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Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen

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ABSTRACT

The objective of this study was to evaluate the effects of reduced glutathione (GSH) and catalase (CAT) supplementation on the kinematics and membrane functionality of sperm during the liquid storage of ram semen, cooled at 5 °C, for up to 24 h. Semen samples from four rams were pooled, diluted with Tris-egg yolk extender without antioxidants (control) or supplemented with either CAT (100, 200, and 400 U/mL) or GSH (100, 200, and 400 mM) at a final concentration of 50×10^6 sperm/mL. Sperm kinematics, which was analyzed by computer-assisted sperm analysis (CASA), and membrane functionality, which was analyzed using the hypo-osmotic swelling test (HOST), were determined after the addition of the semen samples at different processing times (fresh/diluted, 1.5, 6, 12, and 24 h, at 5 °C). No significant differences were recorded in the kinematics or membrane functionality between treatments at different times. The supplementation of diluents with 100 and 200 U/mL of CAT prevented the harmful effects of cooling on total sperm motility. No significant differences were observed in progressive sperm motility throughout processing, regardless of the treatment and time of evaluation. Supplementation with 400 mM GSH resulted in an earlier reduction ($P < 0.05$) of total sperm motility, a decrease in rapid sperm rate and a reduction in curvilinear velocity during incubation, at 5 °C. The cooling induced a reduction ($P < 0.05$) in the percentage of sperm with a functional plasma membrane (HOST), especially after 1.5 h of incubation. Based on the results of the present study, the addition of CAT (100 and 200 U/mL) reduced the deleterious effects of cooling on total motility in ram sperm maintained at 5 °C for 24 h, although it did not affect the functionality of the sperm membranes. However, the addition of 400 mM GSH caused negative effects on the velocity parameters of the sperm.

1. Introduction

According to Bucak et al. (2008), semen cryopreservation is useful to the livestock industry, as artificial insemination (AI) can substantially increase the rate of genetic progress, compared to natural selection (O'Hara et al., 2010). However, cervical insemination with frozen-thawed semen generally results in low pregnancy rates, and the storage of ram semen in a chilled state may serve as an alternative technique when AI is to be performed within a short time after collection (King et al., 2004). However, despite cooling, sperm exhibit an irreversible reduction in motility, morphological integrity and fertility (Maxwell and Salamon, 1993).

Supplementation with antioxidants could reduce the impact of oxidative stress induced by reactive oxygen species (ROS) generally during sperm storage (Michael

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et al., 2009). Small amounts of ROS act as mediators of normal sperm function (Aitken, 1995). However, high concentrations of ROS are responsible for oxidative damage to the sperm, due to the polyunsaturated fatty acid content in the plasma membrane (Taylor, 2001). Thus, it is important that spermatozoa and seminal plasma have enzymatic and non-enzymatic defenses against the ROS (Kankofer et al., 2005).

In addition to catalase (CAT), the antioxidants glutathione reductase (GR) and glutathione peroxidase (GPx) and its substrates (i.e. reduced and oxidized glutathione) play important roles in lipid peroxidation and the detoxification of hydrogen peroxide, which is a precursor molecule of the hydroxyl radical (Storey et al., 1998; Engel et al., 1999). The imbalance between the scavenging and generation of ROS results in oxidative stress, which is the detrimental to the sperm cells and sperm cell functionality parameters, such as motility, acrosome reaction and sperm–oocyte fusion (Engel et al., 1999). Despite the presence of seminal antioxidants, such as superoxide dismutase (SOD), GPx and CAT (Mann and Lutwak-Mann, 1981), semen processing decreases the level of these antioxidant defenses (Bilodeau et al., 2000).

Various studies have been conducted to prevent oxidative stress during liquid storage of ram semen with antioxidant supplementation (Maxwell and Stojanov, 1996; Upreti et al., 1997; Bucak and Tekin, 2007; Çoyan et al., 2010). However, contradictory results have been reported. The aim of this work was thus to evaluate the effects of reduced glutathione (GSH) and CAT supplementation on kinematics and membrane functionality during liquid storage of ram semen cooled at 5 °C, for up to 24 h.

