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Combined addition of superoxide dismutase, catalase and glutathione peroxidase improves quality of cooled stored stallion semen

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ABSTRACT

During cold storage stallion spermatozoa experience undergo oxidative stress, which can impair sperm function and fertilizing capacity. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are the main endogenous enzymatic antioxidants in stallion seminal plasma, and counteract reactive oxygen species. Semen dilution reduces the endogenous antioxidant concentrations. The aim of this study was to investigate whether addition of 15 IU/mL each of SOD, CAT, and GPX to diluted stallion semen would ameliorate a reactive oxygen-mediated decrease in semen quality during 72 h of storage at 5 °C. Ejaculates ($n = 7$) were divided in two aliquots and diluted in INRA 96 without (control) or with addition of antioxidants. Semen analysis was performed at the time of dilution and every 24 h during chilled storage. Antioxidant supplementation completely inhibited the storage-dependent increase in activated caspase 3 ($P < 0.05$). Concomitantly, the antioxidant-supplemented samples had a greater percentage of viable, motile and rapidly moving sperm than control samples after 72 h storage ($P < 0.05$). The DNA damage, as evaluated by TUNEL assay and SCSA, increased with storage time ($P < 0.05$). Antioxidant supplementation did not prevent, but did significantly reduce the increase in DNA strand breakage. The results indicate part of the intrinsic apoptotic pathway leading to effector caspase activation was inhibited, although an activation of molecules with endonuclease activity still occurred. In conclusion, adding equal concentrations of SOD, CAT and GPX to a semen extender suppressed caspase-3 activation and improved preservation of stallion sperm motility and viability during 72 h of storage at 5 °C.

1. Introduction

Artificial insemination (AI) with cooled transported semen has become a major part of horse breeding management (Aurich and Aurich, 2006). One of the challenges when preserving semen for AI is that, during cooled storage, the viability, motility and fertilizing

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capacity of the spermatozoa decrease progressively, especially when semen is preserved for more than 24 h (Jasko et al., 1992; Pagl et al., 2006; Gallardo Bolaños et al., 2014). The mechanisms underlying this decrease in semen quality are thought to include to an increase in reactive oxygen species (ROS) production by dying and damaged spermatozoa (Ball et al., 2001; Johannisson et al., 2014). An imbalance in the production and degradation of ROS, leading to a deregulation of redox signaling, creates a condition described as oxidative stress (Sikka et al., 1995; Sies, 2015; Aitken et al., 2016).

Mammalian spermatozoa are very sensitive to peroxidative damage, due to the high concentration of polyunsaturated fatty acids (PUFA) in the plasma membrane (Aitken, 1995). Freshly collected stallion spermatozoa are, however, thought to be relatively resistant to spontaneous or induced lipid peroxidation when compared to frozen-thawed sperm cells (Baumber et al., 2000; Neild et al., 2005). Treatments that result in oxidative stress of stallion semen samples result primarily in a reduction in sperm motility (Baumber et al., 2000). In chilled as well as in frozen-thawed spermatozoa, superoxide leakage as a result of the mitochondrial electron transport chain reactions may result in lipid peroxidation, which increases gradually over time. Subsequent aldehyde adduct formation on proteins in the electron transport chain induces increased hydrogen peroxide release from the mitochondria which, in turn, leads to oxidative damage to the DNA in the spermatozoa with single- and double-strand breakage being indicative of damage to the DNA (Duru et al., 2000; Aitken et al., 2013; Gibb and Aitken, 2016). Indeed, oxidative stress is considered the primary cause of DNA damage in spermatozoa (Aitken and De Iuliis, 2007). Sperm DNA damage is correlated with a decreased likelihood of fertilization, lesser incidence of zygote cleavage, embryo death and delayed embryo and fetal development (Sakkas and Alvarez, 2010). In stallion sperm, experimentally induced increases in ROS concentrations were reported to result in a concentration-dependent increase in DNA damage (Baumber et al., 2003a).

A second consequence of mitochondrial-derived ROS and the resulting oxidative stress is direct compromise of the integrity of the inner and outer mitochondrial membranes. Oxidative stress promotes induction of the intrinsic apoptotic pathway, which is characterized by the release of factors from the intermembrane space and mitochondrial matrix into the cytoplasm of the spermatozoa (e.g., apoptosis inducing factor; AIF), endonucleases and cytochrome c (Candé et al., 2002; Paasch et al., 2004; Koppers et al., 2011). Cytochrome c combines with other co-factors and the initiator caspase 9 to form the apoptosome, which in turn activates the effector caspases 3, 6, or 7 (McIlwain et al., 2013).

Even though there is the destructive potential on sperm integrity of ROS at relatively greater concentrations, relatively lesser concentrations have regulatory actions in many physiological sperm functions such as capacitation-related increases in tyrosine phosphorylation (Baumber et al., 2003b). Stallion spermatozoa and seminal plasma are contain enzymatic and non-enzymatic antioxidants that function to maintain basal concentrations of ROS. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are among the primary enzymatic antioxidants in seminal plasma of men and stallions (Ball et al., 2000; Baumber and Ball, 2005; Hammadeh and Hamad, 2009), although the exact contribution of non-enzymatic antioxidants to the total antioxidant capacity of stallion seminal plasma has not been ascertained (Ball, 2008). The endogenous antioxidant components in stallion seminal plasma function to protect the spermatozoa against ROS-mediated lipid peroxidation until approximately 24 h of cooled storage (Kankofer et al., 2005). After more than 24 h of storage at 5 °C, there is an increase in lipid peroxidation (Ball and Vo, 2002). To avoid oxidative stress-induced damage to spermatozoa during cooled storage (i.e., premature capacitation, motility loss, DNA damage), ROS concentrations in diluted semen should be maintained at optimal concentrations for maintenance of sperm integrity.

