Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry

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ABSTRACT: The use of frozen semen in the swine industry is limited by problems with viability and fertility compared with liquid semen. Part of the reduction in sperm motility and fertility associated with cryopreservation may be due to oxidative damage from excessive or inappropriate formation of reactive oxygen species (ROS). Chemiluminescence measurements of ROS are not possible in live cells and are problematic because of poor specificity. An alternative approach, flow cytometry, was developed to identify viable boar sperm containing ROS utilizing the dyes hydroethidine and 2′, 7′-dichlorodihydrofluorescein diacetate as oxidizable substrates and impermeant DNA dyes to exclude dead sperm. The percentage of sperm with high mitochondrial transmembrane potential was determined by flow cytometry using the mitochondrial probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbo-cyanine iodide with propidium iodide staining to exclude nonviable cells. Sperm were incubated with and without ROS generators and free radical scavengers. Basal ROS formation was low (less than 4%) and did not differ (P = 0.26) between viable fresh and frozen-thawed boar sperm. In addition, fresh and frozen-thawed viable sperm were equally susceptible (P = 0.20) to intracellular formation of ROS produced by xanthine/xanthine oxidase (94.4 and 87.9% of sperm, respectively). Menadione increased (P < 0.05) ROS formation, decreased (P < 0.05) JC-1-aggregate fluorescence intensity, and decreased (P < 0.05) motion variables by 25 to 60%. The mechanism of inhibition of motility by ROS formation may be related to a decrease in mitochondrial charge potential below a critical threshold. Catalase and superoxide dismutase treatment in the presence of xanthine/xanthine oxidase indicated that hydrogen peroxide was the primary intracellular ROS measured. Further, catalase, but not superoxide dismutase, was capable of attenuating ROS-induced inhibition of motility. Whereas basal intracellular hydrogen peroxide formation was low in viable fresh and frozen-thawed boar sperm, both were quite susceptible to external sources of hydrogen peroxide.

Key words: 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbo-cyanine iodide, cryopreservation, flow cytometry, hydroethidine, motility

INTRODUCTION

Whereas most swine in the United States are produced by artificial insemination (Levis, 2000), the use of frozen semen in the swine industry is limited by problems with viability and fertility compared with liquid semen (Johnson, 1985; Waberski et al., 1994). Part of the reduction in sperm motility and fertility associated with cryopres-
Chemiluminescence measurements of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are problematic because of poor specificity (Fridovich, 1997, 2003). As an alternative approach, flow cytometric procedures have been used to identify ROS in leukocytes by utilizing the dyes hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) as ROS substrates (Bass et al., 1983; Bucana et al., 1986; Carter et al., 1994). The percentage of cells with a high MMP can be estimated flow cytometrically using the mitochondrial probe, 5, 5′, 6, 6′-tetrachloro-1, 1′, 3′, 3′-tetrakis(4-dimethylaminophenyl)carbazole iodide (JC-1; Cossarizza et al., 1993). The objective of this study was to validate the use of flow ofometry as a method to measure ROS and MMP in viable fresh and frozen-thawed sperm.

MATERIALS AND METHODS

Boars used in this study were treated under experimental protocol #04-031 approved by the Beltsville Area Animal Care and Use Committee.

Sperm Processing

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, semen collection, and determination of sperm concentration were conducted as previously described (Guthrie and Welch, 2005). Before initiation of incubation treatments, fresh and thawed sperm were extended to a concentration of 150 × 10⁶/mL in Beltsville Thawing Solution (BTS; Guthrie and Welch, 2005) and 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). The sperm pellets were washed and diluted to a suitable concentration (30 to 60 × 10⁶ sperm/mL) in a modified noncapacitating Tyrode’s medium (TYR; Guthrie and Welch, 2005). The Percoll isolation procedure is required because constituents of the freezing extenders interfere with the use of JC-1 for determination of MMP in boars (H. D. Guthrie, unpublished data) and bull sperm (Garner et al., 1999).

Cryopreservation

The boar semen cryopreservation protocol (Guthrie and Welch, 2005) was based on one published previously (Almlid and Johnson, 1988). Sperm (1 × 10⁹ 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, St. Louis, MO) and 0.5% Equex STM Paste (vol/vol; Nova Chemical Sales, Scituate, MA). The contents of the straws were frozen in liquid N₂ vapor in a programmable freezer (IceCube 1810, Minitube of America, Verona, WI), 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 2-chamber MicroCell glass counting chambers (Conception Technologies, San Diego, CA) using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd., Sheffield, England) set for boar sperm analysis (Holt et al., 1996). Sperm were extended to 30 × 10⁶/mL in BTS containing 0.3% BSA (A2153, Sigma-Aldrich, Cohn Fraction V) and warmed in a heating block at 38°C for 30 min before analysis. The sperm motion variables recorded for statistical analysis were percent motile (motility); curvilinear velocity (VCL), μm/sec; cross-beat frequency (BCF), Hz; and percent linear motion (LIN).

ROS-Generating Systems

Effects of ROS formation on motility, viability, and MMP were investigated utilizing the following treatments: xanthine/xanthine oxidase (XA/XO) treatment to generate O₂⁻ and H₂O₂ extracellularly (Britigan et al., 1990), menadione (2-methyl-1, 4-naphthoquinone) that generates O₂⁻ intracellularly as a consequence of disruption of mitochondrial NADH-ubiquinone oxidoreductase (Floreani et al., 2002), and direct treatment with H₂O₂.