2. Materials and methods

2.1. Animals and semen processing

This study used 4 Santa Inês rams, a Brazilian hair breed, 3–4 years of age, that were raised in confinement at Ceará, Brazil (03.43 S; 38.30 W) with uniform feeding, housing and lighting conditions. The rams had been previously deemed fit for breeding (Fonseca et al., 1992) and were routinely used for semen collection (i.e. artificial vagina and teaser female) of two ejaculates per day, three times weekly within a few minutes of each other. Immediately after collection, the second semen samples from all 4 rams were pooled to minimize individual differences (Bucak and Tekin, 2007). All rams were collected to provide three high-quality semen replicates of the pool (total sperm motility ≥80%; sperm concentration $\geq 3 \times 10^9$ sperm/mL). The sperm motility was subjectively assessed with the aid of a phase-contrast microscope (Olympus, Tokyo, Japan; 400×), following dilution (1:1, v/v) in a sodium citrate solution (2.94%). The sperm concentration was determined by measuring the optical density with the aid of a spectrophotometer, calibrated with a hemocytometer after determining a standard curve for ram semen (Mocé and Graham, 2008). Three pooled ejaculates were evaluated in this study.

The Tris-based extender was composed of Tris 3.605 g, fructose 1.488 g, citric acid 2.024 g, distilled water (q.s.p. 100 mL) and egg yolk 12% (v/v). Each sperm pool was split into 7 equal aliquots, and diluted (37 °C) with a base extender containing CAT (100, 200 and 400 U/mL; C100, C200 and C400, respectively), GSH (100, 200 and 400 mM; G100, G200 and G400, respectively) or no antioxidants (control), with a final concentration of 50×10^6 sperm/mL. The diluted semen samples were stored in glass tubes and cooled at a cooling rate of approximately 0.3 °C/min from 37 to 5 °C and maintained at 5 °C for up to 24 h. Sperm evaluations were performed immediately after dilution (fresh and diluted) and after 1.5, 6, 12 and 24 h of storage, at 5 °C.

2.2. Sperm evaluation

2.2.1. Analysis of sperm kinematics using CASA

Sperm kinematics were assessed using a computer-assisted sperm analyzer (CASA) system. The CASA system consisted of an optical phase-contrast microscopy system (Nikon™ H5505, Eclipse 50i, Japan), with stroboscopic illumination and a warm stage at 37 °C, a video camera (Basler Vision Tecnologie™ A312FC, Ahrensburg, Germany) and a PC with Sperm Class Analyzer™ software (Microptics, S.L., Version 3.2.0, Barcelona, Spain). The kinematic parameters of the sperm were assessed following the dilution of the sample in 2.94% sodium citrate (v/v) and incubation in a water bath at 37 °C for 5 min – to minimize the occurrence of collisions between the sperm cells, as well as the inclusion of debris in the estimate (Verstegen et al., 2002). A pre-warmed Makler Chamber® (Sefi Medical Instrument, Haifa, Israel) was loaded with 5 μL of diluted semen sample, and at least 4 non-consecutive, randomly selected microscopic fields per sample were scanned in which at least 400 motile sperm were recorded. Events not related to the sperm were removed; image sequences were saved for later analysis.

The following sperm characteristics were analyzed: total motility (TM, %), progressive motility (PM, %), rapid sperm (RS, %), curvilinear velocity (VCL, μm/s), progressive velocity (VSL, μm/s), path velocity (VAP, μm/s), amplitude of lateral head displacement (ALH, μm) and beat frequency of the tail (BCF, Hz). All kinematic parameters were measured at a temperature of 37 °C, 25 frames acquired, a frame rate of 25 s, a minimal contrast of 75, a frame number of 25 per field, a sperm velocity ranging from 0 to 180 μm/s and threshold STR of 75%.

2.2.2. Analysis of sperm membrane functionality using the hypo-osmotic swelling test (HOST)

Sperm membrane functionality was evaluated using the HOST test. In brief, firstly 50 μL diluted semen was added to 50 μL hypo-osmotic solution (fructose 1.351 g, sodium citrate 0.735 g and bi-distilled water q.s.p. 100 mL) in microtubes and incubated at 37 °C for 1 h. Immediately after incubation, the samples were fixed with 50 μL formol saline solution. A drop of fixed semen was placed on a microslide, covered with a cover slide and 200 cells were evaluated under 1000× magnification, using a phase-contrast microscope (Nikon™ H5505, Eclipse 50i, Japan). Sperm with swollen or coiled tails were considered to have functional membranes (Correa et al., 1997).