When semen is processed for artificial insemination, the concentrations of seminal plasma are reduced and may be insufficient to counteract oxidative stress during cooling and subsequent storage. There have been attempts to increase the capacity of the intrinsic antioxidant defense mechanisms by addition of catalase to the semen extender, but this did not lead to improved preservation of semen quality (Aurich et al., 1997; Ball et al., 2001). In the physiological situation, stallion spermatozoa and seminal plasma contain all three major enzymatic antioxidants (Ball et al., 2000; Baumber and Ball, 2005). The aim of the present study, therefore, was to investigate whether adding a combination of SOD, CAT and GPX to a skim milk-based semen extender ameliorates the decrease in stallion semen quality during storage at 5 °C. In particular, the effects on motility variables, DNA fragmentation and activation of caspase-3 were assessed during a 72 h period.

2. Material and methods

2.1. Semen collection and processing

Two ejaculates from each of seven stallions (2–15 years old) were included in the study. There was a regular semen collection schedule (three times/week) and all ejaculates contained a minimum of 50% motile spermatozoa. Semen collection was performed with a pre-warmed, lubricated Missouri type artificial vagina. Immediately after collection, the semen was filtered through a semen filter pouch (Minitube, Germany). From each ejaculate, two portions of 8 mL were separated and diluted with semen extender (INRA96, IMV technologies, Italy) without (control) or with the addition of antioxidants to obtain a final concentration of 50×10^6 spermatozoa/mL. The final concentrations of antioxidants in the diluted semen were 15 IU/ml of superoxide dismutase (SOD from cattle erythrocytes, S5395-15KU), 15 IU/ml of catalase (CAT from cattle liver, C1345-1 G) and 15 IU/ml of glutathione peroxidase (GPX from cattle erythrocytes, G6137-200UN), respectively. All antioxidant preparations were purchased from Sigma–Aldrich (Steinheim, Germany). Control and treated samples were transferred to 20 mL rubber-free syringes without air and stored at 5 °C for 72 h. At predetermined intervals, subsamples were removed for analysis of sperm viability, motility, DNA damage and relative quantification of cleaved caspase 3. The time for withdrawing subsamples was kept to an absolute minimum to avoid major temperature fluctuations.

2.2. Semen evaluation

Semen evaluation was performed within 3 h after semen collection, but before addition of antioxidants (0 h), and subsequently at 24 h intervals during cooled storage (24, 48, 72 h). At each time point, viability and motility were evaluated. In addition, 500 µL aliquots of each control and treated subsample were washed twice (600 *g*, 10 min at room temperature) with 4.5 ml of PBS (Dulbecco's Phosphate Buffered Saline, D8537, Sigma-Aldrich, Steinheim, Germany). The supernatants were discarded, and the pellets re-suspended in 300 µL PBS. Subsequently, the sperm suspension was divided into three aliquots of 100 µL each. One aliquot was fixed in 2% paraformaldehyde (in 0.1 M PBS; pH 7.6) for 15 min at room temperature (RT). The cells were washed in PBS and maintained in 5 ml of 70% ethanol at −20 °C until DNA damage was assessed using terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labelling (TUNEL). The other two aliquots were stored at −80 °C until processing for (i) the sperm chromatin structure assay (SCSA) and (ii) Western blotting for detection and quantification of cleaved caspase 3.

2.3. Viability

Sperm viability was evaluated using eosin staining (Eosin viability stain, Europath, Naples, Italy). A diluted sperm sample (5 µl) was mixed with the eosin stain (10 µl) at 37 °C, incubated for 30 s, smeared on a slide and dried on a warm plate at 37 °C. Slides were evaluated using bright field microscopy at 400x magnification. Live spermatozoa remained unstained, whereas dead spermatozoa were red-pink. At least 200 sperm cells were scored for each sample. The percentage of live (i.e., eosin negative), spermatozoa is reported as the viability (%).

2.4. Sperm motility evaluation with a computer-assisted sperm analysis (CASA) system

Sperm motility was assessed immediately after semen dilution and at each time point during storage at 5 °C. Semen (500 µL) was incubated for 10 min at 37 °C and 10 µL were then placed in a pre-warmed Makler chamber with a depth of 20 µm. Samples were analyzed using a SCA (Sperm Class Analyzer by Microptic S:L:, Barcelona, Spain) at 100x magnification. At least five representative fields were evaluated at a frame rate of 25 Hz. Kinematic variables of the spermatozoa such as VCL (curvilinear velocity; µm/s), VSL (straight-line velocity; µm/s), VAP (average path velocity; µm/s), LIN (linearity; VSL/VCL in %), STR (straightness; VSL/VAP in %), WOB (wobble; VAP/VCL in %), ALH (amplitude of lateral head-displacement; µm), and BCF (beat cross frequency; Hz) were assessed. Percentages of sperm having any (total) motility (VCL > 10 µm/s) and progressive motility (VCL > 10 µm/s and at least 75% straightness) were evaluated. Furthermore, spermatozoa were classified into subpopulations of rapid (VCL ≥ 90 µm/s), medium (90 µm/s > VCL ≥ 45 µm/s), slow (45 µm/s > VCL ≥ 10 µm/s), and static (VCL < 10 µm/s) sperm.

