Effects of Water- and Lipid-Soluble Antioxidants on Turkey Sperm Viability, Membrane Integrity, and Motility During Liquid Storage

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ABSTRACT Aerobic conditions are required to maintain the viability of turkey sperm *in vitro*. In mammalian sperm, excess oxygen during *in vitro* storage results in lipid peroxidation, causing membrane damage and reduced sperm motility and subsequent fertility. The effect of adding antioxidants to turkey sperm during liquid storage was studied. Semen was collected and pooled from 20 toms and antioxidants were tested at a minimum of six concentrations, n = 6 observations per concentration. Semen was diluted into Beltsville Poultry Semen Extender. Extended semen served as a control; treatments were extended semen supplemented with tocopherol (vitamin E, 1 to 80 μg/mL); butylated hydroxytoluene (BHT, 0.02 to 1.25 m*M*); Tempo (0.039 to 1.25 μM); or vitamin C (1 to 400 μg/mL) and stored at 5 C for 48 h. Sperm viability in extended semen was evaluated after 0, 24, or 48 h storage. Membrane integrity and motility were also measured. Flow cytometric analysis was done using the live/dead stain combination (SYBR-14/propidium iodide) for sperm viability, and membrane integrity was assessed using a hypo-osmotic stress test. Sperm motility was evaluated subjectively. Control sperm viability was reduced almost 50% between 0 and 48 h. However, supplementation with vitamin E, Tempo, and BHT maintained populations of viable sperm similar to the 0 h levels at 48 h. Hypo-osmotic membrane integrity in the control sperm was reduced to approximately 22% (at 24 h, $P \leq 0.05$) and 5% (at 48 h, $P \leq 0.05$) of the total sperm population. Similar to controls after 24 h *in vitro* storage, sperm treated with the antioxidants vitamin E, Tempo, and BHT had reduced hypo-osmotic membrane integrity compared to 0 h samples. However, many of these treated samples maintained hypo-osmotic membrane integrity observed from 24 through 48 h (range, 21.5 to 44.6%), whereas hypo-osmotic membrane integrity fell to 4.6% at 48 h for the control ($P < 0.05$). Vitamin C treatments were similar to controls at all time points. Addition of the antioxidants vitamin E, BHT, and Tempo to extended turkey semen improves sperm survival, membrane integrity, and reduces the loss of motility after 48 h storage.

(*Key words*: antioxidants, lipid peroxidation, sperm, flow cytometry, turkey)

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INTRODUCTION

Hypothermic storage of semen is used to reduce metabolism and maintain sperm viability over extended periods of time. Although extensive effort has been made to develop liquid storage procedures for holding turkey sperm for 24 h or longer, fertility is generally lower when hens are inseminated with semen stored more than 6 h (Sexton, 1988a,b; Lake, 1989; Wishart, 1989; Bakst *et al.*, 1991, 1994; Thurston, 1995). Turkey sperm cells require oxygen for maintenance of fertilizing ability *in vitro* (Wishart 1981; Sexton and Giesen, 1982). However, sperm are subject to oxygen toxicity resulting from lipid peroxidation, which can result in membrane damage, reduced motility, and lower fertility (Jones and Mann, 1973, 1977; Fujihara and Howarth, 1978; Fujihara and Koga, 1984; Wishart, 1984; Ravie and Lake, 1985).

Suppression of lipid peroxidation through addition of antioxidants, which block the production of reactive oxygen species or counteract oxygen toxicity, has been achieved with mammalian sperm with some success (Alvarez and Storey, 1983; Alverez *et al.*, 1987; Aitken and Clarkson, 1988; Maxwell and Stojanov, 1996). In this study, we evaluated three classes of antioxidants based on solubility characteristics (Hammerstedt, 1993). Antioxidants that are water-soluble, e.g., vitamin C, reduce peroxidation outside the cell but have little effect in the membrane or inside of the cell. Lipid-soluble compounds, such as vitamin E and butylated hydroxytoluene (BHT), minimize damage to the plasma membrane, as do compounds such as Tempo, which are lipid- and water-soluble (Lindermann and Kanous 1991; Hammerstedt, 1993). Our objective was to determine whether antioxidant supplementation could improve

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turkey sperm viability, membrane integrity, and sperm motility during *in vitro* storage for up to 48 h.

