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Influence of catalase and pre-freezing equilibration on post-thaw semen quality and conception rate in ewes laparoscopically inseminated

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Abstract

The aim of this study was to investigate the effects of catalase and pre-freezing equilibration during ram sperm cryopreservation on motility and membrane and acrosomal integrity of frozen-thawed semen, as well as conception rate following laparoscopic timed-insemination. Semen was collected from four mature Dorper rams, pooled and diluted in Tris egg-yolk extender basic solution (CON), or this solution supplemented with catalase (CAT; 20 U/100 × 10^6 sperm). Extended semen was packaged in 0.25 ml mini straws (25 × 10^6 sperm/straw), chilled (to 5°C), and then either frozen immediately (CON and CAT) or maintained at 5°C for 12 h of pre-freezing equilibration (CON12 and CAT12). Immediately after thawing and at 1 h after incubation at 37°C, kinematic parameters (CASA), plasma membrane integrity (PI-FITC), and acrosomal status (FITC-PNA) of sperm were assessed. There were no significant differences among the four groups on sperm traits evaluated immediately post-thaw. However, after 1 h of incubation, total motility (46.7 and 25.0%) and plasma membrane integrity (38.7 and 25.7%) were higher ($P < 0.05$) in CAT12 than CON. When these two treatments were used for laparoscopic timed artificial insemination of ewes (with synchronized ovulation), conception rate was similar for CAT12 and CON (32.8%, n = 61 *vs*. 27.3%, n = 55). In conclusion, the combination of catalase and pre-freezing equilibration resulted in significantly improved quality of post-thawed ram semen without affecting conception rate in fixed-time laparoscopically intrauterine inseminated ewes.

Keywords: antioxidant, cryopreservation, equilibration time, ram, spermatozoa.

Introduction

Semen is an inherently complex redox system, with a balance between antioxidant properties of seminal plasma/sperm and the oxidant potential of sperm metabolites, which are particularly activated in non-physiological conditions and control sperm lipoperoxidation rate (Stradaioli *et al*., 2007). It is noteworthy that low concentrations of reactive oxygen species (ROS) are involved in numerous signaling pathways (Aitken and Bennetts, 2006) that support normal sperm function (Aitken, 1999) without affecting sperm integrity (Wang *et al*., 2003). However, if the oxidative balance became pro-oxidant, due to excessive ROS production or decreased antioxidant activity, the biological system is under oxidative stress (Sikka, 1996), damaging sperm and reducing fertility (Wang *et al*., 2003).

Although lipids with gel and fluid phases can co-exist at conventional environmental temperatures, temperature variations modify the physical state of sperm-membrane lipids. The cooling process (during the initial stage of sperm cooling, prior to cryopreservation) causes a transition from fluid to gel phases and decreases membrane fluidity (Holt, 2000), which is probably responsible for thermal shock that may occur at this time. Furthermore, in addition to cooling, sperm must retain their potential viability at -196°C and subsequently overcome stresses associated with thawing (Söderquist *et al*., 1997). It is believed that pre-freezing equilibration can help sperm reach an osmotic equilibrium following the addition of cryoprotectant (Emmens and Blackshaw, 1955).

In the last few years, we have reported the effects of antioxidants on cryopreserved ram semen (Câmara *et al*., 2011a; Silva *et al*., 2011, 2012). Furthermore, we recently reported that catalase and pre-freezing equilibration may improve some *in vitro* aspects of frozen-thawed ram sperm (Câmara *et al*., 2011b). Although both catalase and glutathione are enzymatic scavengers of hydrogen peroxide, catalase is able to act on higher oxidative stress than glutathione (Baker and Aitken, 2004), presenting a selective action able to reduce lipid peroxidation induced by hydrogen peroxide, but not affecting spontaneous lipid peroxidation in frozen-thawed ram sperm (Maia *et al*., 2010). It is noteworthy that catalase can act by itself in cases of oxidative stress, whilst the glutathione system depends on a group of cofactors to succeed in its metabolic properties (Silva, 2006).