2.3. Statistical analyses

The values of each parameter were expressed as the mean ± SD. The experimental unit consisted of three semen pools, each formed by the combined ejaculates of 4 rams. The variables used for comparison purposes were the various treatments (control, C100, C200, C400, G100, G200 and G400) and incubation times (fresh/diluted, 1.5, 6, 12 and 24 h). Percentage data were transformed to arc-sin data, before functional statistical analyses were performed. Data normality was tested using the Kolmogorov–Smirnov test. Differences between treatments or equilibration times were assessed using a one-way ANOVA, followed by the Tukey's test, with the aid of SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Correlations between parameters were determined by combining data from all treatments with the assistat statistics package for Windows (DEAG-CTRN-UFCG, Campina Grande – PB, Brazil, Version 7.5). A *P*-value of <0.05 was considered to be significant.

3. Results

The statistical analyses revealed no difference between treatments during incubation at 5 °C, with respect to PM (motility data not shown). Likewise, no differences were recorded between treatments for TM (total motility) or RS (rapid sperm) at any incubation time (fresh/diluted, 1.5, 6, 12 and 24 h) (Table 1). However, CAT at a concentration of 100 and 200 U/mL preserved the TM, thereby maintaining a value similar to that observed in the fresh/diluted samples over the full 24 h storage period. Conversely, C400, G100 and G200 treatments exhibited a reduced ($P < 0.05$) TM at 24 h. The control and G400 treatments led to an

Table 1
Mean (±SD) percentages for total motility (TM) and rapid sperm (RS) of ram semen following dilution in a Tris-egg yolk extender (control), supplemented with different concentrations of antioxidants for 24 h at 5 °C.

Parameter	Treatment	Refrigeration time at 5 °C				
		Fresh/diluted	1.5 h	6 h	12 h	24 h
TM (%)	Control	89.5 ± 1.4^{a}	89.5 ± 1.0^{a}	88.3 ± 0.4^{ab}	86.7 ± 1.3^{b}	79.6 ± 0.2^{c}
	CAT 100 U/mL	89.5 ± 0.3^{a}	89.2 ± 0.3^{a}	87.5 ± 4.5^{a}	87.5 ± 6.0^{a}	81.0 ± 6.8^{a}
	CAT 200 U/mL	89.5 ± 0.2^{a}	88.3 ± 1.8^{a}	87.4 ± 3.2^{a}	87.9 ± 2.1^{a}	81.2 ± 6.6^{a}
	CAT 400 U/mL	89.2 ± 0.3^{a}	88.1 ± 2.2^{a}	89.4 ± 0.3^{a}	87.3 ± 1.9^{ab}	82.2 ± 3.4^{b}
	GSH 100 mM	89.3 ± 0.3^{a}	88.6 ± 2.1^{a}	88.2 ± 1.8^{a}	87.4 ± 0.9^{ab}	78.5 ± 6.8^{b}
	GSH 200 mM	89.6 ± 0.4^{a}	87.2 ± 4.0^{a}	87.2 ± 2.4^{a}	86.4 ± 3.3^{a}	75.8 ± 2.0^{b}
	GSH 400 mM	89.5 ± 0.3^{a}	86.9 ± 3.3^{ab}	81.6 ± 2.9^{ab}	77.8 ± 7.3^{bc}	69.9 ± 4.9^{c}
RS (%)	Control	48.8 ± 7.3^{a}	50.5 ± 8.2^{a}	44.0 ± 4.8^{a}	44.8 ± 7.5^{a}	35.9 ± 4.6^{a}
	CAT 100 U/mL	56.2 ± 3.8^{a}	53.7 ± 5.4^{a}	45.3 ± 4.9^{ab}	42.4 ± 2.3^{b}	34.6 ± 3.6^{b}
	CAT 200 U/mL	55.6 ± 3.7^{a}	49.8 ± 8.9^{ab}	46.6 ± 4.5^{abc}	41.1 ± 3.5^{bc}	33.2 ± 2.8^{c}
	CAT 400 U/mL	54.4 ± 2.0^{a}	48.4 ± 9.2^{a}	39.6 ± 1.5^{ab}	42.6 ± 6.4^{ab}	35.7 ± 6.1^{b}
	GSH 100 mM	55.0 ± 0.6^{a}	49.4 ± 7.8^{ab}	45.2 ± 1.1^{ab}	40.4 ± 1.9^{bc}	33.0 ± 2.0^{c}
	GSH 200 mM	53.3 ± 5.2^{a}	45.6 ± 6.4^{ab}	42.8 ± 7.9^{ab}	35.4 ± 3.9^{bc}	27.1 ± 2.3^{c}
	GSH 400 mM	49.7 ± 2.6^{a}	45.3 ± 5.5^{ab}	40.4 ± 1.9^{b}	36.7 ± 1.8^{bc}	27.9 ± 1.9^{d}