A 2-step procedure was used to expose the sperm to various levels ROS through the use of ROS-generating systems and ROS scavengers. For the XA/XO oxidase system, a set of tubes containing the 0.5-mL sperm suspensions were warmed for 5 min at 38°C at a concentration of 60 × 10⁶ sperm/mL with or without the ROS scavengers superoxide dismutase (SOD; S-8160, Sigma) and catalase (CAT; 0, 200, and 600 U/mL; C-1345, Sigma). A second set of tubes containing 0 or 2 mM XA (X0626, Sigma) or 0, 0.02, 0.06, or 0.2 U/mL of XO (X4500, Sigma), or both XA and the various doses of XO, were also warmed for 5 min. After warming, 0.5-mL volumes of the XA/XO system components from the second set of tubes were mixed with 0.5-mL volumes of sperm, providing the following final concentrations: sperm at 30 × 10⁶/mL; SOD and CAT at 0, 100, or 300 U/mL; XA at 1 mM; XO at 0, 0.01, 0.03, or 0.1 U/mL; or XA and the various doses of XO. The mixtures were allowed to incubate an additional 30 min at 38°C before their preparation for motion and flow cytometric analysis.

A 2-step procedure was also used for generation of ROS by menadione (M5625, Sigma). Aliquots containing 0, 20, 60, or 200 μM of menadione were removed from a 200 mM working solution in DMSO, transferred into of 0.5 mM of TYR, and warmed to 38°C for 5 min. After warming, 0.5-mL suspensions containing 60 × 10⁶ sperm were added and incubated at 38°C for 30 min.
Staining Sperm for Flow Cytometric Analysis of ROS and MMP

The dye H$_2$DCFDA is freely permeable across cell membranes and is incorporated into hydrophobic regions of the cell (Bass et al., 1983). The acetate moiety is cleaved by cellular esterases, leaving impermeant, non-fluorescent, 2′,7′-dichlorodihydrofluorescein (H$_2$DCF). The H$_2$DCF is oxidized by H$_2$O$_2$ to dichlorofluorescein (DCF), which emits fluorescence at 530 nm in response to 488 nm excitation (Carter et al., 1994).

Hydroethidine is the sodium borohydride-reduced form of ethidium (Bucana et al., 1986). Hydroethidine is freely permeable to cells and is oxidized by O$_2$ to ethidium (Rothe and Valet, 1990; Carter et al., 1994) and other products (Fridovich, 2003; Zhao et al., 2003). Ethidium binds to DNA with fluorescent emission at 610 nm in response to 488-nm excitation.

The probe JC-1 is freely permeable to cells and undergoes reversible transformation from a monomer to an aggregate form (J$_{agg}$) when it binds to membranes having an MMP of 80 to 100 mV (Cossarizza et al., 1993). The J$_{agg}$ form fluoresces at 590 nm in response to 488-nm excitation. A flow cytometric JC-1 protocol for sperm (Garner et al., 1997) was modified to enumerate viable sperm with high MMP by electronically gating on viable sperm that were negative for propidium iodide (PI).

Preliminary testing was conducted to optimize the temperature and duration of the staining protocols for maximum fluorescence development (data not shown). Working solutions for HE (20 mM; D-1168, Molecular Probes Inc., Eugene, OR), H$_2$DCFDA (20 mM; D-399, Molecular Probes), Yo Pro-1 (0.1 mM; Y-3603, Molecular Probes), and JC-1 (0.15 mM; T-3168, Molecular Probes Inc.) were prepared in DMSO. The exclusion of the DNA dye Yo Pro-1 from plasma membrane intact sperm identifies them as viable cells. The working solution for PI (2.4 mM; P-4170, Sigma Chemical Co., St. Louis, MO) was prepared in TYR.

After the incubation treatments, aliquots of 1 × 10$^6$ sperm (33 μL) were transferred to 12 × 75-mm polypropylene tubes containing 467 μL of TYR prepared for each stain combination at the following final concentrations: 1) HE and Yo Pro-1, 4 and 0.05 μM, respectively; 2) H$_2$DCFDA and PI, 200 and 9.6 μM, respectively; and 3) JC-1 and PI, 0.3 and 9.6 μM, respectively. Staining times and temperatures for the stain combinations were as follows: HE and Yo Pro-1, 40 min and 25°C; H$_2$DCFDA and PI, 60 min and 25°C; and JC-1 and PI, 10 min and 38°C.

Flow Cytometric Analysis

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. Fluorescence from ethidium- and Yo Pro-1-stained sperm was collected in fluorescence detector 3 with a 620 nm band-pass (BP) filter and fluorescence detector 1 with a 525 nm BP filter, respectively. Statistical regions were set manually to enumerate the total viable sperm population (Yo Pro-1-negative), as shown in Figure 1 (quadrants 3 and 4 in panels A and B), and the viable subpopulation containing ROS, designated ethidium-positive (E+), as shown in quadrant 4 of panels A and B. Data in quadrants 1 and 2 represented nonviable sperm penetrated and stained by Yo Pro-1. The log of the mean ethidium fluorescence intensity/sperm of the viable sperm population was recorded from the fluorescence detector 3 output. For example, in Figure 1 sperm were incubated in the absence (panel A) or in the presence (panel B) of 1 mM XA and 0.1 U/mL XO. In the absence of XA/XO, only 1.4% of this population contained sufficient ROS to oxidize HE to ethidium (as shown in quadrant 4 of Figure 1), whereas in the presence of XA/XO, 94.4% of the viable sperm contained the oxidized form.