2.5. Assessment of sperm DNA fragmentation

Sperm DNA damage was evaluated using two different methods. The sperm chromatin structure assay (SCSA) was performed to detect single-strand breakage, while the APO-BrdU terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labelling (TUNEL) assay was used to detect single- and double-strand breakage in the DNA.

2.5.1. TUNEL assay

An APO-BrdU™ TUNEL Assay Kit with Alexa Fluor® 488anti-brdU (Invitrogen, Molecular Probes) was used. This kit allows detection of 3′-hydroxyl ends in the DNA that serve as starting points for TdT, which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analogue BrdUTP to the TdT reaction labels the breakage sites. Once incorporated into the DNA, BrdUTP is detected by an anti-BrdUTP antibody, which is conjugated to Alexa Fluor® 488 (excitation: 495 nm, emission: 519 nm). The DNA was counterstained using propidium iodide (PI; excitation: 535 nm, emission: 617 nm).

Thawed spermatozoa were washed twice in a wash buffer and resuspended in the DNA-labelling solution for 60 min at 37 °C in a water bath. The suspensions were washed twice in a rinse buffer and the Alexa Fluor® 488-labelled anti-BrdU antibody was added to each suspension. The samples were incubated for 30 min at RT in the dark. There was subsequently addition of 0.5 ml of PI/RNase A staining buffer. After an additional 30 min of incubation in a darkened area at RT, the sperm suspension was pipetted onto three microscope slides and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). At least 100 sperm cells were scored per slide under a fluorescence microscope (Nikon Eclipse 90, Firenze, Italy) equipped with a mercury lamp (100 W) using a 488 nm excitation filter and a 530 long-pass filter. The heads of all sperm cells were stained red with use of PI and only spermatozoa with fragmented DNA were additionally stained green (Fig. 1). The average for three slides that were evaluated was considered to be the final value of TUNEL positive spermatozoa in a given sample.

2.5.2. SCSA

The SCSA is a technique used to evaluate the susceptibility of sperm DNA to acid-induced DNA denaturation in situ, by staining with the fluorescent DNA-binding dye acridine orange (AO). The metachromatic shift of AO from green (stable, double-stranded DNA) to red (denatured, single-stranded DNA) is detected using flow cytometry. The assay was conducted as previously describe by Evenson and Jost (2000). The stained samples were processed using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). For each sample, 10,000 events were analyzed at low speed. Acridine orange was excited with an argon ion laser at 488 nm (200 mW) and filters for green fluorescence (530 ± 30 nm BP) and red fluorescence (630 nm LP) were used. The DNA fragmentation index

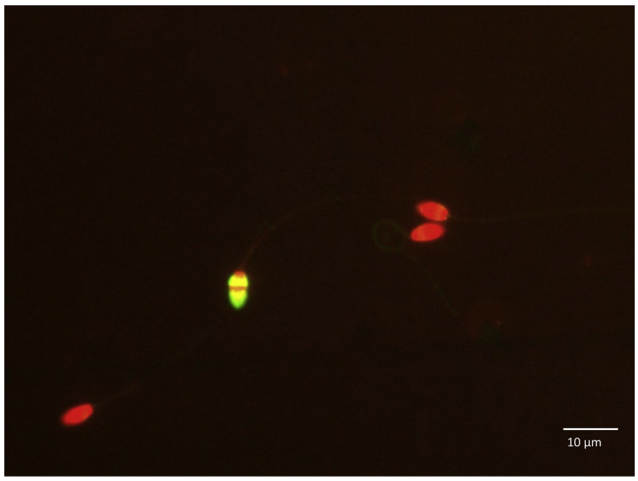

Fig. 1. Representative image of stallion spermatozoa stained using the APO-BrdU™ TUNEL Assay Kit with anti-BrdU Alexa Fluor 488® and assessed with a fluorescence microscope at 400x magnification; Propidium iodide (PI) was used to stain the nucleus of all sperm cells red while anti-BrdU Alexa Fluor 488® was used to stain spermatozoa with fragmented DNA green (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

(DFI; formerly: alpha t) was calculated and the percentage of spermatozoa with an increased amount of singled stranded DNA was expressed as the %DFI (formerly: comp alpha t). A reference sample was processed after assessment of every six samples to evaluate machine performance.

2.6. Assessment of the apoptosis marker cleaved caspase 3 by Western blotting

Activation of the apoptosis pathway was detected by Western blot analysis of activated (cleaved) caspase 3. Sperm pellets were re-suspended in a lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% deoxycholic acid, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate and 50 mM sodium fluoride) and then homogenized using the TissueLyser LT (Qiagen, Hilden, Germany). After determining the protein concentration using a modified Bradford method (Protein Assay kit, Bio-Rad, Segrate, Italy), the samples were boiled and 25 µg protein per lane loaded on an acrylamide gradient gel (Mini-PROTEAN® TGX™ Precast Gel, Biorad) and electrophoresis procedures were conducted. A dilution series confirmed that the standardized protein concentration resulted in a band intensity for the internal reference (alpha tubulin) that was in the linear range for signal quantification. The proteins were then blotted from the gel onto polyvinylidene difluoride (PVDF) membranes, which were blocked with Tris-buffered saline (12.5 mM TrisHCl, pH 7.4; 125 mM NaCl) containing 5% milk at RT for 60 min. Membranes were incubated overnight with a primary antibody at 4 °C. The primary antibody was a rabbit anti-caspase 3 (Cell Signaling Technologies, catalog no. 9662; 1:1000 dilution) which was used to detect both the full length caspase-3 and the large fragment of caspase-3 resulting from cleavage. Membranes were incubated for at least 1 h with a secondary HRP-conjugated anti-rabbit IgG antibody (catalog no. NA934, GE Healthcare, Buckinghamshire, UK; 1:1000 dilution) at RT. The bound antibodies were subsequently visualized using enhanced chemiluminescence (ECL, Clarity ECL, Western Blotting Substrate, Bio-Rad).