$^{a–d}$Within a row, means with different superscript differ significantly ($P < 0.05$).
There is no significant difference within column ($P > 0.05$).

inferior TM, showing a significant reduction at 12 h storage (Table 1). The percentage of RS gradually decreased ($P < 0.05$) with time in all of the treatments with antioxidants at 6 h (G400), 12 h (C100, C200, G100 and G200) and 24 h (G400) of incubation at 5 °C. Only the control RS remained relatively stable for storage up to 24 h, as set out in Table 1.

The mean values for curvilinear velocity (VCL) in the control decreased ($P < 0.05$) only after 24 h of storage at 5 °C. Conversely, treatments supplemented with antioxidants recorded a reduction ($P < 0.05$) in VCL after 1.5 h (G400), 6 h (C100) and 12 h (C200, C400, G100, and G200) of storage (Table 2). Regarding the control, the C400 and G400 samples, no differences were recorded between the fresh/diluted sperm and sperm stored for 24 h for progressive velocity (VSL). There was however, a significant reduction in VSL after 12 h (C100, G100 and G200) and 24 h (C200) of storage. No significant differences in path velocity (VAP) for the control and G400 samples were recorded between fresh/diluted sperm and sperm stored for 24 h. The other treatments exhibited decreased VAP values ($P < 0.05$) at 6 h (C100 and G100), 12 h (C200 and G200) and 24 h (C400) at 5 °C (Table 2). There was however no effect on the amplitude of lateral head displacement (ALH) or beat frequency of the tail (BCF) under any treatment or incubation time, at 5 °C. The ALH values ranged from 3.53 ± 0.36 μm (C400, 24 h) to 4.93 ± 0.23 μm (C200, fresh/diluted), while the BCF values ranged from 7.07 ± 0.42 Hz (control, 1.5 h) to 8.43 ± 0.06 Hz (C100, 24 h).

Following 1.5 h of incubation at 5 °C, HOST values (Table 2) were lower ($P < 0.05$) than for values of fresh/diluted sperm in all treatments, but they remained relatively stable from 1.5 h to 24 h of storage, except for C100, which decreased ($P < 0.05$) at 6 h.

As kinematics and HOST values did not differ between treatments for any incubation period, the mean values of all treatments ($n = 7$) at each incubation time ($n = 5$) were used in the correlation analyses. Table 3 demonstrates significant ($P < 0.05$ to $P < 0.01$) positive correlations between the sperm parameters evaluated. Notably, BCF was negatively correlated with TM ($r = -0.46$; $P < 0.01$), RS ($r = -0.24$; $P < 0.05$), VCL ($r = -0.34$; $P < 0.01$) and VAP ($r = -0.21$, $P < 0.05$), but recorded no correlation with VSL or ALH. HOST on the other hand correlated positively with TM ($r = 0.31$; $P < 0.01$), PM ($r = 0.29$; $P < 0.01$), RS ($r = 0.60$; $P < 0.01$), VCL ($r = 0.64$; $P < 0.01$), VSL ($r = 0.54$; $P < 0.01$), VAP ($r = 0.59$; $P < 0.01$) and ALH ($r = 0.34$; $P < 0.01$), but showed no correlation with BCF. Conversely, no significant correlation was recorded between TM and PM ($r = 0.12$), while the percentage of RS recorded a strong positive correlation with VCL ($r = 0.94$; $P < 0.01$), VSL ($r = 0.81$; $P < 0.01$) and VAP ($r = 0.95$; $P < 0.01$).