MATERIALS AND METHODS

Animals and Semen Collection

Commercial Large White breeder turkey toms were purchased from a primary breeder and maintained under standard husbandry conditions during their brooding and growing periods. Toms were photostimulated (0300 to 1700 h) at 26 wk of age and semen was collected by abdominal massage (Burrows and Quinn, 1935) beginning 2 wk later. Preliminary studies to determine optimal concentrations of antioxidants to test were initiated when toms were 30 wk old. Toms (n = 20) were 42 to 48 wk old during the study and housed in two pens. Semen was collected once a week for the 6 wk of the study.

Antioxidant Treatments

Ejaculates were pooled, mixed thoroughly, and diluted to 4×10^9 sperm per milliliter with Beltsville Poultry Semen Extender (BPSE).[3] Extended semen served as a control, treatments were extended semen supplemented with tocopherol[4] (vitamin E, 1 to 80 μg / mL); BHT[4] (0.02 to 1.25 m*M*); Tempo[4] (0.039 to 1.25 μ*M*); or vitamin C[4] (1 to 400 μg / mL) and stored at 5 C for 48 h. Because of low water solubility, stocks of BHT (100 m*M*) and Tempo (20 m*M*) were prepared in anhydrous dimethyl sulfoxide[5] (DMSO). Working solutions for BHT and Tempo were prepared by a 1:10 dilution of the stock solution to BPSE supplemented with 6% BSA.[4] Because DMSO has been shown to have some antioxidant effects in rooster sperm (Fujihara and Koga, 1984), a second control containing DMSO and BSA at concentrations equal to those which were in the highest antioxidant treatments was run with all experiments. Semen treatments were placed in 24-well flat bottom tissue culture plates[5] and room temperature water was placed between wells to the upper level of semen in the well. A 250-μL aliquot of semen suspension was diluted 1:1 with either BPSE or BPSE and antioxidant solution for a final volume of 500 μL and a sperm concentration of 2×10^9 sperm per milliliter. Each antioxidant was tested at a minimum of six concentrations, n = 6 replicate observations per concentration.

Semen Evaluation and Storage

For storage, tissue culture plates were placed on an orbital shaker (150 rpm) in a refrigerator at 5 C (Sexton, 1988a). An aliquot of semen from each treatment was evaluated for sperm viability, membrane integrity, and motility at 0, 24, and 48 h of *in vitro* storage.

To assess sperm viability, a combination of live / dead stains previously validated for use on turkey sperm was used (Donoghue *et al*., 1995). SYBR-14, a membrane-permeant DNA probe (FertiLight kit),[6] stained only living sperm, resulting in the nuclei fluorescing bright green when excited at 488 nm. Propidium iodide (PI) stained membrane-damaged cells red. The SYBR-14 (MW 565) was dissolved in DMSO at a concentration of 1 mg / mL. The PI was dissolved in BPSE at 4 mg / mL. Aliquots of diluted semen (500 μL) were stained with 0.27 μL of a SYBR-14 stock (diluted 1:10 with DMSO was used for staining, 0.1 mg / mL) and 8 μL of PI stock. All samples were incubated at 37 C for 15 min. Immediately after incubation and removal of a portion of sample for microscopic evaluation, samples were diluted 1:3 in PBS and analyzed by flow cytometry. To determine membrane integrity, a hypo-osmotic H_2O test was used (Donoghue *et al*., 1996). For both fresh and *in vitro* stored samples, 50-μL aliquots of diluted semen was added to 450 μL of either PBS or H_2O (osmolality 297 and 19 mosm / kg H_2O, respectively). Samples were stained and evaluated microscopically and flow cytometrically as described above for sperm viability. For both viability and hypo-osmotic membrane integrity, 10,000 individual sperm were assessed by flow cytometry for each replicate. Therefore, means represent data from the evaluation of 1.2 million individual sperm (six observations in replicate) for each treatment and control group.