Fluorescence from DCF- and PI-stained single sperm was collected in fluorescence detector 1 (525 nm BP) and fluorescence detector 3 (620 nm BP), respectively. Statistical regions were set manually to enumerate the total viable sperm population (PI-negative) shown in Figure 2 (quadrants 3 and 4 in panels A and B) and the viable subpopulation containing ROS, designated DCF-positive (DCF+), shown in quadrant 4 of panels A and B. Data in quadrants 1 and 2 of Figure 2 represent nonviable sperm penetrated and stained with PI. The mean log of DCF fluorescence intensity/sperm was recorded for viable sperm from the fluorescence detector 1 output. In the absence of XA/XO, only 0.1% of this population contained sufficient ROS to convert H$_2$DCFDA to DCF (as shown in quadrant 4 of Figure 1), whereas in the presence of XA/XO, 73.3% of the viable sperm contained the oxidized form.

The standard filter configuration of the flow cytometer was altered for analysis of MMP (JC-1-fluorescence detector) by using a 645 nm dichroic filter to reflect wavelengths shorter than 645 nm to a 595 BP filter (fluorescence detector 2) and pass wavelengths longer than 645 nm to a 675 nm BP filter (fluorescence detector 4). The fluorescence detector 4 collected PI fluorescence to distinguish between the viable, PI-negative (PI−), and nonviable, PI-positive (PI+), sperm populations, which are illustrated in Figure 3, panels A and D, for fresh and frozen-thawed sperm, respectively. The percentage of viable sperm (panels A and D) was 84.1 and 43.3%, respectively.

The J$_{agg}$ fluorescence (red-orange) for viable sperm was collected in the fluorescence detector 2. The lower limit of the region attributed to the J$_{agg}$ fluorescence (the top region marked J$_{agg}$+ in Figure 3, panels B, C, E, and F) was delineated by a statistical marker set just above the dead sperm J$_{agg}$− fluorescence level (see panels C and F). The dot-plots (panels B and E), showing the J$_{agg}$ fluorescence (vertical axis) distribution of viable sperm, were derived by gating on the PI- populations (panels A and D), respectively. The dot-plots in panels C and F,
Figure 1. Flow cytometric, 2-dimensional dot-plots of fluorescence intensities of 10,000 individual boar sperm from fresh semen stained with hydroethidine (HE) and Yo Pro-1 (Y-3603, Molecular Probes Inc., Eugene, OR). Sperm were incubated for 30 min at 38°C in the absence of xanthine and xanthine oxidase as controls (panel A), and in the presence of 1 mM xanthine and 0.1 U of xanthine oxidase/mL (panel B). Data in quadrants 3 and 4 represent viable sperm with no Yo Pro-1 fluorescence. Only 1.4% of the control sperm population (panel A, quadrant 4) contained sufficient reactive oxygen species to oxidize HE to ethidium, compared with 94.4% of the sperm incubated with xanthine and xanthine oxidase (panel B, quadrant 4). The fluorescence of Yo Pro-1 and ethidium were collected in fluorescence detectors 1 (FL1) and 3 (FL3), respectively.

showing the $J_{agg}$ fluorescence (vertical axis) distribution of nonviable sperm, were derived by gating on the PI+ populations in panels A and D, respectively. The percentage of viable sperm and the percentage of $J_{agg}$+viable sperm, and the log of the mean $J_{agg}$ fluorescence detector intensity/sperm, were recorded from the fluorescence detector 2 output. The percentage of $J_{agg}$+ sperm shown in panels B, C, E, and F of Figure 3 was 99, 30, 93, and 9%, respectively.

The epifluorescence images of the 3 fluorescent labeling patterns of JC-1 and PI in 3 unfixed boar sperm are shown in Figure 4. The staining pattern for the 3 sperm from left to right are a viable sperm with a high MMP (nonred fluorescent nucleus with a $J_{agg}$+ “red-orange” fluorescent midpiece), a nonviable sperm with a low MMP (red fluorescent nucleus with no $J_{agg}$+ “red-orange” midpiece), and a viable sperm with a low MMP (nonred fluorescent nucleus with no $J_{agg}$+ “red-orange” midpiece).

The negative regulation of MMP was demonstrated in preliminary experiments (data not shown). Analysis of dead sperm killed by repeated freeze-thawing in the absence of cryoprotectants decreased the percentage of sperm with high MMP ($J_{agg}$ fluorescence) to zero. In a second experiment, boar sperm were incubated aerobically for 4 h at 38°C in TYR without or with 5 $\mu$M calcium ionophore A23187 to uncouple respiration and oxidative phosphorylation. The percentage of viable sperm, 80% overall, was not affected by A23187 under these conditions. However, in comparison with no A23187, the presence of A23187 decreased ($P < 0.05$) the percentage of sperm with high MMP from 79 to 1%.

Experiment 1. XA/XO ROS-Generation Studies

Preliminary experiments were conducted to establish the concentrations of XA and XO and incubation times that increased intracellular ROS formation and decreased motility without decreasing viability (data not shown). Incubation of sperm with up to 300 U of SOD/mL had no effect ($P = 0.43$) on ROS formation in the presence or absence of XA/XO (data not shown).

Experiment 1 was conducted to determine the effects of XA/XO on intracellular ROS content and the effects of ROS on motion variables after a 30-min aerobic incubation in the absence or presence XA/XO and CAT. One ejaculate was collected from each of 5 boars, and each sperm sample was divided into 6 portions. Two XA/XO-free portions served as detached controls, one without CAT and the other with CAT at 300 U/mL. The remaining 4 parts received XA at a dose of 1 mM and were in a $2 \times 2$ factorial arrangement of XO at 0.03 and 0.1 U/mL and CAT at 0 and 300 U/mL. The first
Figure 2. Flow cytometric, 2-dimensional dot-plots of fluorescence intensities of 10,000 individual boar sperm from fresh semen stained with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and propidium iodide (PI). Sperm were incubated for 30 min at 38°C in the absence of xanthine and xanthine oxidase as controls (panel A), and in the presence of 1 mM xanthine and 0.1 U/mL xanthine oxidase (panel B). Data in quadrants 3 and 4 represent viable sperm with no PI fluorescence. Only 0.1% of the control sperm population (panel A, quadrant 4) contained sufficient reactive oxygen species to convert H2DCFDA to 2′,7′-dichlorofluorescein (DCF), compared with 70.3% of the viable sperm incubated with xanthine and xanthine oxidase (panel B, quadrant 4). The fluorescence of DCF and PI were collected in fluorescence detectors 1 (FL1) and (FL3), respectively.