4. Discussion

Storage of ram semen reduces the fertilizing capacity of sperm due to changes in the motility parameters and damage to the sperm membrane integrity (Maxwell and Watson, 1996). The reduction in the percentage of motile sperm obtained during refrigeration in the present study was similar to previous studies (Salamon and Maxwell, 2000; Paulenz et al., 2002; Bucak and Tekin, 2007). However, the control treatment showed a significant reduction in TM after 12 h of refrigeration, when compared to a fresh/diluted sample, while CAT addition (100 and 200 U/mL) preserved the TM during refrigeration. Likewise, the addition of 400 U/mL CAT resulted in better storage of TM, compared to the control (12 h versus 6 h). These findings are in agreement with those reported by Maxwell and Stojanov (1996), who demonstrated a positive effect of CAT, on TM of ram sperm stored at 5 °C. One explanation for this finding may be the fact that the use of antioxidants restores the equilibrium between the amounts of ROS produced and scavenged. Which in turn can preserve the metabolic activity of the sperm cell (De Lamirande and Gagnon, 1995) and prevent sperm cryodamage (Bucak et al., 2008). The lower efficacy of treatment with 400 U/mL CAT compared to 100 and 200 U/mL could be related to the toxic effects of this antioxidant at high concentrations, as previously reported by Maxwell and Stojanov (1996). This

Table 2
Mean (±SD) percentages for curvilinear velocity (VCL), progressive velocity (VSL), path velocity (VAP) and hypo-osmotic swelling test (HOST) of ram semen following dilution in a Tris-egg yolk extender (control), supplemented with different concentrations of antioxidants for 24 h at 5 °C.

Parameter	Treatment	Storage time at 5 °C				
		Fresh/diluted	1.5 h	6 h	12 h	24 h
VCL (μm/s)	Control	130.1 ± 18.0^{a}	131.6 ± 13.3^{a}	114.5 ± 9.6^{ab}	109.2 ± 11.9^{ab}	93.3 ± 2.9^{b}
	CAT 100 U/mL	140.3 ± 6.7^{a}	131.4 ± 11.2^{ab}	113.9 ± 12.7^{bc}	106.7 ± 2.5^{c}	93.1 ± 3.5^{c}
	CAT 200 U/mL	141.3 ± 1.7^{a}	125.6 ± 18.1^{ab}	115.3 ± 10.0^{ab}	102.5 ± 3.4^{bc}	88.7 ± 6.7^{c}
	CAT 400 U/mL	138.6 ± 6.5^{a}	124.3 ± 19.4^{ab}	114.2 ± 7.4^{abc}	101.8 ± 2.5^{b}	91.3 ± 6.7^{c}
	GSH 100 mM	137.5 ± 3.7^{a}	126.1 ± 17.8^{ab}	113.6 ± 10.7^{ab}	97.8 ± 2.8^{b}	88.9 ± 5.7^{b}
	GSH 200 mM	134.8 ± 5.9^{a}	123.7 ± 21.6^{ab}	107.9 ± 12.1^{ab}	91.3 ± 6.4^{b}	76.6 ± 2.6^{b}
	GSH 400 mM	129.5 ± 11.6^{a}	111.9 ± 9.9^{b}	104.6 ± 5.5^{bc}	93.0 ± 9.0^{bc}	74.6 ± 6.9^{c}
VSL (μm/s)	Control	52.0 ± 9.4^{a}	52.7 ± 8.4^{a}	45.3 ± 2.6^{a}	44.0 ± 5.5^{a}	35.4 ± 4.3^{a}
	CAT 100 U/mL	60.0 ± 7.3^{a}	57.1 ± 3.0^{ab}	47.3 ± 1.2^{abc}	45.5 ± 7.3^{bc}	36.5 ± 4.9^{c}
	CAT 200 U/mL	57.3 ± 9.7^{a}	54.9 ± 5.8^{ab}	48.4 ± 0.9^{ab}	43.6 ± 5.4^{ab}	39.1 ± 5.3^{b}
	CAT 400 U/mL	55.7 ± 5.5^{a}	52.7 ± 11.2^{a}	41.0 ± 2.2^{a}	44.34 ± 6.8^{a}	40.7 ± 9.9^{a}
	GSH 100 mM	56.2 ± 2.2^{a}	51.3 ± 3.7^{ab}	47.5 ± 2.7^{abc}	42.2 ± 2.3^{bc}	39.7 ± 6.7^{c}
	GSH 200 mM	53.3 ± 5.8^{a}	48.4 ± 2.2^{ab}	46.3 ± 6.4^{abc}	38.2 ± 3.5^{bc}	34.5 ± 4.7^{c}
	GSH 400 mM	49.9 ± 4.2^{a}	52.5 ± 3.9^{a}	48.4 ± 11.4^{a}	45.4 ± 10.4^{a}	42.4 ± 13.9^{a}
VAP (μm/s)	Control	83.8 ± 12.2^{a}	86.1 ± 12.4^{a}	75.4 ± 7.9^{a}	74.5 ± 10.7^{a}	63.9 ± 5.9^{a}
	CAT 100 U/mL	95.6 ± 8.1^{a}	90.9 ± 7.4^{ab}	76.8 ± 5.7^{bc}	72.5 ± 5.4^{c}	61.5 ± 3.5^{c}
	CAT 200 U/mL	92.3 ± 9.4^{a}	86.1 ± 11.1^{ab}	78.0 ± 3.9^{abc}	70.9 ± 5.4^{bc}	60.7 ± 2.6^{c}
	CAT 400 U/mL	90.5 ± 5.9^{a}	83.9 ± 15.2^{ab}	69.2 ± 1.6^{ab}	72.9 ± 8.2^{ab}	64.0 ± 9.6^{b}
	GSH 100 mM	91.3 ± 1.5^{a}	82.8 ± 7.9^{ab}	76.3 ± 0.5^{bc}	68.1 ± 2.3^{cd}	60.5 ± 5.5^{d}
	GSH 200 mM	87.5 ± 7.5^{a}	78.4 ± 4.8^{ab}	73.0 ± 9.8^{ab}	62.1 ± 5.3^{bc}	51.7 ± 3.3^{c}
	GSH 400 mM	82.0 ± 4.1^{a}	79.7 ± 6.2^{a}	58.9 ± 8.3^{a}	67.9 ± 4.9^{a}	61.7 ± 13.6^{a}
HOST (%)	Control	44.6 ± 3.4^{a}	40.1 ± 0.8^{b}	35.1 ± 0.4^{b}	36.1 ± 2.8^{b}	36.3 ± 2.7^{b}
	CAT 100 U/mL	48.2 ± 4.1^{a}	41.7 ± 0.2^{b}	33.0 ± 1.9^{c}	32.7 ± 1.6^{c}	35.2 ± 4.4^{c}
	CAT 200 U/mL	46.5 ± 7.3^{a}	39.4 ± 0.6^{b}	36.3 ± 6.1^{b}	35.7 ± 1.7^{b}	34.6 ± 2.4^{b}
	CAT 400 U/mL	44.8 ± 8.4^{a}	35.1 ± 0.4^{b}	35.2 ± 1.9^{b}	34.4 ± 0.9^{b}	32.3 ± 5.6^{b}
	GSH 100 mM	44.7 ± 6.5^{a}	37.9 ± 1.0^{b}	36.6 ± 1.7^{b}	33.5 ± 1.8^{b}	36.7 ± 0.9^{b}
	GSH 200 mM	47.9 ± 2.4^{a}	36.9 ± 3.6^{b}	34.8 ± 4.9^{b}	34.1 ± 1.3^{b}	35.2 ± 1.9^{b}
	GSH 400 mM	46.6 ± 5.1^{a}	34.6 ± 1.5^{b}	39.1 ± 7.5^{b}	36.4 ± 8.1^{b}	37.0 ± 0.6^{b}