Sperm were evaluated subjectively for percentage motility and forward progressive motility (scale of 0 to 5: 0 = no forward movement and 5 = rapid linear forward progression). A fixed volume of sperm suspension (10 μL) was placed on a microscope slide and evaluated at room temperature. A sperm motility index (SMI) was calculated to provide an overall estimate of sperm motility characteristics: SMI = (sperm percentage motility + [forward progressive motility $\times$ 20]) $\div$ 2 (Howard *et al*., 1986).

Flow Cytometry

Quantitative assessment of fluorescently-stained sperm was determined using an EPICS Profile II.[7] The flow cytometer utilizes an air-cooled Argon laser at (488 nm) and was equipped with the PowerPak option to provide for three-color fluorescence detection in addition to the side and forward light scatter parameters. Data were collected as log of fluorescence on 10,000 sperm per sample. The green wavelength fluorescence (LFL1) was collected through a 525 nm band pass filter, and the red fluorescence parameters fluorescence 2 (LFL2) and fluorescence 3 (LFL3) were gathered through 575- and 635-nm band pass filters, respectively. Data were analyzed using the Coulter Histogram Analysis[7] program.

Statistical Analysis

Data sets were analyzed by ANOVA using the least squares procedure and the General Linear Models Procedure of SAS® (SAS Institute, 1985). Data expressed as percentages were arc sine transformed before analysis.

[3]Continental Plastic Corp., Delavan, WI 53115-9916.
[4]Sigma Chemical Co., St. Louis, MO 63178-9916.
[5]PGC Scientifics, Gaithersburg, MD 20898.
[6]Molecular Probes, Eugene, OR 97402.
[7]Coulter Electronics, Inc., Hialeah, FL 33196.

RESULTS

For most treatments, no differences were observed between antioxidant treatments and controls at 0 h for any of the sperm variables evaluated (Tables 1 to 3). For all time points, controls with and without DMSO and BSA did not differ and therefore were combined for analysis. Addition of vitamin C did not produce differences in sperm viability, membrane integrity, or sperm motility index over controls at any concentration or time period tested ($P \geq 0.05$, data not shown).

When evaluated 24 h after initiation of *in vitro* storage, control sperm viability, hypo-osmotic membrane integrity, and sperm motility indexes were lower ($P \leq 0.05$, Tables 1 to 3) than for the 0 h control. These parameters in control samples were further reduced from 24 to 48 h ($P \leq 0.05$, Tables 1 to 3). Although sperm viability was reduced almost 50% by 48 h in the Control treatments, supplementation with vitamin E, Tempo, and BHT maintained sperm viability similar to 0 h levels at 48 h (Table 1, Figure 1a,b).

When sperm were subjected to hypo-osmotic stress, approximately 60% of the 0 h control sample maintained membrane integrity (Table 2). Hypo-osmotic membrane integrity in the control was reduced to approximately 22% at the 24 h ($P \leq 0.05$) and 5% of the total sperm population at 48 h ($P \leq 0.05$, Table 2). Similar to controls, after 24 h of storage, sperm supplemented with antioxidants vitamin E, Tempo, and BHT had reduced hypo-osmotic membrane integrity compared to 0 h samples (Table 2; Figure 2a,b). However, many of these treated samples maintained hypo-osmotic membrane integrity (observed at 24 h) through 48 h (range, 21.5 to 44.6%), whereas hypo-osmotic membrane integrity fell to 4.6% at 48 h for the Control (Table 2, Figure 2 a,b).

Unlike the control, for which the sperm motility index declined from 56.4% at 24 h to 17.8% at 48 h, treatments supplemented with vitamin E (5 to 80 μg/mL), Tempo (0.039 to 0.156 μM), and BHT (0.02 to 1.25 mM) maintained motility over the 48-h period (Table 3, Figure 3 a,b).

