Skim milk-egg yolk based semen extender compensates for non-enzymatic antioxidant activity loss during equine semen cryopreservation

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Abstract

Cryopreservation exposes spermatozoa to stressful conditions, leading to reduced cell viability. Several studies propose that overproduction of reactive oxygen species and decreased antioxidant capacity of semen may increase the damaging effects of the technique. The objective of this work was to evaluate the influence of a skim milk-egg yolk based semen extender on enzymatic and non-enzymatic antioxidant activity in equine semen cryopreservation. Fifteen ejaculates from six fertile Criollo stallions were cryopreserved using a commercial citrate-Hepes, egg yolk, skim milk and glycerol extender. Activities of catalase, glutathione peroxidase and superoxide dismutase and total radical-trapping antioxidant potential were assessed in raw semen, semen diluted in extender and thawed semen. All three enzymes showed higher activities in raw semen than in diluted or in thawed semen (P < 0.01), but enzyme activities did not differ significantly between diluted and thawed semen samples (P > 0.05). Non-enzymatic antioxidant defenses did not differ among any of the stages in the cryopreservation process (P > 0.05). In conclusion, the present study shows that dilution of semen with skim milk-egg yolk based extender after centrifugation compensates for the non-enzymatic antioxidant protection (but not enzymatic antioxidant defense) lost with seminal plasma removal. The absence of correlation between seminal and antioxidant parameters suggests that the compensation was enough for semen protection against oxidative stress, or antioxidant protection plays a minor role on semen from fertile stallions.

Keywords: antioxidants, cryopreservation, equine semen, extender, oxidative stress.

Introduction

The storage of cryopreserved spermatozoa is associated with a reduction in cell viability and fertilizing capacity. The quality of stored semen is affected by handling procedures such as dilution, centrifugation, dilution in semen extender and freezing. These procedures are associated with the generation of and imbalance among reactive oxygen species (ROS; Twigg *et al.*, 1998; Bilodeau *et al.*, 2000; Ball *et al.*, 2001; Chateerjee and Gagnon, 2001; Baumber *et al.*, 2005).

Although oxidative stress was suggested as an important contributor to disruption of sperm function over 50 years ago, the importance of oxidative stress has gained a wider understanding in the last decade (Sharma and Agarwal, 1996). In normal physiological functions, there is a balanced generation of ROS and antioxidant enzymes (Kovalski *et al.*, 1992; Plante *et al.*, 1994; Aitken *et al.*, 1995). ROS have a physiological role in signaling events controlling sperm capacitation and induction of the acrosome reaction in many species including equine (De Laraminde and Gagnon, 1993; De Laraminde *et al.*, 1993, 1997; Griveau *et al.*, 1994; Leclerc *et al.*, 1997; Baumber *et al.*, 2003). However, overproduction of ROS and decreased antioxidant defense activity cause low sperm motility and viability, DNA fragmentation and protein denaturation (Aitken *et al.*, 1994; Halliwell and Gutteridge, 1999; Baumber *et al.*, 2002; Agarwal and Said, 2005; Kankofer *et al.*, 2005).

The cell structure of spermatozoa makes them potentially susceptible to damage from free radicals (De Laraminde and Gagnon, 1995; Sikka, 2004). Sperm membranes are rich in polyunsaturated fatty acids and can easily undergo lipid peroxidation in the presence of ROS, leading to changes in membrane fluidity (Alvarez and Storey, 1982), which finally results in decreased fertilizing capacity. In addition, low cytoplasm content remaining after spermatogenesis contributes to sperm cell fragility, limiting the potential for DNA and protein repair (Bustamante Filho *et al.*, 2005).