After quantification of the signal for activated caspase 3, the blots were stripped and probed with an anti- α-tubulin antibody (catalog no. 2144, Cell Signaling Technologies; 1:1000 dilution). The abundances of activated caspase 3 were quantified using densitometry, normalizing to the corresponding band intensity of α-tubulin using the ChemiDoc Gel scanner (Bio-Rad). Ratios obtained for samples immediately after collection (0 h) were considered to represent 100%.

2.7. Statistical analysis

Statistical analyses were performed using the IBM® SPSS® Statistics software (version 22.0, IBM Corporation, Armonk, New York). For all semen variables, the average value of both ejaculates per stallion was calculated ($n = 7$). All data are expressed as mean ± SD. Data were not normally distributed (Shapiro-Wilk test). Consequently, non-parametric tests were used for evaluation. The effect of storage time on sperm analysis data was evaluated in each group (control and treatment) using a Friedman test. If significant, a *post-hoc* analysis with Wilcoxon's signed rank test was conducted to compare individual storage times. The same test was used to compare differences between control and antioxidant-supplemented samples at a given time point. Data from the densitometric analysis of Western Blots were normally distributed. A one-way ANOVA and Tukey's tests were used, therefore, to compare the relative abundance of activated caspase 3 between control and treated semen samples. The significance level was set at $P < 0.05$.

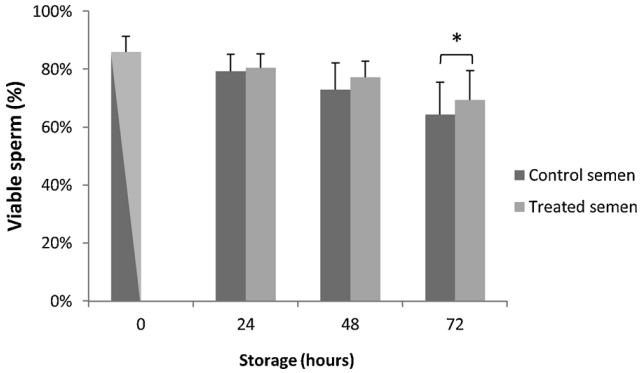

Fig. 2. Sperm viability (i.e., percentage of eosin-negative spermatozoa) in stallion semen without (control) or with antioxidant supplementation (treated: 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) during cooled (5 °C) storage ($n = 7$); An asterisk (*) indicates differences between the control and treated sample at given time point ($P < 0.05$).

3. Results

3.1. Sperm viability

The percentage of viable spermatozoa decreased with storage time in both groups ($P < 0.01$, Supplementary Table 1). Percentage of viable spermatozoa was greater, however, in the treated semen samples after 72 h storage ($P < 0.05$; Fig. 2).

3.2. Spermmotility

The percentages of sperm with total and progressive motility decreased during cold storage ($P < 0.01$; Supplementary Table 1). Percentage of motile sperm was greater in treated than control samples after 48 h and 72 h storage ($P < 0.05$; Fig. 3). In addition, proportion of progressively motile sperm tended to be greater in treated than in control semen after 48 h cold storage ($P = 0.063$).

The values for VSL, LIN, STR, WOB and BCF variables did not differ between control and antioxidant-supplemented semen samples ($P > 0.05$; Table 1). The VCL and VAP were greater, however, in treated semen than in the control group at 24 h and 72 h of cooled storage ($P < 0.05$; Table 1). The ALH of treated semen was greater than in control samples only at 24 h ($P < 0.05$).

The percentage of rapidly motile sperm cells decreased in both groups during cooled storage ($P < 0.01$, Supplementary Table 1), while the percentage of static sperm cells increased concomitantly ($P < 0.01$). A decrease in spermatozoa with medium velocity was evident in treated ($P < 0.05$) but not control samples. Storage time had no effect on the sperm in the slow moving population ($P > 0.05$).

Antioxidant supplementation resulted in a larger population of rapid sperm than in control samples at 24 and 72 h of cooled storage ($P < 0.05$, Fig. 4). There was no difference in the percentage of medium and slow sperm cells between the two groups ($P > 0.05$). In antioxidant-supplemented samples the percentage of static spermatozoa was less ($P < 0.05$), however, than in control semen at 24 h and 48 h of storage.