TABLE 1. Turkey sperm viability for samples supplemented with vitamin E and Tempo evaluated at 0, 24, and 48 h of *in vitro* storage

Treatment	Time of *in vitro* storage		
	0 h	24 h	48 h
	(%)		
Control	74.0 ± 4.0^{a,x}	59.2 ± 6.5^{b,x}	40.5 ± 6.4^{c,x}
Vitamin E			
1 μg/mL	90.6 ± 1.8^{a,xy}	83.7 ± 0.9^{b,y}	78.3 ± 1.5^{b,y}
2 μg/mL	85.7 ± 6.3^{a,xy}	85.2 ± 3.1^{a,y}	83.2 ± 3.0^{a,y}
5 μg/mL	88.1 ± 2.0^{a,xy}	67.5 ± 11.7^{b,x}	78.7 ± 6.2^{ab,y}
10 μg/mL	83.7 ± 5.0^{a,xyz}	60.7 ± 9.0^{b,x}	84.3 ± 3.7^{a,y}
20 μg/mL	74.3 ± 10.2^{a,xy}	72.3 ± 7.2^{a,xy}	73.2 ± 14.7^{a,y}
40 μg/mL	67.2 ± 17.3^{a,xyz}	83.1 ± 3.7^{a,y}	67.0 ± 16.3^{a,xy}
80 μg/mL	77.2 ± 4.2^{a,xy}	70.8 ± 12.8^{a,xy}	68.6 ± 11.6^{a,xy}
Tempo			
0.039 μM	71.4 ± 9.6^{ab,x}	58.9 ± 10.3^{b,x}	78.6 ± 1.4^{a,y}
0.078 μM	83.4 ± 1.5^{a,y}	74.1 ± 12.3^{a,xy}	79.3 ± 7.1^{a,y}
0.156 μM	73.3 ± 6.8^{a,x}	89.4 ± 0.7^{b,y}	61.4 ± 6.5^{a,y}
0.312 μM	80.2 ± 5.8^{a,xy}	73.3 ± 8.6^{a,xy}	77.8 ± 9.2^{a,y}
0.625 μM	79.9 ± 5.4^{a,xy}	72.3 ± 5.7^{a,xy}	69.5 ± 8.1^{a,y}
1.250 μM	77.2 ± 4.2^{a,xy}	50.3 ± 12.3^{b,x}	76.7 ± 8.5^{a,y}

$^{a-c}$Means in a row with no common superscript differ significantly ($P \leq 0.05$).

$^{x-z}$Means in a column with no common superscript differ significantly ($P \leq 0.05$).

DISCUSSION

Addition of appropriate concentrations of lipid- and amphipathic-soluble antioxidants maintained viability, membrane integrity, and motility of turkey sperm after 48 h *in vitro* storage better than our control system. It is speculated that the improvement in sperm parameters are a result of antioxidants suppressing or limiting the damaging effects of lipid peroxidation *in vitro.* Possibly the improvements to turkey sperm are at the level of the membrane, as lipid- and water-and-lipid-soluble antioxidants maintained these sperm parameters but the water-soluble antioxidant tested did not.

Vitamin E, Tempo, and BHT maintained turkey sperm membrane integrity during 48 h of *in vitro* storage at levels fivefold higher than controls. Because of their lipid solubility, these antioxidants can permeate plasma membranes and suppress free radical damage (Aitken and Clarkson, 1988). In the present study, these antioxidants appear to maintain membrane integrity and