To counteract oxidative damage, spermatozoa and seminal plasma have several mechanisms to neutralize free radicals. Enzymatic and non-enzymatic

antioxidant systems work synergistically to prevent harmful effects of byproducts from aerobic metabolism (De Laraminde and Gagnon, 1993; De Laraminde *et al.*, 1993). For example, mammalian semen (mainly seminal plasma) has many compounds with non-enzymatic antioxidant activity (e.g., ascorbic acid, α-tocopherol, taurine and albumin; Alvarez and Storey, 1983). However, the presence of specific antioxidant enzymes suggests that they also play a major role in the protection of spermatozoa against ROS. Three enzyme system (catalase, glutathione peroxidase, and superoxide dismutase) have superoxide radicals and hydrogen peroxides as substrates (Alvarez and Storey, 1989; Zini *et al.*, 1993; Ball *et al.*, 2000). These antioxidants act by reducing the production of deleterious residues from oxidative physiological metabolism. In bovine semen, a decrease in antioxidant activity following cryopreservation has been reported (Bilodeau *et al.*, 2000). Furthermore, freeze–thawing of equine and bovine spermatozoa has been associated with an increase in ROS generation (Bilodeau *et al.*, 2000).

Little is known about the dynamics of enzymatic or non-enzymatic antioxidant defense systems during cryopreservation of stallion semen and the influence of semen extenders. Comprehension of their impact during procedures such as centrifugation, removal of seminal plasma dilution and freezing might lead to improved fertilizing capacity of semen through use of antioxidants in semen extenders.

In the present study we investigated antioxidant defenses status of equine semen during cryopreservation. To accomplish that, the effect of a routinely used semen freezing protocol was studied on total radical-trapping antioxidant potential (TRAP), which evaluates non-enzymatic antioxidant defenses and activities of the antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx).

Materials and Methods

Animals

Six fertile Criollo stallions between six and nine years of age were used. The stallions belong to two stud farms in Rio Grande do Sul, Brazil and were on a routine semen collection schedule. They were stabled with access to an outdoor paddock from 8 AM to 6 PM and were fed hay and a concentrate ration balanced to provide their daily requirements for energy, protein and micro-nutrients twice daily. Water and mineral supplementation were freely available.

Experimental design

In the experiment, non-enzymatic antioxidant activity and activities of superoxide dismutase, catalase and glutathione peroxidase were monitored on semen samples from different stages of the cryopreservation procedure. Stallions had a phase of sexual rest for one week before the first semen collection. A total of fifteen ejaculates were used in the experiment (three ejaculates of three stallions and two ejaculates of three stallions). Freezing was performed by a standard technique comprising dilution of semen (1:1) with FR-1 extender (raffinose, lactose, glucose, potassium citrate and Hepes; Nutricell, Campinas, SP, Brazil) at 30°C, centrifugation at 400 x *g* for 10 min and removal of 90 – 95% of the supernatant (extender plus seminal plasma). All samples were extended to a final concentration of 100×10^6 sperm/mL in FR-5 extender (FR-1 plus skim milk, glucose, egg yolk and glycerol; Nutricell, Campinas, SP, Brazil), reaching a final glycerol concentration of 2.5%, packaged into 0.5 mL straws (IMV International Corporation, Minneapolis, MN, USA) and directly frozen 4 cm above the liquid nitrogen surface for 20 min (Martin *et al.*, 1979; Klug *et al.*, 1992; Alvarenga *et al.*, 2005). Semen was thawed after seven days by plunging the straw in a water-bath at 37°C during 30 s.

For the oxidative stress assays, samples were obtained from three cryopreservation stages: (1) raw semen, (2) extended semen prior to freezing and (3) post-thawed semen. From each stage, 100 μL semen samples were suspended in 600 μL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl and stored at -20°C.

Experimental procedures

Semen was collected with an artificial vagina (Hannover model, Minitüb GmbH, Germany) on an estrous Criollo mare. After collection, the gel fraction was removed and semen was filtered through sterile gauze. Progressive, total motility and morphology were evaluated after collection and after thawing. In addition, after thawing, structural and functional integrity of spermatozoa membranes were evaluated by fluorescent stain (CFDA + PI; Kneissl, 1993) and hypoosmotic swelling tests (Lagares *et al.*, 2000), respectively.