Experiment 2. Effects of H2O2 on Fresh Sperm

The oxidation of HE was selected to determine ROS accumulation in subsequent experiments because in preliminary experiments both dyes were oxidized by 300 μM H2O2, and HE required a shorter incubation period than H2DCFDA for maximum fluorescence development. In addition, results from Exp. 1 showed that CAT inhibited ROS formation generated by XA/XO, measured by either ethidium or DCF fluorescence detection.

Experiment 2 was conducted to confirm that HE could be used for the measurement of H2O2, and to confirm that CAT and not SOD would attenuate H2O2-induced oxidation of HE and inhibition of motility. One ejaculate was collected from each of 6 boars, and each sperm sample was divided into 7 portions for aerobic incubation with 7 treatment combinations. Treatments 1 through 7 consisted of the following dosages for H2O2 (μM), CAT (U/mL), and SOD (U/mL), respectively: 1) 0, 0, 0; 2) 0, 0, 300; 3) 0, 300, 0; 4) 300, 0, 0; 5) 300, 0, 300; 6) 300, 300, 0; and 7) 300, 300, 300. The experimental design was a randomized complete block with the 7 treatments as fixed effects. The error variances for 2 variables, percentage E+ sperm and motility, were partitioned into 2 variance groups to compensate for heterogeneity of treatment variance; one group contained the 2 treatment combinations incubated with 300 μM H2O2 and no CAT, and the other group contained the other 5 treatment combinations.

Experiment 3. ROS in Fresh and Thawed Sperm

This experiment was conducted to determine the intracellular amounts of ROS in fresh and frozen-thawed sperm in the absence and presence of XA/XO. Fresh semen was analyzed on the day of collection, and cryopreserved semen was thawed and analyzed during the following week. Eight ejaculates were used, and each sperm sample, fresh or frozen-thawed, was divided into 3 parts and assigned to one of three 30-min incubation treatments: 25°C, no XA/XO; 38°C, no XA/XO; and 38°C, XA at 1 mM and XO at 0.1 U/mL. The experimental design was a randomized complete block with semen treatment (fresh or frozen-thawed) as the whole plot.
Figure 3. Flow cytometric histograms and 2-dimensional dot-plots of fluorescence intensities of boar sperm stained with 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetrathylbenzimidazoylcarbocyanine iodide (JC-1) and propidium iodide (PI) for fresh (panels A–C) and frozen-thawed (panels D–F) semen. The PI− and PI+ peaks represent viable and nonviable sperm, respectively. The dot-plots in panels B and E, showing the JC-1 aggregate (Jagg) fluorescence (vertical axis) distribution of viable sperm, were derived by gating on the PI− populations in panels A and D, respectively. The dot-plots in panels C and F, showing the Jagg fluorescence (vertical axis) distribution of nonviable sperm, were derived by gating on the PI+ populations in panels A and D, respectively. The fluorescence of JC1 monomer, JC1 aggregates, and PI were collected in fluorescence detectors 1 (FL1), 2 (FL2), and 4 (FL4), respectively.

and the 3 incubation treatments as the subplots. The error variances of 2 variables, percentage of E+ sperm and ethidium fluorescence intensity, were partitioned into 2 variance groups; one group incubated in the absence of XA/XO, and the other group in the presence of XA/XO. The error variance of percentage of Jagg+ sperm was partitioned into 2 variance groups: the fresh and frozen-thawed treatments.

Experiment 4. Menadione Studies

Preliminary experiments were conducted by incubating sperm for 5 min to 4 h using doses of menadione ranging from 30 μM to 3 mM (data not shown). Incubation for 30 min with a dose of 100 μM was chosen as the greatest concentration in subsequent experiments because it did not decrease (P = 0.37) viability compared with no menadione and reduced motility to a range of 40 to 60% compared with a total inhibition of motility after a 30- to 60-min exposure to doses of >100 μM menadione.

Experiment 4 was conducted to determine the effects of menadione dose on ROS formation, MMP, and motion variables. Each semen sample was divided into 4 portions for aerobic incubation with menadione at 0, 10, 30, or 100 μM at 38°C for 30 min. The experimental design was a randomized complete block with dose of menadione as the fixed effect. Data for the variable percentage of E+ sperm were transformed to arcsine square roots to compensate for a positive correlation between the means and variances.

Statistical Analysis

Data were analyzed using the MIXED model procedure of SAS (release 8.02, SAS Inst. Inc., Cary, NC), as
Figure 4. The epifluorescence images of the 3 fluorescence labeling patterns of 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolycarbocyanine iodide and propidium iodide in unfixed boar sperm. The staining pattern from left to right represents a viable sperm with a high mitochondrial transmembrane potential, a nonviable sperm with a low mitochondrial transmembrane potential, and a viable sperm with a low mitochondrial transmembrane potential.

described for each experiment. The model diagnostics included testing for a normal distribution of 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

The effects of XA/XO and CAT on sperm ethidium fluorescence intensity are shown in Table 1. Basal, spontaneous ROS formation in the absence XA/XO was low in viable boar sperm; only 3.8 and 1% of the sperm were E+ or DCF+, respectively. Compared with the detached controls (no XA/XO), XA/XO treatment increased \( (P < 0.01) \) the percentage of E+ sperm to 90.1 and 95.5% and increased \( (P < 0.05) \) ethidium fluorescence intensity by 3- and 4.6-fold, respectively.