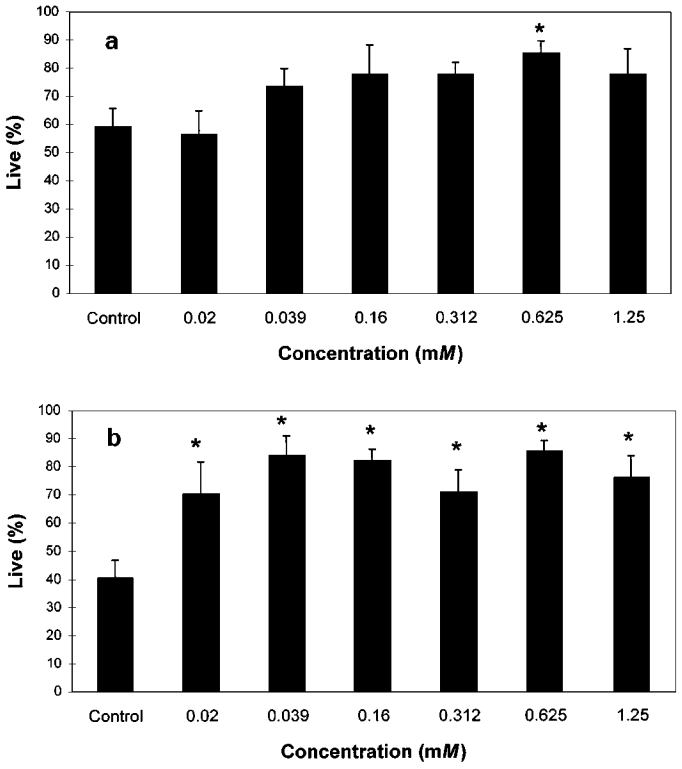

FIGURE 1. Viability (percentage) of sperm treated with butylated hydroxytoluene at 24 (a) and 48 h (b) *in vitro* storage at 5 C. *Value significantly different from control ($P \leq 0.05$).

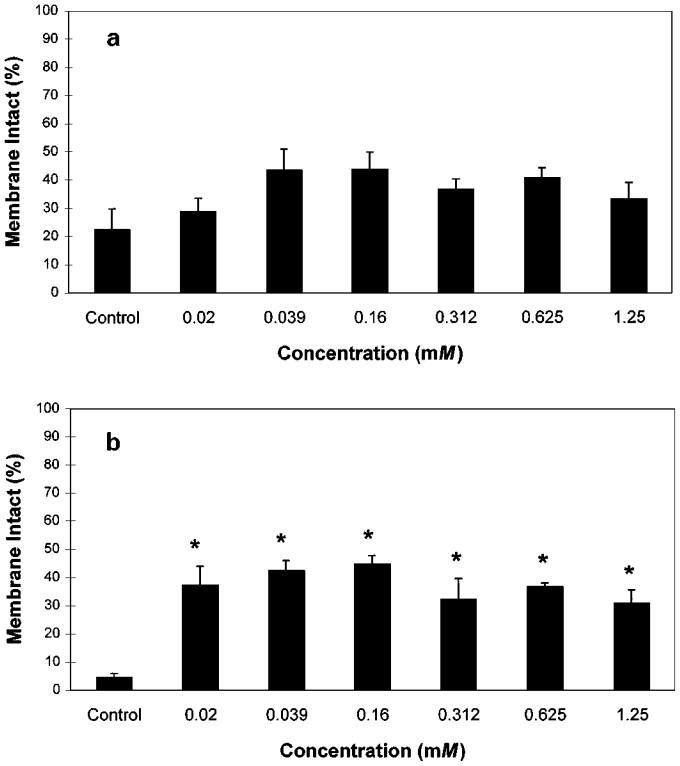

FIGURE 2. Hypo-osmotic membrane integrity (percentage) of sperm treated with butylated hydroxytoluene at 24 (a) and 48 h (b) *in vitro* storage at 5 C. *Value significantly different from control ($P \leq 0.05$).

TABLE 2. Membrane integrity of semen samples supplemented with vitamin E and Tempo subjected to hypoosmotic stress test and evaluated at 0, 24, and 48 h of *in vitro* storage