Catalase activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001®). Thirty microliters of semen sample was added to 720 μL of reaction medium consisted of 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer pH 7.0. One unit is defined as 1 μmol of hydrogen peroxide consumed per minute (read at 240 nm), and specific enzyme activity is reported as units per milligram protein (Aebi, 1984; Banerjee *et al.*, 2002; Kasahara *et al.*, 2002; Cortassa *et al.*, 2004;

Khosrowbeygi and Zarghami, 2007).

Glutathione peroxidase activity was measured using *tert*-butyl-hydroperoxide as substrate (Wendel, 1981; Munz *et al.*, 1997; Cortassa *et al.*, 2004). Ninety microliters of semen sample were added to an incubation medium containing 790 µL of 100 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.7, 20 µL of 2 mM glutathione, 30 µL of 0.15 U/mL glutathione reductase, 10 µL of 0.4 mM azide, 10 µL of 0.1 mM NADPH and 50 µL of 0.5 mM *tert*-butyl-hydroperoxide. NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). One GPx unit is defined as 1 µmol of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) consumed per minute, and specific enzyme activity is represented as units per mg protein.

The assay of SOD activity was carried out as described (Marklund, 1985; Silva *et al.*, 2005; Bhandari *et al.*, 2007) based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide radicals. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity was indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). In a quartz cuvette were added 30 µL of semen samples, 4 µL of 30 µM catalase, 958 µL 50 mM Tris 1 mM EDTA pH 8.2 buffer and 8 µL of 24 mM pirogalol prepared in 10 mM HCl. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. Results were reported as units of SOD/mg protein.

Non-enzymatic antioxidant defenses were assessed by the total radical-trapping antioxidant potential (TRAP) method (Lissi *et al.*, 1992; Rhemrev *et al.*, 2000; Evelson *et al.*, 2001), based on chemoluminescent intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane; ABAP) thermolysis in a Wallac 1409 Scintilator Counter. The initial chemoluminescence value was obtained by adding 3 mL of ABAP 10 mM dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 µL of luminol (5.6 mM) to a glass scintillation vial. Ten microliters of 300 µM Trolox (water soluble α-tocopherol analogue) or sample were then added to the vial, and the chemoluminescence was monitored until it achieved the initial levels. The time required for this to occur is called induction time, which is directly proportional to the antioxidant capacity of the sample. The induction time of the sample was compared to that presented by Trolox. Results were reported as nmol Trolox/mg protein.

Protein concentration was determined using bovine serum albumin as standard (Lowry *et al.*, 1951).

Statistical analysis

Statistical analysis was performed by repeated measures ANOVA, followed by the Tukey test for multiple comparisons when the *F* value was significant. Catalase and SOD values were transformed to logarithms to normalize (ln) the distributions. All analyses were performed using the Graphpad Prisma 5 software. Pearson correlation coefficients were calculated to quantify associations between semen characteristics (motility, structural and functional integrity of the spermatozoa membranes), TRAP activity and enzymatic activities (catalase, SOD and GPx). Values of $P < 0.05$ were considered to be significant.

Results

Parameters for raw semen (mean ± S.D.) were 45.7 ± 7.8 mL for semen volume, 71.3 ± 20.0% for total motility, 57 ± 21.9% for progressive motility and 258.3 ± 51.1 x 10^6 spermatozoa per milliliter for sperm concentration. At post-thawing evaluation, total motility averaged 28.7 ± 17.5%, progressive motility averaged 18.3 ± 14.6%, membrane structural integrity averaged 19.4 ± 12.5% and membrane functionality averaged 24.2 ± 15.9%. The percentage of morphologically normal spermatozoa was 54.5 ± 9.1%. Activities of the antioxidant enzymes and of non-enzymatic antioxidant potential (TRAP) in raw, diluted and thawed semen are shown in Fig. 1. Antioxidant activities for raw, extended and frozen semen were respectively: catalase: 2.8 ± 0.75 ln U/mg protein, 1.33 ± 0.64 ln U/mg protein, 1.25 ± 0.69 ln U/mg protein; SOD: 0.76 ± 0.47 U/mg protein, 0.22 ± 0.25 U/mg protein, 0.05 ± 0.14 U/mg protein; GPx: 12.75 ± 4.98 U/mg protein, 7.26 ± 2.97 U/mg protein, 6.56 ± 1.92 U/mg protein; TRAP: 1.58 ± 1.04 nmol Trolox/mg protein, 1.08 ± 0.67 nmol Trolox/mg protein, 1.40 ± 0.39 nmol Trolox/mg protein.