The pattern of DCF fluorescence in response to XA/XO treatment differed from that of the ethidium response to XA/XO. Whereas both 0.03 and 0.1 U/mL of XO increased the percentage of DCF+ sperm and intensity of DCF fluorescence, compared with no XO, the response to 0.1 U/mL of XO was less than that of 0.03 U/mL of XO (Table 1). The presence of CAT during incubation with XA/XO attenuated the XA/XO-induced increases in ethidium and DCF fluorescence intensity for both XO concentrations.

The effects of XO and CAT concentration on motion variables are shown in Table 2. Motility in the presence of 0.1 U/mL XO (33%) was decreased \( (P < 0.05) \) compared with 0.03 U/mL of XO (62%) and restored when CAT was present with 0.1 U/mL of XO (72%). Compared with no CAT, the values of VCL, BCF, and LIN were increased \( (P < 0.01) \) when CAT was present with either 0.03 or 0.1 U/mL of XO. The percentage of viable sperm present after treatment, as estimated by negative PI and Yo Pro-1 staining, was 60.3 and 61.0%, respectively, and in neither case was viability affected by incubation treatment (data not shown).

Experiment 2

Effects of \( \text{H}_2\text{O}_2 \), CAT, and SOD concentrations on sperm ethidium fluorescence intensity and motility are shown in Table 3. Incubation with \( \text{H}_2\text{O}_2 \) for 30 min increased \( (P < 0.05) \) the percentage of E+ sperm, increased \( (P = 0.041) \) ethidium fluorescence intensity, and decreased \( (P < 0.01) \) motility. Presence of CAT during the incubation with \( \text{H}_2\text{O}_2 \) completely blocked the \( \text{H}_2\text{O}_2 \) stimulatory effect on ethidium fluorescence and the \( \text{H}_2\text{O}_2 \) inhibitory effect on motility. By contrast, the incubation of SOD with \( \text{H}_2\text{O}_2 \) had no significant effect on the development of ethidium fluorescence or the inhibitory effect on motility. Percentage of viable sperm estimated

<table>
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<tr>
<th>XA, mM</th>
<th>XO, U/mL</th>
<th>CAT, U/mL</th>
<th>Sperm with ethidium fluorescence, %</th>
<th>Mean ethidium fluorescence intensity/sperm</th>
<th>Sperm with DCF fluorescence, %</th>
<th>Mean DCF fluorescence intensity/sperm</th>
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<td>3.8 ± 1.9a</td>
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<td>0</td>
<td>90.1 ± 2.8a</td>
<td>14.5 ± 1.9c</td>
<td>96.5 ± 1.0b</td>
<td>21.7 ± 2.8b</td>
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<td>95.5 ± 2.6b</td>
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<td>300</td>
<td>60.4 ± 11.8d</td>
<td>9.3 ± 0.7b</td>
<td>4.4 ± 0.8a</td>
<td>6.7 ± 1.3a</td>
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</table>

\( a-d \)Means within a column with no superscript letter in common differ \( (P < 0.05) \) by LSMEANS using the DIFF option of SAS (SAS Inst. Inc., Cary, NC).

1Values are means ± SEM for an ejaculate from each of 5 boars.
Effects of catalase (CAT) and xanthine (XA)/xanthine oxidase (XO) on motion variables of sperm of boars (Exp. 1)

<table>
<thead>
<tr>
<th>XA, mM</th>
<th>XO, U/mL</th>
<th>CAT, U/mL</th>
<th>Motility, %</th>
<th>Curvilinear velocity, μm/sec</th>
<th>Cross beat frequency, Hz</th>
<th>Linearity, %</th>
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</table>

Values are means ± SEM for an ejaculate from each of 5 boars.

Effects of freeze-thawing and incubation with XA/XO on sperm ethidium fluorescence and Jagg+ formation are shown in Table 4. Incubation treatment was a source of variation for the development of ethidium fluorescence (P < 0.01), whereas storage treatment and the interaction of storage and incubation treatments were not. In the absence of XA/XO, the percentage E+ viable sperm after 30-min incubation at 25°C was very low in fresh and frozen-thawed semen (1.1 and 1.9%, respectively). Compared with 25°C, incubation at 38°C increased (P < 0.05) the percentage of E+ viable sperm in both fresh and frozen-thawed semen (3.5 and 2.8%, respectively). Ethidium fluorescence intensity followed a pattern similar to that of the percentage E+ sperm; there was no significant difference between fresh or frozen-thawed semen at either incubation temperature. Compared with no XA/XO, the presence of XA/XO increased (P < 0.05) the percentage of E+ viable sperm and ethidium fluorescence intensity for both fresh and frozen-thawed semen (E+ sperm: 94.4 and 87.9%, respectively), and the effect did not differ significantly between fresh and frozen-thawed semen.

Storage treatment influenced (P < 0.01) the percentage of Jagg+ viable sperm, whereas incubation treatment and the interaction of storage and incubation treatment had no effect (Table 4). The incidence of Jagg+ viable sperm was >90% over all treatment combinations; however, values for frozen-thawed semen were less (P < 0.05) than those of fresh semen. The Jagg+ fluorescence intensity was increased (P < 0.05) in frozen-thawed sperm after incubation at 38°C compared with incubation at 25°C; Jagg fluorescence intensity did not differ (P = 0.11) between the absence and presence of XA/XO (Table 4).

