reaction with the production the hydroxyl radical (Aitken, 1995; Storey, 1997; Halliwell and Gutteridge, 1999). Lipid peroxidation is triggered by oxidative activity of hydroxyl radicals and by existing lipid hydroperoxides after conversion to alkoxyl radicals by the Fenton reaction (Aitken, 1995; Storey, 1997; Halliwell and Gutteridge, 1999; Spiteller, 2006).

One of the major findings of this study and an earlier study (Guthrie and Welch, 2006) was that basal ROS formation, in the absence of a ROS-generating system, in boar sperm is very low. This is important, because not all cells are endowed with the enzymes that scavenges for ROS (Storey, 1997; Halliwell and Gutteridge, 1999). Theoretically, the first 2 check points in the ROS defense system are dismutation and then catalase or glutathione peroxidase to catalyze the reduction of H₂O₂ to water (Aitken, 1995; Storey, 1997). In fresh boar semen, the rate of basal or spontaneous lipid peroxidation formation was low (MDA formation: Mennella and Jones, 1980; Comaschi et al., 1989; Cerolini et al., 2000; BODIPY oxidation: current study). Based on the low basal levels of ROS formation and lipid peroxidation in fresh boar sperm or during hypothermic liquid storage, the endogenous ROS defense system is either successful or essentially unchallenged, indicating a low level of mitochondrial superoxide formation or sufficient enzymatic activity to neutralize H₂O₂.

Low basal or spontaneous lipid peroxidation in freshly collected sperm appears to be true for many mammalian species: bovine as measured by BODIPY oxidation (Brouwers and Gadella, 2003), human as measured by MDA formation (Storey, 1997), ovine as measured by MDA formation and BODIPY oxidation (Christova et al., 2004), and equine sperm as measured by MDA formation (Baumber et al., 2000) and BODIPY
oxidation (Ball and Vo, 2002; Almeida and Ball, 2005; Neild et al., 2005).

The negative effects of some ROS-generating systems do not require lipid peroxidation to induce cytotoxic changes in spermatozoa. Hypoxanthine-xanthine oxidase treatment inhibits sperm motion but has no effect on boar sperm MDA formation (Mennella and Jones, 1980). Menadione and H₂O₂ also decreased the percentage of motile sperm but had no effect on BODIPY oxidation in the current study. The use of HE and BODIPY can capture a wide range of ROS formed during exposure to oxidative stress: the oxidation of HE by superoxide or H₂O₂ (Guthrie and Welch, 2006) and the oxidation of BODIPY by the action of hydroxyl, alkoxyl, and peroxyl radicals and peroxynitrite (Drummen et al., 2002).

Reactive oxygen species inhibit sperm motion and become cytotoxic through damage to proteins, nucleic acids, and membrane lipids if ROS concentrations become high enough to overcome or saturate the natural defense mechanisms of the cell and extending medium (Aitken, 1995; Storey, 1997). Seminal plasma contains many protective enzymatic antioxidants such as superoxide dismutase, the glutathione peroxidase-glutathione reductase system, and catalase and a variety of nonenzymatic antioxidants such as ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (Storey, 1997). Potentially, the protective effect of these antioxidants or free radical scavengers is reduced when seminal plasma is diluted to varying degrees during liquid hypothermic storage or during freezing and thawing procedures. Although the rate of spontaneous lipid peroxidation was low (1%) in viable sperm stored up to 5 d in BTS, viability did decrease during storage. We found that during FeAc treatment, BODIPY fluorescence intensity in individual sperm increased approximately 2-fold from d 0 to 5. This increasing susceptibility to FeAc during liquid in BTS has also been reported by the measurement of MDA formation in boar sperm on d 0, 3, and 5 (Cerolini et al., 2000). The addition of α-tocopherol to BTS attenuates viability loss and FeAc-induced lipid peroxidation in sperm stored for 5 d (Cerolini et al., 2000), so it is possible that oxidative stress is a possible cause of increased cell death during hypothermic liquid storage.