Treatment	Time of *in vitro* storage 0 h	24 h	48 h
	(%)		
Control	62.5 ± 5.2^{a,x}	22.4 ± 7.4^{b,x}	4.6 ± 1.4^{c,x}
Vitamin E			
1 μg/mL	80.1 ± 6.4^{a,y}	39.0 ± 4.5^{b,x}	27.0 ± 6.6^{b,y}
2 μg/mL	75.4 ± 10.9^{a,xy}	43.6 ± 3.0^{b,y}	29.1 ± 1.8^{c,y}
5 μg/mL	73.0 ± 5.9^{a,xy}	33.6 ± 6.2^{b,x}	32.7 ± 3.9^{b,y}
10 μg/mL	69.0 ± 7.6^{a,xy}	28.1 ± 3.0^{b,x}	38.4 ± 3.3^{b,y}
20 μg/mL	66.9 ± 10.1^{a,xy}	39.9 ± 2.8^{b,y}	39.3 ± 9.1^{b,y}
40 μg/mL	61.0 ± 12.7^{a,xy}	42.4 ± 2.6^{b,y}	29.0 ± 8.3^{b,y}
80 μg/mL	56.3 ± 6.3^{a,x}	41.4 ± 12.1^{ab,xy}	29.5 ± 4.0^{bc,y}
Tempo			
0.039 μM	54.9 ± 8.1^{a,x}	30.4 ± 5.0^{b,x}	38.7 ± 1.6^{b,y}
0.078 μM	52.1 ± 13.4^{a,x}	40.8 ± 6.7^{a,x}	37.4 ± 5.5^{a,y}
0.156 μM	53.7 ± 5.3^{a,x}	47.0 ± 1.4^{a,x}	34.9 ± 3.3^{b,y}
0.312 μM	62.7 ± 6.4^{a,xy}	39.1 ± 6.0^{b,xy}	35.1 ± 4.8^{b,y}
0.625 μM	59.7 ± 6.0^{a,x}	32.6 ± 4.2^{b,x}	21.5 ± 5.6^{b,y}
1.25 μM	55.8 ± 4.9^{a,x}	20.7 ± 4.5^{b,x}	23.2 ± 3.8^{b,y}

$^{a-c}$Means in a row with no common superscript differ significantly ($P \leq 0.05$).

^{x,y}Means in a column with no common superscript differ significantly ($P \leq 0.05$).

sperm motility. Vitamin E is naturally present in chicken (Blesbois *et al*., 1993) and turkey sperm (Surai, 1989; Surai and Ionov, 1992). Furthermore, Fujihara and Koga (1984) suggested that seminal plasma may contain an antioxidant factor that counteracts the toxic effects of endogenous lipid peroxide on sperm. However, results from the present study with turkey sperm demonstrated that the antioxidant activity in seminal plasma and sperm is not high enough to prevent lipid peroxide damage after extension and *in vitro* storage, and that supplemental antioxidants could improve semen shelf life. The addition of vitamin E to chicken semen also has been shown to improve the fertilizing ability of semen stored for 24 h over controls (Blesbois *et al*., 1993).

Turkey sperm remained motile after 48 h storage when the optimal concentrations of vitamin E, BHT, or Tempo were present in the extender. In ram sperm, organic peroxides were formed during *in vitro* storage at 5 C and this accumulation was linked to loss in motility (Jones and Mann, 1973). Lipid peroxidation reduced chicken sperm motility in some studies (Fujihara and Howarth, 1978) but not in others (Wishart, 1984). When chicken semen was supplemented with vitamin E and stored 24 h *in vitro*, no difference was observed in motility between controls and treated samples (Blesbois *et al*., 1993). In the present study, sperm motility indexes for controls averaged 56% at 24 h but declined to approximately 18% after 48 h of storage for turkey sperm, whereas in treatments with lipid-soluble-antioxidants, sperm motility indexes were as high as 84% at 48 h. Possibly, if chicken semen was held for an additional 24 h, vitamin E might have contributed towards maintaining motility.