The effects of freeze-thawing and incubation with XA/XO on sperm motion variables and viability are shown in Table 5. Motility, VCL, BCF, and viability decreased (P < 0.05) in frozen-thawed compared with fresh sperm. Compared with incubation at 25°C, incubation at 38°C did not affect motility (P = 0.22) and BCF (P = 0.10); however, VCL and viability were decreased (P < 0.05). The presence of XA/XO decreased motility and BCF compared with no XA/XO at 38°C in fresh sperm and compared with no XA/XO at 25°C in frozen-thawed sperm. Incubation at 38°C decreased (P < 0.05) VCL and viability compared with incubation at 25°C for both fresh and frozen-thawed sperm, but XA/XO at 38°C had no additional effect on either variable.

The concentration of menadione was a significant source of variation for the percent of E+ viable sperm.
(P < 0.01), ethidium fluorescence intensity (P < 0.01), and Jagg fluorescence intensity (P < 0.01; Table 6). The percentage of E+ viable sperm was low for 0 and 10 μM menadione, approximately 1.5%. However, increasing the menadione concentration to 30 and 100 μM increased the percentage of E+ viable sperm to 24.6 and 92.1%, respectively. Ethidium fluorescence intensity tended to increase between 10 and 30 μM menadione, but only the increase between 30 and 100 μM menadione was significant (P < 0.05). The percentage of Jagg+ viable sperm after 30-min incubation averaged 95% and increased the percentage of E+ viable sperm to 24.6 and 92.1%, respectively. Ethidium fluorescence intensity decreased in a dose related fashion from 28.3 with 0 μM menadione to 11.6 with 100 μM of menadione.

Concentration of menadione was a significant source of variation for motility, (P < 0.05), VCL (P < 0.01), BCF (P < 0.05), and LIN (P = 0.056; Table 7). Motion variables did not vary among menadione concentrations ranging from 0 to 30 μM, but all were decreased at 100 μM compared with the other menadione concentrations. Incubation of sperm with increasing concentrations of menadione for 30 min at 38°C had no effect (P = 0.44) on the percentage of viable sperm with a mean for the experiment of 78% (data not shown).

### DISCUSSION

Treatment of boar sperm with either XA/XO or H₂O₂ caused the development of ethidium and DCF intracellular fluorescence. The relatively short exposure to ROS, an incubation period of 30 min was sufficient, to decrease percent of motile sperm but not to decrease viability compared with control incubations at the same temperature. Some sources have indicated that HE and H₂DCFDA were specifically oxidized by ·O₂ and H₂O₂, respectively (Carter et al., 1994; Zamzami et al., 1995; Haugland, 2005), whereas others have concluded that under certain conditions HE can serve as a substrate for oxidation by either H₂O₂ or ·O₂ (Kobzik et al., 1990; Rothe and Valet, 1990).

In agreement with the results of others using the XA/XO ROS-generating system in human (Aitken et al., 1993; Griveau et al., 1995) and stallion (Baumber et al., 2000) sperm, and in other cell types (Zamzami et al., 1995; Halliwell and Gutteridge, 1999), results from the current study provide evidence that H₂O₂ is the primary source of oxidative damage in boar sperm. The presence of CAT during incubation with XA/XO or H₂O₂ decreased intracellular fluorescence of both DCF and ethidium, whereas SOD had no effect, indicating that both probes were measuring intracellular H₂O₂. Even

### Table 4. Effects of semen storage (fresh and frozen-thawed) and xanthine (XA)/xanthine oxidase (XO) treatment on the oxidation of hydroethidine to ethidium and on mitochondrial transmembrane potential as measured by J-aggregate (Jagg) fluorescence in viable sperm of boars (Exp. 3)¹

<table>
<thead>
<tr>
<th>Storage</th>
<th>Incubation</th>
<th>Sperm with ethidium fluorescence, %</th>
<th>Mean ethidium fluorescence intensity/sperm</th>
<th>Sperm with Jagg fluorescence, %</th>
<th>Mean Jagg fluorescence intensity/sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>25°C</td>
<td>1.1 ± 0.3ᵃ</td>
<td>3.0 ± 0.3ᵇ</td>
<td>98.1 ± 0.5ᵃ</td>
<td>33.3 ± 2.7ᵃ</td>
</tr>
<tr>
<td></td>
<td>38°C</td>
<td>3.5 ± 0.9ᵇ</td>
<td>3.6 ± 0.3ᵇ</td>
<td>98.0 ± 0.3ᵇ</td>
<td>33.6 ± 1.9ᵃ</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>38°C, XA/XO</td>
<td>94.4 ± 2.9ᵃ</td>
<td>12.6 ± 1.8ᵇ</td>
<td>97.8 ± 0.5ᵃ</td>
<td>36.8 ± 3.0ᵃ</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>1.9 ± 0.3ᵃ</td>
<td>3.4 ± 0.2ᵇ</td>
<td>95.8 ± 1.6ᵇ</td>
<td>34.7 ± 2.4ᵇ</td>
</tr>
<tr>
<td></td>
<td>38°C</td>
<td>2.8 ± 0.3ᵇ</td>
<td>3.7 ± 0.2ᵇ</td>
<td>91.1 ± 2.8ᵇ</td>
<td>40.8 ± 1.4ᵇ</td>
</tr>
<tr>
<td></td>
<td>38°C, XA/XO</td>
<td>87.9 ± 4.4ᵃ</td>
<td>15.1 ± 2.7ᵇ</td>
<td>95.0 ± 1.3ᵇ</td>
<td>41.7 ± 2.2ᵇ</td>
</tr>
</tbody>
</table>

ᵃᵇMeans within a column with no superscript letter in common differ (P < 0.05) by LSMEANS using the DIFF option of SAS (SAS Inst. Inc., Cary, NC).

¹Values are means ± SEM for an ejaculate from each of 8 boars.