There was a tendency for reduction of the three enzyme activities through stages of the cryopreservation process. Conversely, total antioxidant potential did not differ between stages of cryopreservation. There was no catalase or SOD activity in analysis of the extender alone; however, glutathione peroxidase activity and TRAP were detected (4.23 U/mg protein and 0.22 ± 0.08 nmol Trolox/mg protein, respectively). No significant correlations were observed between superoxide dismutase, catalase, glutathione peroxidase and TRAP and any of the semen variables ($P > 0.05$).
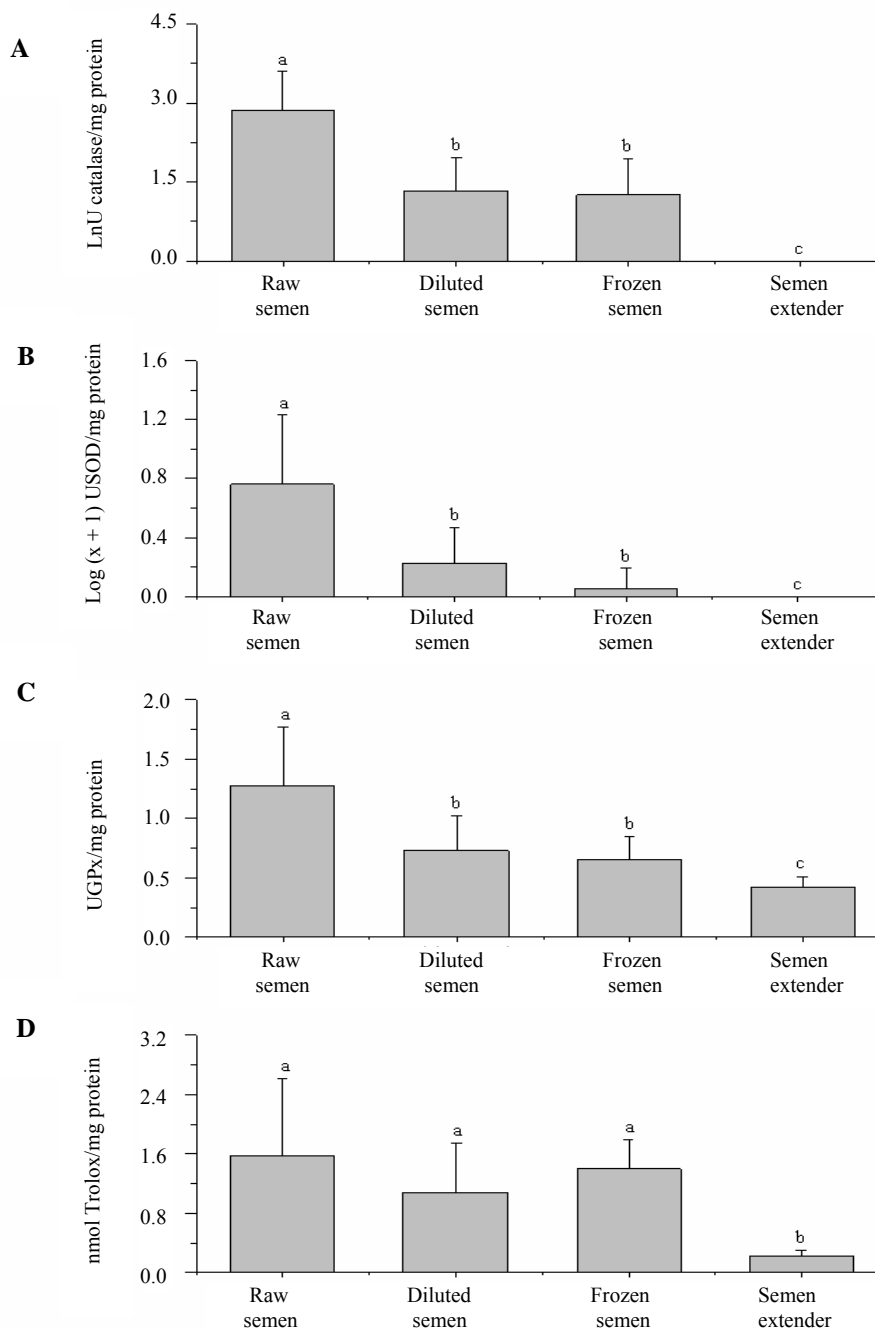
Figure 1. Activities of (A) catalase, (B) superoxide dismutase (C) glutathione peroxidase and (D) TRAP value in raw, diluted and frozen-thawed semen. Means with different superscripts (a, b, c) differ ($P < 0.05$). Data are mean ± SEM.