Vitamin C is a powerful antioxidant when peroxyl radicals are located in the aqueous phase, but the vitamin is a poor scavenger for radical oxygen species

TABLE 3. Sperm motility index for samples supplemented with vitamin E and Tempo evaluated at 0, 24, and 48 h of *in vitro* storage

Treatment	Time of *in vitro* storage 0 h	24 h	48 h
	(%)		
Control	73.9 ± 2.1^{a,x}	56.4 ± 3.0^{b,x}	17.8 ± 3.5^{c,x}
Vitamin E			
1 μg/mL	75.0 ± 5.0^{a,x}	81.7 ± 7.3^{a,y}	31.7 ± 22.4^{b,xy}
2 μg/mL	75.0 ± 5.0^{a,x}	76.7 ± 4.4^{a,y}	40.0 ± 20.8^{b,xy}
5 μg/mL	76.3 ± 3.0^{ab,x}	80.0 ± 4.0^{a,y}	63.0 ± 8.4^{a,yz}
10 μg/mL	76.3 ± 3.0^{a,x}	76.0 ± 2.3^{a,y}	73.5 ± 4.1^{a,z}
20 μg/mL	76.3 ± 3.0^{a,x}	76.0 ± 5.5^{a,y}	69.5 ± 3.7^{a,z}
40 μg/mL	76.3 ± 3.0^{a,x}	77.5 ± 5.1^{a,y}	73.8 ± 3.0^{a,z}
80 μg/mL	78.3 ± 3.0^{a,x}	83.3 ± 5.5^{a,y}	75.0 ± 3.8^{a,z}
Tempo			
0.039 μM	76.9 ± 2.6^{a,x}	73.1 ± 4.9^{a,y}	68.8 ± 0.7^{a,xy}
0.078 μM	76.9 ± 2.6^{a,x}	75.6 ± 4.8^{a,y}	66.3 ± 3.0^{a,xy}
0.156 μM	76.9 ± 2.6^{a,x}	75.6 ± 4.6^{a,y}	68.8 ± 3.9^{a,xy}
0.312 μM	77.5 ± 2.1^{a,x}	67.5 ± 11.3^{ab,xy}	41.0 ± 13.1^{b,y}
0.625 μM	77.5 ± 2.1^{a,x}	45.0 ± 11.6^{b,x}	6.0 ± 6.0^{c,wx}
1.25 μM	77.5 ± 2.1^{a,x}	35.0 ± 8.9^{b,x}	0 ± 0^{c,w}

$^{a-c}$Means in a row with no common superscript differ significantly ($P \leq 0.05$).

$^{w-z}$Means in a column with no common superscript differ significantly ($P \leq 0.05$).

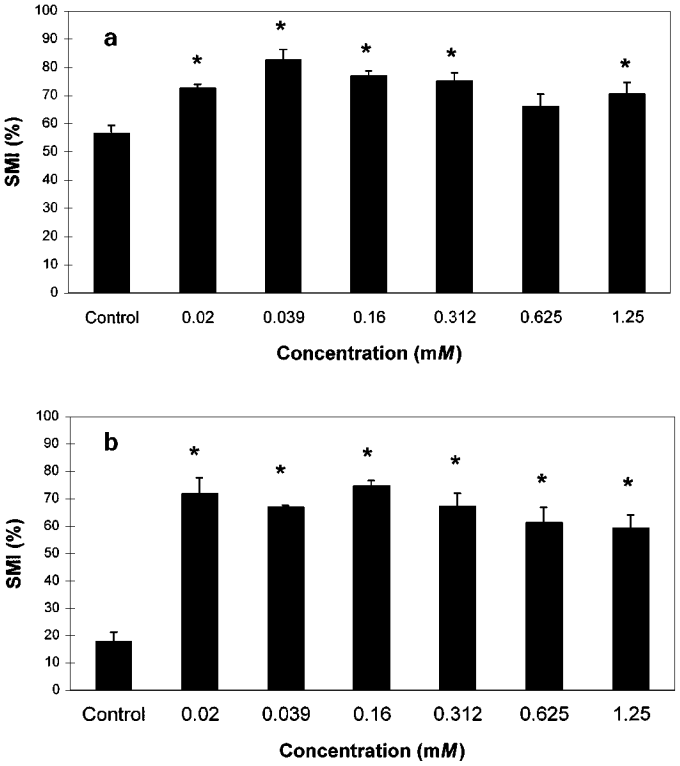

FIGURE 3. Sperm motility indexes (percentage) of sperm treated with butylated hydroxytoluene at 24 (a) and 48 h (b) *in vitro* storage at 5 C. *Value significantly different from control ($P \leq 0.05$).