A factor that could contribute to efficacy of BTS in boar sperm hypothermic liquid storage even in the absence of α-tocopherol or other free radical scavengers is the composition of BTS. An important constituent of BTS, EDTA (3.35 mM), was found to be inhibitory of FeSO₄-catalyzed lipid peroxidation at 0.3 μM in ram (Jones and Mann, 1976) and at 0.28 μM in boar sperm (Comaschi et al., 1989). In the current study, during liquid storage, the presence of EDTA in BTS did not convey any advantage over its absence. However, the presence of either EDTA or the Fe chelator DFM during incubation of boar sperm at 37°C with FeAc was effective in blocking lipid peroxidation and maintaining motility. Perhaps the presence of seminal plasma during liquid storage provided sufficient protection from lipid peroxidation even in the absence of EDTA.

Thawed sperm appeared to be more sensitive to the presence FeAc than fresh sperm. The presence of FeAc during incubation of thawed sperm not only increased lipid peroxidation and decreased motility but also decreased viability from 64 to 49%. Incubation of fresh or liquid stored sperm with FeAc did not reduce viability. Although the low dose of EDTA, 1 μM, did not block FeAc-lipid peroxidation or maintain motility, it did maintain viability. Thawed and fresh sperm may have also differed with respect to expression of basal lipid peroxidation. The percentage of sperm with spontaneous lipid peroxidation appeared to be slightly greater in thawed sperm compared with freshly collected sperm (3.4 vs. ≤1%). In addition, the presence of 9 μM EDTA decreased the percentage of high intensity BODIPY fluorescent thawed sperm from 3.4 to 1.2%. A possible conclusion is that the antioxidant defenses in thawed sperm may have been slightly lower compared with fresh sperm, and the addition of EDTA during sperm incubation compensated for this deficiency. Iron and other transition metals are likely present in seminal plasma and egg yolk and are theoretically available to undergo a Fenton reaction if not chelated by EDTA.

Freeze-thawing increased boar sperm basal lipid peroxidation measured by MDA formation by 3-fold compared with fresh semen (Roca et al., 2004). In contrast, the level of spontaneous lipid peroxidation was low in viable sperm. However, if the results for viable and nonviable sperm had been combined, the total BODIPY fluorescence intensity/cell would have shown a considerable increase in thawed compared with fresh semen. Nonviable sperm comprised approximately 36% of the thawed population in our study, and the BODIPY fluorescence intensity of nonviable sperm was similar to that in viable sperm treated with FeAc. Therefore, the high rate of basal lipid peroxidation thawed boar sperm reported previously (Roca et al., 2004) was likely due to the presence of nonviable, freeze-thaw-damaged cells.

In contrast to thawed boar sperm, thawed bovine sperm underwent a high rate of lipid peroxidation when incubated for 12 h with BODIPY at 38°C in TYR (Brouwers and Gadella, 2003). Basal lipid peroxidation is low in thawed bovine sperm only when the egg yolk-based freezing extender is retained during the 12-h incubation with BODIPY (Brouwers and Gadella, 2003). Egg yolk is an important component of freezing extenders. Dialyzed egg yolk offers almost complete protection from Fe²⁺-catalyzed lipid peroxidation in ram sperm (Jones and Mann, 1977) and an egg yolk Tris-glycerol freezing extender scavenged ROS produced by xanthine-xanthine oxidase incubated with bull sperm (Chatterjee and Gagnon, 2001).

In conclusion, basal or spontaneous membrane lipid peroxidation was low in viable fresh, liquid stored, and frozen-thawed boar sperm, and all these forms of boar sperm were susceptible to FeAc. The advantages of the flow cytometric BODIPY assay over measurement of
MDA formation is the ability to determine the percentage of the viable sperm population affected by lipid peroxidation and to measure changes in lipid peroxidation on a per cell basis over time. It is important to distinguish between viable and nonviable cells, because nonviable cells have greater BODIPY fluorescence intensity than viable sperm.

LITERATURE CITED