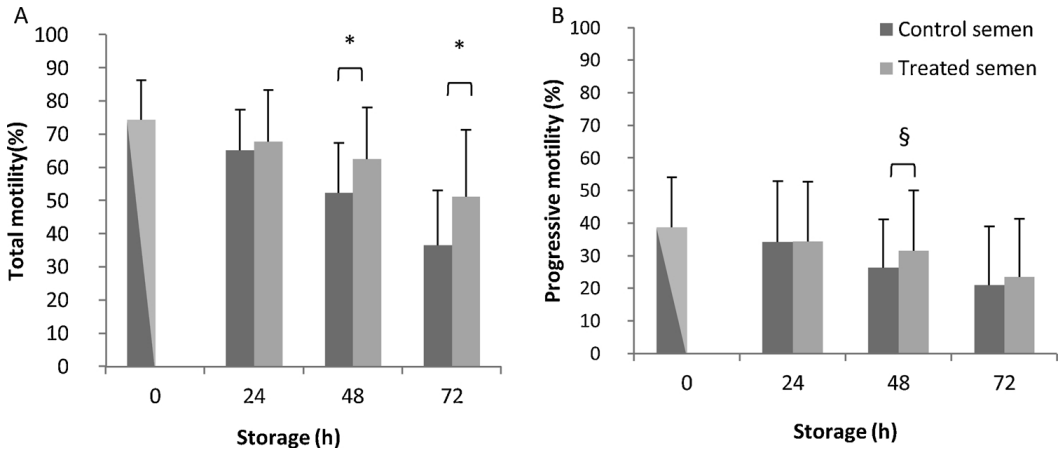

Fig. 3. (A) Percentages of total motile and (B) progressively motile spermatozoa in control and antioxidant-supplemented (treated; 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) semen at different times during storage at 5 °C ($n = 7$); An asterisk (*) indicates differences between a control and a treated sample at a given time point ($P < 0.05$); "§" indicates the tendency for a difference between control and treated semen ($P = 0.063$).

Table 1
Values for motility variables of stallion spermatozoa in extenders containing antioxidants (treated: 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) or not containing antioxidants (control) at different times during storage at 5 °C (n = 7); Different small letters (a, b) indicate differences between control and treated samples at a given time point ($P < 0.05$).

CASA variable	Time of storage at 5 °C (h)						
	0	24		48		72	
	Control	Control	Treated	Control	Treated	Control	Treated
VCL (μm/s)	106.8 ± 45.3	77.7 ± 30.0[a]	94.9 ± 34.3[b]	93.1 ± 40.4	96 ± 48.7	85.5 ± 51.3[a]	99.5 ± 51.3[b]
VSL (μm/s)	60.6 ± 31.0	44.1 ± 17.7	50.9 ± 24.1	45.3 ± 23.4	48.8 ± 28.5	36.8 ± 25.4	43.3 ± 24.8
VAP (μm/s)	76.5 ± 34.6	56.0 ± 21.9[a]	67.4 ± 27.6[b]	60.4 ± 29.2	82.1 ± 68.0	49.6 ± 31.9[a]	58.7 ± 29.6[b]
LIN (%)	52.8 ± 10.3	53.0 ± 7.9	50.6 ± 9.7	43.5 ± 10.6	46.4 ± 11.6	39 ± 10.4	40.1 ± 12.4
STR (%)	74.4 ± 6.2	75.1 ± 6.1	73.1 ± 9.0	70.3 ± 9.7	71.8 ± 10.5	69.2 ± 8.6	67.6 ± 10.3
WOB (%)	68.8 ± 8.6	67.8 ± 7.0	67.3 ± 5.5	59.1 ± 8.0	61.9 ± 7.8	54.3 ± 8.3	56.4 ± 10.2
ALH (μm)	3.8 ± 1.3	3.0 ± 0.8[a]	3.8 ± 0.8[b]	3.7 ± 1.3	3.8 ± 1.5	3.4 ± 1.6	4.2 ± 1.5
BCF (Hz)	14.2 ± 6.9	12.5 ± 5.2	12.8 ± 5.1	10.8 ± 3.6	13 ± 4.4	9.2 ± 5.3	9.4 ± 2.5

VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; STR = straightness coefficient; WOB = wobble; ALH = amplitude of lateral head displacement; BCF = beat/cross frequency.

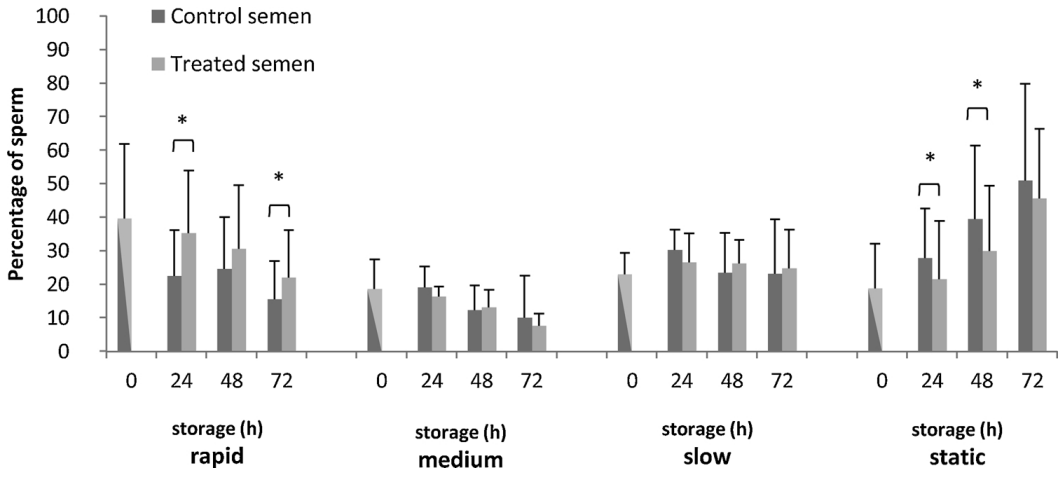

Fig. 4. Comparison of stallion sperm classified into different motility categories (rapid, medium, slow and static) in control and treated (antioxidant supplemented; 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) semen at different times during storage at 5 °C (n = 7); An asterisk (*) indicates differences between a control and a treated sample at a given time point ($P < 0.05$).