$^{a–d}$Within a row, means with different superscript differ significantly ($P < 0.05$).
There is no significant difference within column ($P > 0.05$).

toxicity is due to the action of the antioxidants, related to the type and concentration of ROS generated (Peña et al., 2003). Nonetheless, treatment with 400 mM GSH caused an early reduction in VCL (1.5 h) and RS (6 h), compared to the other treatments. Additionally, TM significantly decreased after 12 h of incubation at 5 °C, resulting in a value that was similar to the control sample, but inferior to the other treatments (Tables 1 and 2). Thus, it is possible that the use of higher levels (400 mM) of GSH can influence the cellular Ca^{2+} homeostasis (Sikka, 1996). In addition, high concentrations of Ca^{2+} may make sperm susceptible to cryodamage (Meseguer et al., 2004).

The maintenance of the RS percentage in the control sample during refrigeration did not necessarily reflect better sperm preservation. It is known that thermal shock, which occurs during the cooling process, stimulates the capacitation and ROS generation in sperm sub-populations more sensitive to temperature decreases (Watson, 2000; Paulenz et al., 2002). This response is likely due to the loss of proteins and glycoproteins from the sperm plasma membrane (Flesch and Gadella, 2000), resulting in increased metabolic activity (Iqbal and Hunter, 1995) and the stimulation of hyperactivation and capacitation (De Lamirande et al., 1997). Notably, there was a direct relationship

Table 3
Correlation (r) following the hypo-osmotic swelling test (HOST) and kinematic end points as determined by CASA of ram semen following dilution for 24 h at 5 °C.