### Table 5. Effects of semen storage (fresh and frozen-thawed) and xanthine (XA)/xanthine oxidase (XO) treatment on sperm motion variables and sperm viability of boars (Exp. 3)¹

<table>
<thead>
<tr>
<th>Semen storage</th>
<th>Incubation treatment</th>
<th>Motility, %</th>
<th>Curvilinear velocity, μm/sec</th>
<th>Cross beat frequency, Hz</th>
<th>Viability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>25°C</td>
<td>90.9 ± 2.3ᵃ</td>
<td>105.7 ± 1.5ᵇ</td>
<td>18.6 ± 0.9ᵃ</td>
<td>92.5 ± 0.7ᵃ</td>
</tr>
<tr>
<td></td>
<td>38°C</td>
<td>80.0 ± 3.4ᵇ</td>
<td>88.0 ± 2.5ᵇ</td>
<td>14.7 ± 1.1ᵃ</td>
<td>76.7 ± 3.6ᵇ</td>
</tr>
<tr>
<td></td>
<td>38°C, XA/XO</td>
<td>52.1 ± 7.3ᵇ</td>
<td>73.3 ± 7.8ᵇ</td>
<td>5.6 ± 1.9ᵇ</td>
<td>76.2 ± 2.3ᵇ</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>25°C</td>
<td>33.5 ± 7.4ᵇ</td>
<td>76.9 ± 4.8ᵇ</td>
<td>8.3 ± 1.5ᵇ</td>
<td>45.2 ± 4.8ᵇ</td>
</tr>
<tr>
<td></td>
<td>38°C</td>
<td>20.3 ± 4.4ᵈ</td>
<td>66.8 ± 4.3ᵈ</td>
<td>5.5 ± 1.0ᵇ</td>
<td>32.2 ± 3.1ᵈ</td>
</tr>
<tr>
<td></td>
<td>38°C, XA/XO</td>
<td>7.9 ± 3.0ᵈ</td>
<td>52.2 ± 13.5ᵈ</td>
<td>2.5 ± 1.8ᵇ</td>
<td>32.6 ± 1.4ᵈ</td>
</tr>
</tbody>
</table>

ᵃᵇMeans within a column with no superscript letter in common differ (P < 0.05) by LSMEANS using the DIFF option of SAS.

¹Values are means ± SEM for an ejaculate from each of 8 boars.
measurements can be made exclusively in the viable methods for ROS measurement because intracellular tric assays have an advantage over chemiluminescence because of intracellular ROS formation. Flow cytometric assays should be useful in identifying populations of sperm that may be dysfunctional accumulation in frozen-thawed relative to fresh sperm. Moreover, it is possible that incubation at °C for longer periods of time would increase ROS formation in the absence of SOD. In boar sperm after a 30-min incubation of fresh or frozen-thawed semen, it is possible that incubation at °C for longer periods of time would increase ROS formation in the absence of SOD. The effects of storage-associated or spontaneous accumulation of ROS in boar sperm is difficult to evaluate portion of the sperm population, and chemiluminescence methods lack specificity (Fridovich, 1997; 2003). Specificity is reduced using lucigenin, luminol, and tetrazolium because they must be univalently reduced to the corresponding monocation before reacting with -O2 and producing a luminescent signal. The lucigenin monocation is capable ofautoxidation and produces -O2 even in cases where no -O2 was being produced in the absence of lucigenin. Luminal and tetraborn radicals can also spontaneously reduce O2 to -O2 in the presence of a variety of oxidants, and in all cases SOD has an inhibitory effect.

Membrane lipid peroxidation is one outcome of ROS formation in sperm (Aitken, 1995). Others have shown that freshly collected boar sperm are susceptible to FeSO4-ascorbate catalyzed lipid peroxidation as measured by malondialdehyde formation (Roca et al., 2004) and become more susceptible during hypothermic liquid storage (Cerolini et al., 2000) and with increased time after thawing (Breininger et al., 2005). Freeze-thawing increased boar sperm lipid peroxidation compared with fresh semen, but this effect was partially attenuated by the presence of butylated hydroxytoluene (Roca et al., 2004). However, the chemiluminescence methods for ROS measurement or the malondialdehyde method for lipid peroxidation measurement do not distinguish between viable and nonviable sperm. Thus, the formation of ROS and subsequent lipid peroxidation might have occurred only in a small portion of the population or primarily in nonviable sperm.

The effects of storage-associated or spontaneous accumulation of ROS in boar sperm is difficult to evaluate

<table>
<thead>
<tr>
<th>Menadione, µM</th>
<th>Sperm with ethidium fluorescence, %</th>
<th>Mean ethidium fluorescence intensity/sperm</th>
<th>Mean Jagg fluorescence intensity/sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.5 ± 1.9⁶</td>
<td>2.2 ± 0.6⁶</td>
<td>28.3 ± 0.9⁶</td>
</tr>
<tr>
<td>10</td>
<td>1.6 ± 2.7⁶</td>
<td>2.3 ± 0.4⁶</td>
<td>23.2 ± 0.8⁵</td>
</tr>
<tr>
<td>30</td>
<td>24.6 ± 0.8⁶</td>
<td>3.4 ± 0.4⁶</td>
<td>16.9 ± 0.8³</td>
</tr>
<tr>
<td>100</td>
<td>92.1 ± 2.8⁶</td>
<td>9.6 ± 1.9⁵</td>
<td>11.6 ± 2.8⁴</td>
</tr>
</tbody>
</table>

a,bMeans within a column with no superscript letter in common differ (P < 0.05) by LSMEANS using the DIFF option of SAS (SAS Inst. Inc., Cary, NC).