3.3. Sperm DNA damage

After 72 h storage, control samples had a greater percentage of TUNEL-positive spermatozoa than samples supplemented with antioxidants ($P < 0.05$; Fig. 5). The %DFI, however, did not differ between the two groups at any time ($P > 0.05$). In general, the % DFI increased compared to the control at 0 h during cooled storage in both control and antioxidant-supplemented samples ($P < 0.05$; Supplementary Table 2). Likewise, the percentage of TUNEL-positive spermatozoa increased in control and treated semen during storage ($P < 0.01$; Supplementary Table 2).

3.4. Detection of cleaved caspase 3

Cold storage at 5 °C for 72 h resulted in an increase in activated caspase 3 in control but not in treated semen ($P < 0.01$; Supplementary Table 2). After 72 h of storage, the amount of activated caspase 3 was greater in control than in treated semen ($P < 0.05$; Fig. 6).

4. Discussion

The results of the current study indicate that the combined addition of SOD, CAT and GPX to a commercial semen extender inhibited the activation of caspase 3 and larger populations of rapidly moving spermatozoa were sustained during semen storage at 5 °C. A storage-dependent increase in spermatozoa with DNA damage, as detected using the TUNEL assay, was also reduced to some extent when there were longer storage times.

The major finding was the observation that the mixture of antioxidants prevented an increase in cleaved (i.e., activated) caspase

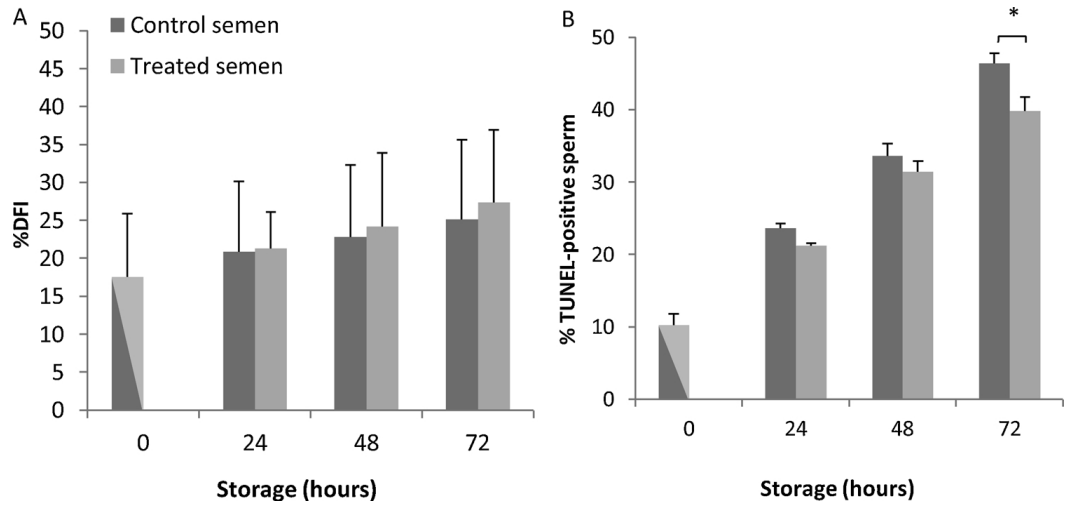

Fig. 5. Percentage of control or treated (antioxidant supplemented extender; 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) spermatozoa with either (A) increased amount of single-stranded DNA (%DFI; SCSA) or (B) single and/or double DNA strand breaks (% TUNEL-positive sperm; APO-BrdU-TUNEL assay) at different times during storage at 5 °C ($n = 7$); An asterisk (*) indicates differences between the control and treated sample at a given time point ($P < 0.05$).