	PM	RS	VCL	VSL	VAP	ALH	BCF	HOST
TM	0.12	0.67**	0.70**	0.30**	0.56**	0.53**	−0.46**	0.31**
PM	–	0.49**	0.30**	0.81**	0.55**	−0.33**	0.13	0.29**
RS	–	–	0.94**	0.81**	0.95**	0.45**	−0.24*	0.60**
VCL	–	–	–	0.71**	0.90**	0.66**	−0.34**	0.64**
VSL	–	–	–	–	0.86**	0.02	−0.10	0.54**
VAP	–	–	–	–	–	0.37**	−0.21*	0.59**
ALH	–	–	–	–	–	–	−0.17	0.34**
BCF	–	–	–	–	–	–	–	−0.16

TM: total motility (%), PM: progressive motility (%), RS: rapid sperm (%), VCL: curvilinear velocity (μm/s), VSL: progressive velocity (μm/s), VAP: path velocity (μm/s), ALH: amplitude of lateral head displacement (μm) and BCF: beat frequency of the tail (Hz).
* $P < 0.05$.
** $P < 0.01$.

between RS and VAP (Verstegen et al., 2002), as it is a motility end point, which is indicative of the highly active sperm sub-population (Favareto et al., 2010).

The reduction in the percentage of sperm with a functional plasma membrane during cold storage in the present study is in agreement with the data reported by Paulenz et al. (2002).

It also showed a reduction in the percentage of cells with an intact plasma membrane in ram semen stored at 5 °C, for up to 30 h. Therefore, the current results demonstrate a significant reduction in the percentage of sperm with a functional plasma membrane to occur in the interval between dilution and 1.5 h incubation. This was likely due to the semen heterogeneity (Grasa et al., 2005; Favareto et al., 2010), as sperm are highly polarized cells with a compartmentalized distribution of lipids and proteins in their plasma membrane (James et al., 2004). In that regard, it was concluded that the changes in the membrane lipids and phospholipids from sperm sensitive to thermal shock, occurred at the beginning of storage, followed by a tendency to decrease to the loss in osmotic resistance (White, 1993). Furthermore, both CAT and glutathione scavenge the hydrogen peroxide (H_2O_2) generated by sperm cells, preventing the lipid peroxidation cascade (Chatterjee and Gagnon, 2001; Brouwers and Gadella, 2003; Baker and Aitken, 2004; Maia et al., 2010). However, in this study, the addition of either CAT or GSH did not appear to have any specific protective effect on the functionality of the sperm plasma membrane, as determined by HOST. The values being similar to those observed in the control sample throughout the refrigeration process. The absence of a direct relationship between lipid peroxidation and sperm quality has previously been reported for rams (Bucak et al., 2008; Çoyan et al., 2010), bulls (Brouwers and Gadella, 2003) and stallions (Pagl et al., 2006). The protective effect of egg yolk is due to the antioxidant properties of egg yolk proteins, which are capable of preventing the oxidation of polyunsaturated fatty acids (Sakanaba et al., 2004). Together with the presence of lipid aggregates in the egg yolk which are adsorbed on the sperm membrane, preventing the occurrence of peroxidative damage, this characteristic may have contributed to the results obtained in the present study (Brouwers and Gadella, 2003).

The positive correlation between sperm motility end points (VCL, VSL and VAP) and the functionality of the sperm plasma membrane are in agreement with previous data (Favareto et al., 2010) on fresh ram semen – evaluated using a combination of propidium iodide and carboxyfluorescein diacetate. The absence of a significant correlation between TM and PM in the present study corroborates the findings of by Maia et al. (2009). However it stands in contrast to the findings of Anel et al. (2003). Comparing the sperm kinetics in different studies is difficult, because of the variety of sperm concentrations in the samples and diluents used, and these factors may affect the final result (Tardif et al., 1997; Verstegen et al., 2002; Suarez and Ho, 2003; Mortimer and Maxwell, 2004).

Based on the results of the present study, the addition of CAT (100 and 200 U/mL) reduced the negative effect of cooling on the total motility of ram sperm maintained at 5 °C for 24 h. It did not, however, affect the functionality of the sperm membrane. The addition of 400 mM GSH did show a deleterious effects on the sperm velocity parameters.

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