Discussion

The present work documented the maintenance of non-enzymatic antioxidant defenses by skim milk-egg yolk based extender during stallion semen cryopreservation.

Non-enzymatic antioxidant defenses were assessed by the total radical-trapping antioxidant potential (TRAP) method. TRAP results were similar in raw, diluted and frozen-thawed equine semen, indicating a compensatory effect by semen extender on non-enzymatic antioxidant activity after seminal plasma removal.

Non-enzymatic antioxidant defenses comprise a huge number of molecules, including amino acids, peptides, proteins and vitamins bearing different

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reactive centers (e.g., phenols, thiols) with widely different hydrophobicities that allow the trapping of both hydrophobic and hydrophilic radicals (Evelson *et al.*, 2001). These compounds share the role of controlling the oxidative balance of tissues and plasma with enzymatic antioxidant systems. Although equine semen from fertile stallions rarely presents leukocytes and high percentage of abnormal spermatozoa (the main sources of ROS), the cryopreservation process increases ROS generation by spermatozoa (Ball *et al.*, 2001). ROS release in medium by damaged cells is a potential danger for efficiently cryopreserved spermatozoa. After thawing the antioxidant content of medium provided by semen extender contributes avoiding or decreasing the risk of lipid peroxidation of sperm cell membranes.

The semen extender used in our experiment showed an antioxidant activity (0.22 ± 0.08 nmol Trolox/mg protein), which might be explained by its composition. Skim milk and egg yolk are usual components of semen extenders. However, their precise composition is difficult to define. Both components are susceptible to variations in its mineral, lipid and protein content once they are influenced by animal feed, health and management.

Recently, a proteomic approach shed more light on egg yolk composition (Mann and Mann, 2008). Comprising at least 116 proteins, 86 of which were reported to occur in egg yolk for the first time, this article describes several proteins with probable antioxidant activity. Egg yolk consists of approximately 33% lipid and an antioxidant protection is of a paramount importance for embryo development. The presence of metal chelators such as yolk phovitin, ceruloplasmin, ovalbumin and ovotransferrin remove free metal ions which could catalyze the production of ROS. Also, a protein similar to extracellular superoxide dismutase and a protein similar to plasma glutathione peroxidase may contribute to the antioxidative capacity of yolk (Mann and Mann, 2008).

Similarly, bovine skim milk presents antioxidant activity for protection of its high lipid content (Taylor and Richardson, 1980). However, we should consider two steps in skim milk preparation: (1) fat removal also results in loss of fat soluble vitamins (e.g., retinols and tocopherols); (2) ultra-high temperature processing, when milk is heated for a short time at a temperature exceeding 135°C, consequently inactivating enzymes such as catalase, SOD and GPx. Nevertheless, free radical scavenger activity was identified and related to minerals as copper and zinc, which are necessary for proper activity of scavenger enzymes. Also, these minerals have their own antioxidant properties (Przybylska *et al.*, 2007). In addition, protein denaturation exposes sulphydryl groups (Patrick and Swaisgood, 1976; Taylor and Richardson, 1980; Jiménez-Guzmán *et al.*, 2002), enhancing antioxidant capacity of proteins and peptides

in spite of its functional structure.