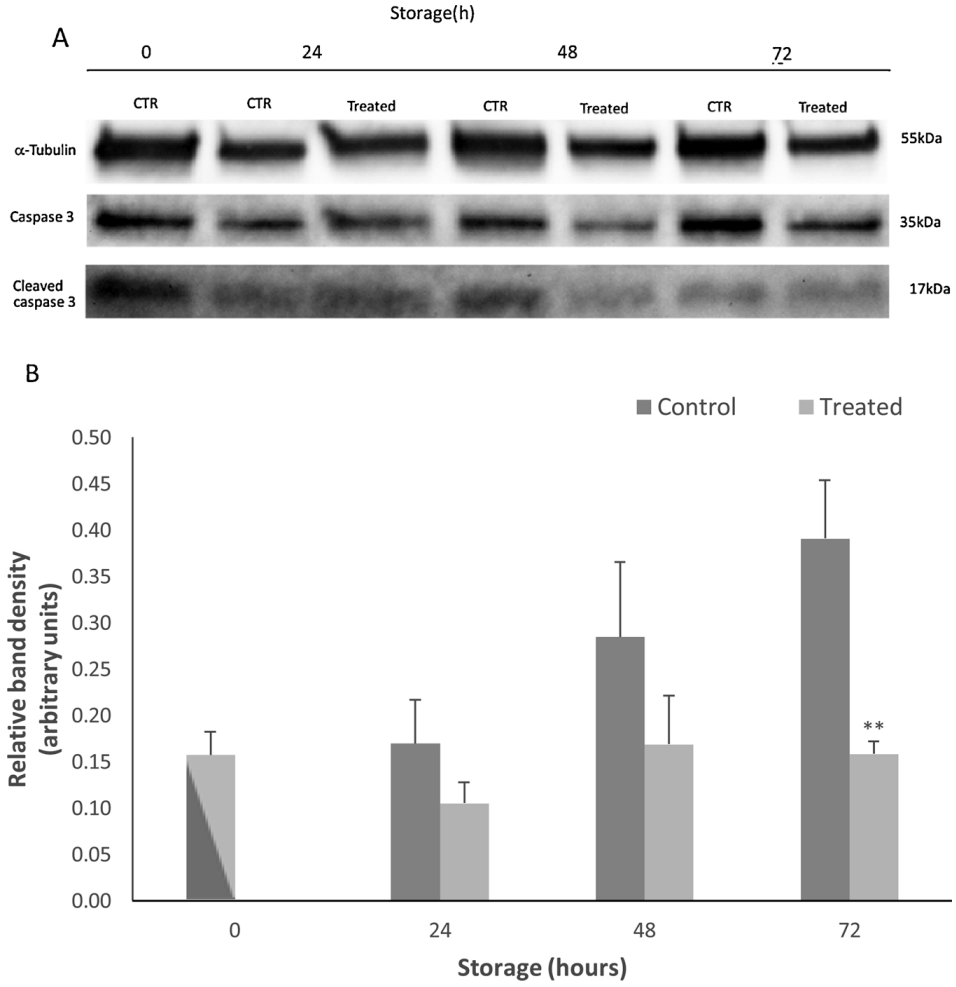

Fig. 6. (A) Representative Western blot of corresponding control and treated (antioxidant supplemented extender; 15 IU/ml each of superoxide dismutase, catalase and glutathione peroxidase) semen samples during storage at at 5 °C; Whole and cleaved caspase 3 were detected using a rabbit anti-caspase-3 antibody (35 kDa and 17 kDa protein band); Alpha- tubulin served as loading control; (B) Bar chart for relative band density amount (arbitrary units) of cleaved caspase-3 in control and treated semen samples at each time point; Values are expressed as intensities relative to the alpha tubulin signal; An asterisk (**) indicates differences between a control sample and a treated sample at a given time point ($P < 0.05$; $n = 7$).

3. Active caspase 3 is primarily located in the sperm midpiece (Marchetti et al., 2004; Espinoza et al., 2009; Muratori et al., 2015), and to a lesser extent in the post-acrosomal region, of human spermatozoa (Kotwicka et al., 2008; Muratori et al., 2015). A similar distribution can be assumed for stallion spermatozoa, as indicated by a simultaneous detection of procaspase 3 and its cleaved form (Ortega Ferrusola et al., 2008). The exact subcellular localization of the procaspase 3 in spermatozoa is not clear, but if similar to somatic cells it is assumed to be located in the cytoplasm and in the mitochondria (Zhivotovsky et al., 1999). Caspase 3 can be activated by either an extrinsic or an intrinsic pathway. The intrinsic pathway involves the release of cytochrome C from the mitochondria in response to cellular stress (e.g., oxidative stress or lipid peroxidation). A subtle increase in lipid peroxidation, therefore, has been detected during liquid storage of stallion semen (Ball and Vo, 2002). Cytochrome C activates initiator caspase 9 which in turn cleaves caspase 3 (McIlwain et al., 2013; Aitken and Baker, 2013). The addition of antixidants to the semen extender may have prevented the destabilization of the mitochondria as a result of oxidative stress and thereby reduced the release of pro-apoptotic factors. Whether the observed effects are a direct action of SOD, CAT and GPX on the spermatozoa or a result of the interaction between the antioxidants and extender components remains unclear.

There was not direct confirmation in the present study of the occurrence of oxidative stress in the diluted semen samples. Lipid peroxidation could not be detected in stallion semen stored at 5 °C for 24 h in a previous study (Kankofer et al., 2005) and it has been suggested lipid peroxidation is not a major cause of sperm damage during a storage period of 24 h. These previous findings are consistent with results in the control samples of the present study. Another possibility is that during prolonged storage the intrinsic antioxidative defense of the spermatozoa may have become exhausted, as indicated by the consistent trend of an increase in cleaved caspase 3 in the control samples.

An association between activation of caspases, a decrease in the percentage of motile spermatozoa and a decrease in spermatozoa with relatively greater mitochondrial membrane potential has been described in frozen-thawed stallion spermatozoa (Ortega Ferrusola et al., 2009; Martín Muñoz et al., 2016). There are indications that there was a similar activation of apoptosis-related pathways during prolonged chilled storage of stallion semen in the control samples of the present study and this is consistent with results of previous studies (Gallardo Bolaños et al., 2012). Supplementation of the INRA96 extender with SOD, CAT and GPX resulted in a suppressed activation of the caspase 3-related pathway in the present study. A similar effect has recently been described for dog spermatozoa (Del Prete et al., 2018). The motility in general and the rapidly motile sperm population in particular were sustained to a greater extent in the samples without mitochondria-dependent caspase 3 activation. This finding indicates preservation of mitochondrial integrity and function might be an important factor in improving chilled storage of stallion semen.