Table 7. Effects of menadione concentration on sperm motion variables of boars (Exp. 4)¹

<table>
<thead>
<tr>
<th>Menadione, µM</th>
<th>Motility, %</th>
<th>Curvilinear velocity, µm/sec</th>
<th>Cross beat frequency, Hz</th>
<th>Linearity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68.2 ± 2.4⁶</td>
<td>81.3 ± 2.1⁶</td>
<td>11.0 ± 1.5⁶</td>
<td>26.9 ± 1.6⁶</td>
</tr>
<tr>
<td>10</td>
<td>67.0 ± 5.8⁶</td>
<td>76.0 ± 3.7⁶</td>
<td>9.9 ± 2.3⁶</td>
<td>26.0 ± 4.5⁶</td>
</tr>
<tr>
<td>30</td>
<td>70.2 ± 3.6⁶</td>
<td>73.5 ± 3.9⁶</td>
<td>6.6 ± 2.6⁶</td>
<td>27.4 ± 5.2⁶</td>
</tr>
<tr>
<td>100</td>
<td>42.8 ± 8.7⁶</td>
<td>60.5 ± 1.9⁶</td>
<td>3.1 ± 0.8⁶</td>
<td>13.7 ± 2.1⁶</td>
</tr>
</tbody>
</table>

a,bMeans within a column with no superscript letter in common differ (P < 0.05) by LSMEANS using the DIFF option of SAS (SAS Inst. Inc., Cary, NC).

¹Values are means ± SEM for an ejaculate from each of 3 boars.
because in many studies lipid peroxidation was not detectable without the use of a catalyst such as Fe\(^{2+}\)/ascorbate or a peroxidized fatty acid, or cold shock treatment (Mennella and Jones, 1980; Breininger et al., 2005). Another factor that could contribute to the low level of ROS formation in the current study is the composition of the cooling and freezing extenders. Dialyzed egg yolk was found to offer almost complete protection from Fe\(^{2+}\) catalyzed lipid peroxidation in ram sperm (Jones and Mann, 1977) and an egg yolk Tris-glycerol freezing extender scavenged ROS produced by XA/XO incubated with bull sperm (Chatterjee and Gagnon, 2001). An important constituent of BTS, EDTA (present at a concentration of 3.35 mM), was found to be inhibitory of Fe\(^{2+}\) catalyzed lipid peroxidation at 0.3 \(\mu M\) in ram (Jones and Mann, 1976) and at 0.28 \(\mu M\) in boar sperm (Comaschi et al., 1989).

Fluorescence-activated flow cytometric assays of J\(_{agg}\) fluorescence have been used to identify sperm with high MMP in a number of experiments with sperm (Garner et al., 1997; Gravance et al., 2001; Love et al., 2003). Results of the current study are novel because J\(_{agg}\) fluorescence was measured in the viable portion of the sperm population. Viability or changes in viability may not be an issue in short-term studies of cultured or dispersed cells. However, the ability to distinguish between viable and nonviable sperm becomes particularly important for studies in which sperm are subject to long-term hypothermic storage or cryopreservation. In a study investigating mitochondrial function in frozen-thawed bovine sperm, the percentage of viable sperm estimated by SYBR-14 staining was positively correlated with the percentage of JC-1 fluorescent sperm in the total population, \(r = 0.99\) (Garner et al., 1997). Similarly in the current study, the incidence of J\(_{agg}\) fluorescence was much less than in nonviable than in viable frozen-thawed sperm. Thus, the actual effect of an experimental treatment or cryopreservation on MMP in a sperm population could be greatly overestimated by the presence of dead cells.

Menadione-induced disruption of mitochondrial electron transport at complex I to produce \(\cdot O_2\) was used to examine the relationship between MPP and sperm motility. Hydrogen peroxide is likely a major source of oxidative damage in menadione-treated boar sperm because of the high levels of SOD activity in boar sperm mitochondria and cytoplasm (Mennella and Jones, 1980). Analysis of motion variables in Exp. 4 showed that 100 \(\mu M\) menadione increased ROS formation to include >90% of treated sperm and decreased motility and measures of sperm velocity compared with no menadione. Maintenance of high MMP (a high proton accumulation in the mitochondrial intramembrane space) is required to drive \(F_0/F_1\) ATPase catalyzed ATP production in somatic cells (Halliwell and Gutteridge, 1999). Others have shown that ATP content of sperm is decreased after XAXO-induced ROS formation (de Lamirande and Gagnon, 1992b; Armstrong et al., 1999). Perhaps decreased MMP reduced ATP production to the point at which the amount of ATP produced is not sufficient to maintain motility. However, ROS damage of the sperm contractile apparatus (de Lamirande and Gagnon, 1992a) is also possible.

In conclusion, basal or spontaneous intracellular ROS formation was low in viable fresh and frozen-thawed boar sperm, and both fresh and thawed sperm were quite susceptible to external sources of hydrogen peroxide. In this study, it was expected that spontaneous ROS formation might increase and trigger a decrease in MMP, followed by decreased ATP production and motility. In the current study, the incidence of basal or spontaneous ROS formation in viable sperm in fresh and frozen-thawed semen was very low and did not vary greatly among boars. For this reason, the measurement of spontaneous ROS formation in fresh or frozen-thawed semen would not likely be predictive of semen quality or fertility independent of motility or viability. However, studies are being designed to investigate long-term hypothermic liquid storage of boar semen, and this is where an association between spontaneous or induced ROS formation and sperm quality and possibly fertility might be found.

**IMPLICATIONS**

The value of these flow cytometric techniques for intracellular ROS formation and MMP is that ROS and MMP can be monitored during the early stages of ROS formation before death occurs in the sperm population. These techniques may be useful for evaluation of long-term hypothermic liquid storage of boar sperm and sperm in other species.

**LITERATURE CITED**