within the lipid membrane (Burton and Ingold, 1988). Interestingly, at low concentrations, vitamin C works as an antioxidant but at high concentrations vitamin C has pro-active qualities (Beconi *et al.*, 1993). At the concentrations tested in the present study, vitamin C was neither helpful nor harmful during *in vitro* storage to the semen parameters measured, suggesting that damage to turkey sperm *in vitro* is not due to peroxyl radicals in the aqueous phase. Other water-soluble antioxidants, such as superoxide dismutase (SOD) and catalase, have recently been shown to improve the viability and fertility of liquid storage of ram sperm (Maxwell and Stojanov, 1996). However, similar to our results with vitamin C, we have observed no benefit of adding SOD or catalase to turkey semen during liquid storage (unpublished data).

Turkey sperm require a highly oxygenated environment for the generation of adenosine triphosphate during low temperature *in vitro* storage (Wishart 1981, Sexton and Giesen, 1982). Although necessary for survival *in vitro*, oxygen also leads to production of free radicals, causing irreversible damage to lipids in sperm membranes (Jones and Mann, 1973, 1977; Fujihara and Howarth, 1978; Froman and Thurston, 1981; Fujihara and Koga, 1984; Wishart 1984; Ravie and Lake, 1985). Because of the metabolic requirements and physical composition of sperm and seminal plasma, *in vitro* storage of turkey sperm appears to be highly susceptible to lipid peroxidation. Chicken and turkey sperm contain high amounts of polyunsaturated fatty acyl groups (Ravie and Lake, 1985) and spontaneous peroxidation occurs during *in vitro* sperm storage in both species (Fujihara and Koga, 1984; Cecil and Bakst, 1993). Cecil and Bakst measured malonaldehyde (MAL, a by-product formed during peroxidation) in turkey semen and found increased MAL concentrations with length of incubation (Cecil and Bakst, 1993). Froman and Thurston (1981) observed lower SOD activity in turkey semen than in chicken sperm and speculated that turkey sperm might be more susceptible to oxygen toxicity during *in vitro* storage. Although the age of the roosters was not reported, Wishart (1984) found that lipid peroxidation differs in individual roosters. Males ranked high in rates of lipid peroxidation produced no fertilized eggs after 5 h of *in vitro* storage at 40 C; however, these same males had similar fertility rates to that of males with low lipid peroxide concentrations when semen was inseminated without prior incubation (Wishart, 1984). In a preliminary study evaluating individual toms at the initiation of semen production (30 wk of age) and the end of semen production (56 wk of age), we found that MAL concentrations were 10-fold higher in the older toms (1.37 ± 0.20 nmol MAL) than in younger toms (0.20 ± 0.02 nmol MAL; A. M. Donoghue and J. Fleming, unpublished data). Malonaldehyde concentrations in semen were not evaluated in the current study, in which the effects of antioxidants were tested on toms 42 to 48 wk of age. However, if lipid peroxidation in semen increases with age in the tom as dramatically as these preliminary data suggest, then the effect of the antioxidants on semen in diluents for *in vitro* storage could differ significantly depending on the age of toms or whether ejaculates from toms of varying age are pooled for *in vitro* storage. Semen from individual toms is generally pooled for long-term storage and free radical generation is autocatalytic (Burton and Ingold, 1988). Thus, mixing semen from a tom with high lipid peroxidation could result in damage to the entire pooled sample.

Developing a defense system against lipid peroxide damage is of practical importance to improving the extended liquid storage of turkey semen. The present study demonstrated improved survival, membrane integrity, and motility after cold storage of turkey sperm with antioxidants that scavenge reactive oxygen species in the lipid membrane. Ultimately, the fertilizability of sperm is most important and future studies will evaluate the effect of these antioxidants on this sperm function.

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