Even though there was an efficient blockage of the activation of caspase 3 in the present study, there was a time-dependent decrease in sperm motility and an increase in TUNEL positive spermatozoa and %DFI in antioxidant-supplemented samples. This may be because mitochondrial function and integrity affects other sperm cell functions that are susceptible to ROS-induced compromise. Mitochondria are cell organelles with a multitude of functions in spermatozoa (Moraes and Meyers, 2018). For example, these organelles serve as calcium-stores and have extensive actions in regulating intra-cellular calcium concentrations. Buffering calcium concentrations in semen extender and/or spermatozoa may preserve mitochondrial integrity and consequently sperm functions such as motility, as recently reported for stallion spermatozoa (Wu et al., 2018). Indeed, super-physiological calcium concentrations can lead to induction of mitochondrial permeability transition pore opening and activation of calpain which in turn activates the apoptosis inducing factor in the intermembrane space of the mitochondria (Orrenius et al., 2015). The destabilization of mitochondria with super-physiological concentrations of calcium may, therefore, have resulted in a release of endonuclease G and apoptosis inducing factor (AIF) from the mitochondrial inter-membranous space in samples in the present study and as a result induced the increase in TUNEL positive spermatozoa in stored samples (Susin et al., 1999; Daugas et al., 2000; van Loo et al., 2001; Bajt et al., 2006). The AIF has been detected in human and bull spermatozoa (Taylor et al., 2004; Martin et al., 2006), however, the function(s) of this factor in inducing DNA damage in stored spermatozoa has not been determined. In addition or as alternative mechanisms, calcium-dependent proteins from the sperm head with endonuclease-activity that are present in mouse, hamster and human spermatozoa (Sotolongo et al., 2005), or proteins with endonuclease-activity from the seminal plasma (Alghamdi and Foster, 2005) may contribute to DNA damage in stored semen samples. The pelleting of intact and defective spermatozoa during storage and the consequent close proximity of midpieces and heads may facilitate endonuclease activation and access to the DNA in the head compartment particularly of plasma membrane damaged cells. To what extent oxidative stress may have contributed to the occurrence of DNA strand breakages in the present study is not clear. Theoretically, the antioxidants should have counteracted any of the actions of ROS. It, however, cannot be discounted the possibility that there was a synergistic action of subtle oxidative stress during cooling and storage that has been reported to induce a truncated base excision repair mechanism in human spermatozoa (Smith et al., 2013), and endonuclease actions on DNA stability (Aitken et al., 2013). Independent of the underlying mechanism, it appears that improvements in liquid semen preservation may depend primarily on the ability to preserve the integrity and functionality of the sperm mitochondria. Future concepts for mitochondrial preservation, and thereby sperm function preservation, therefore, may need to not only include buffering for ROS to prevent oxidative stress, but may also need to ensure buffering of calcium concentrations in extended semen.

The decision to assess the effects of the three antioxidants SOD, CAT and GPX as simultaneous additives to a semen extender was made because these are the primary enzymatic antioxidants in stallion seminal plasma (Brigelius-Flohe, 1999; Mruk et al., 2002). Furthermore, SOD, CAT and GPX function synergistically. The SOD compound functions to dismutate the superoxide radical to hydrogen peroxide while CAT and GPX both catalyze the conversion of hydrogen peroxide to water and oxygen (Aitken and Roman, 2008). While GPX activity is dependent on the availability of reduced glutathione, CAT is not limited by the availability of any other substrate. It is likely that reduced glutathione concentrations varied between samples. Reduced glutathione, therefore, may have been a limiting factor for hydrogen peroxide inactivation because results with experimentally induced increases in ROS indicate hydrogen

peroxide is the ROS with the most deleterious effects on stallion spermatozoa (Baumber et al., 2000). Hydrogen peroxide affects intracellular enzyme systems, induces DNA fragmentation and can lead to a reduction in sperm motility, due to its marked membrane permeability capacity (Baumber et al., 2003b; Ball, 2008).

Another factor that may have biased the results of the present study is the varying abundance of endogenous antioxidant concentration in the diluted samples. Antioxidant concentrations in seminal plasma vary among stallions (Ball et al., 2001; Kankofer et al., 2005; Baumber and Ball, 2005). Considering the simple dilution procedure utilized in the current study, a varying amount of seminal plasma (14%–24%) and thus variable endogenous antioxidant concentrations are likely to have been present in the samples. During the first 24 h of storage, the residual antioxidant concentrations may have been sufficient to mask any effect of antioxidant supplementation on sperm viability and total motility. In support of this possibility, there was no increase in lipid peroxidation detected during a 24 h storage period in a previous study (Kankofer et al., 2005). A lesser percentage of the rapidly motile spermatozoa population and increase in the proportion of static spermatozoa in the control samples after 24 h of storage in the present study may be the first indicators of subtle, sub-lethal ROS-related effects on sperm function. In this respect, a subpopulation analysis proved to be a more sensitive way of detecting subtle changes in sperm function. Notably, antioxidant-supplementation was not sufficient to prevent a general decrease in values for semen quality variables during the first 24 h of storage. Such a decrease may be related to the general effects of the cooling process on lipid phase transitions and thereby lipid organization and stability of the cellular membrane systems, which occur irrespective of ROS (Parks and Lynch, 1992; Oldenhof et al., 2012).

The concentrations of SOD, CAT and GPX used in the current study may appear less as compared to concentrations used in other studies where there was assessment of a single antioxidant supplementation to stallion semen extenders (Aurich et al., 1997; Cocchia et al., 2011). Relatively greater concentrations of individual antioxidants have been reported to have negative effects on sperm motility (Aurich et al., 1997) whereas lesser concentrations had positive effects on acrosome integrity and sperm motility (Cocchia et al., 2011). Future studies, therefore, should be considered to assess the range of concentrations of antioxidants in a system where seminal plasma content is small and standardized.

In conclusion, the results of the present study indirectly confirm that stallion spermatozoa are affected by an increasing imbalance between ROS production and degradation during cold storage. The combined addition of the antioxidants SOD, CAT and GPX to a cooled storage extender prevented the activation of the caspase-based apoptosis pathways in the spermatozoa, and as a result there was maintenance of a larger population of viable and rapidly motile spermatozoa.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

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