Activities of the scavenger enzymes catalase, superoxide dismutase and glutathione peroxidase had similar profiles, being higher in raw semen than in diluted or in frozen-thawed semen. The last step (freeze-thawing) did not reduce the activity of these enzymes, which were stable in the extended and freeze-thawed semen. The decrease of enzyme activity in extended semen was 64% for catalase, 43% for glutathione peroxidase and 78% for superoxide dismutase. However, these enzyme activities did not differ between extended and frozen-thawed semen, indicating that the freezing procedure had no effect on their activity. The decrease of enzyme activities in diluted semen observed in this study was expected and is explained by preparation of equine semen for cryopreservation, which involves the removal of 90 to 95% of seminal plasma and consequently the removal of a dominant source of antioxidant protection (Zini *et al.*, 1993; Ball *et al.*, 2000; Baumber *et al.*, 2005). Evaluation of the commercial extender used in this work did not detect SOD and CAT activity and only minimal GPx activity was found, which shows that the extender did not compensate for loss of enzymatic antioxidant protection caused by removal of seminal plasma.

Spermatozoa are potentially susceptible to damage caused by excess ROS due to their high amount of polyunsaturated fatty acids in membrane phospholipids and to the relatively small volume of cytoplasm. Elimination of most of the cytoplasm during the terminal stages of spermatozoa differentiation results in a limited defense against oxidative stress, making the cell dependent on the antioxidant support of seminal plasma (Baumber *et al.*, 2005). Antioxidant systems control the balance between production and neutralization of ROS and protect spermatozoa against peroxidative damage (Griveau and Le Lannou, 1997a, b).

Numerous studies have evaluated effects of antioxidants on male fertility in several species (Parinaud *et al.*, 1997; Hsu *et al.*, 1998; Bruemmer *et al.*, 2002; Foote *et al.*, 2002). Although many clinical trials demonstrated a beneficial effect of antioxidants in selected cases of male infertility, other studies failed to verify similar benefits. Investigators have used different antioxidants in different combinations, making it difficult to reach a definitive conclusion.

Deichsel *et al.* (2008), working with antioxidant oral supplementation (tocopherol 300 mg/day; ascorbic acid 300 mg/day; L-carnitin 4000 mg/day; folic acid 12 mg/day), have not found a pronounced effect on semen quality of stallions. Conversely, Arlas *et al.* (2008) found a higher total radical trapping potential in stallions supplemented with rice oil containing gama-oryzanol. Animals also presented an increase of total motility and membrane functionality (HOST) on fresh semen.

The addition of antioxidants to cryopreservation extender did not improve the quality of spermatozoa after thawing (Baumber *et al.*, 2005). Similar results were found (Ball *et al.*, 2001) in equine semen stored at 5°C. Conversely, Aurich *et al.* (1997) described a protective effect of ascorbic acid on sperm membrane integrity, in spite of a prejudicial effect on progressive motility. As the addition of antioxidants did not improve frozen semen quality, some authors suggest that causes other than oxidative stress are responsible for sperm damage (Baumber *et al.*, 2005; Pagl *et al.*, 2006). This may explain the similar TRAP values among cryopreservation stages and the absence of correlation between TRAP values and semen variables in our work. Pagl *et al.* (2006) reported that the loss of sperm motility during cooled storage was an effect not only of plasma membrane dysfunction but of mitochondrial membrane dysfunction as well. Addition of antioxidants to semen during cooled storage may have only limited effects.

In conclusion, the present study presents evidence that the composition of skim milk-egg yolk based semen extender provides non-enzymatic antioxidant factors that compensate for loss resulting from seminal plasma removal. However, this compensation was not observed for catalase, superoxide dismutase and glutathione peroxidase. Since no correlation between antioxidant and seminal parameters was found, oxidative stress might play a minor role in semen from fertile stallions or the non-enzymatic antioxidant activity provided by semen extender was enough to avoid deleterious effects caused by ROS.

Acknowledgments

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