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Additionally, modern livestock breeding relies on artificial insemination to hasten genetic improvement (Rodriguez-Martinez, 2013b), although in the sheep industry AI is not done universally, due to inconsistent and often low fertility, especially when frozen-thawed semen is used (Anel *et al*., 2006), which indicate that ram semen cryopreservation still needs improvements (Mata-Campuzano *et al*., 2015). It is noteworthy that the *in vitro* benefits of adding antioxidants during sperm cryopreservation were not consistently manifested by improved fertility *in vivo.* This would be attributed to excessive ROS scavenging, considering that in low concentrations, ROS facilitates normal sperm function (Aitken, 1999), or even due to direct toxic effects of antioxidants (Mata-Campuzano *et al*., 2012).

Therefore, the aim of this study was to evaluate the effects of catalase and pre-freezing equilibration during semen cryopreservation on motility and on membrane and acrosomal integrity of frozen-thawed ram semen, as well as conception rate following laparoscopic timed-insemination.

Materials and Methods

Reagents

All chemicals used were obtained from the Sigma-Aldrich Chemical Company (St Louis, MO, USA).

Rams, semen collection and initial evaluation

Four 1 to 2 year old Dorper rams, raised in a semi-confinement system with natural light (9.1204 S, 35.7368 W), were used. They had *ad libitum* access to hay and good quality drinking water, and received 400 g/head/day of concentrate. All rams were previously deemed breeding sound and were in a semen collection routine three times/week (artificial vagina and teaser female). Ejaculates from all four rams collected on the same day were pooled to eliminate individual differences (Bucak *et al*., 2008). Minimum requirements to freeze pooled ejaculates were volume ≥3.0 ml, motility ≥80%, and a minimum of 3×10^9 sperm/ml. Semen was diluted (1:200) in formol citrate solution, and sperm concentration was calculated using a Neubauer hemocytometer chamber (Menchaca *et al*., 2005) with bright-field microscopy (400X). These procedures were conducted in quadruplicate, resulting in four pooled semen samples from 16 ejaculates.

Semen processing

The base extender medium was Tris-egg yolk extender, supplemented with 6% glycerol and 10% egg-yolk. Each pool of semen was divided into two equal aliquots and diluted (final concentration, 100×10^6 sperm/ml) with base extender containing catalase (20 U/ml; CAT) or no antioxidant (control; CON). Extended semen was then manually loaded into 0.25 ml mini straws (IMV® Technologies, L'Aigle, Cedex, France) at room temperature, and thereafter immediately cooled (~0.2°C/min) to a thermal equilibrium of 5°C, which was reached within approximately 90 min. Thereafter, half of the doses of each treatment (CAT and CON) were placed in a programmable freezer (TK-3000®, TK Tecnologia em Congelação LTDA, Uberaba, MG, Brazil), previously equilibrated at 5°C. Using this machine, semen was cooled from 5 to -120°C at a rate of 12.5°C/min. Once straws reached -120°C, they were plunged directly into liquid nitrogen for storage. The procedure was repeated with the remaining samples after 12 h of equilibration at 5°C (CAT12 and CON12). Following storage for at least 7 days, straws were thawed at 37°C for 30 seconds in a water bath and frozen-thawed semen was evaluated.

Assessment of post-thaw sperm quality using fluorescent probes

Three straws from each treatment (CON, CAT, CON12, and CAT12) were thawed (37°C, 30 sec) and pooled. Immediately and after 1 h of incubation at 37°C, membrane and acrosomal integrity were detected using fluorescence probes. The integrity of the sperm membrane was determined using a combination of propidium iodide (PI) and carboxyfluorescein diacetate (CFDA), as described by Harrison and Vickers (1990) and modified by Coleto *et al*. (2002). Aliquots (50 µl) of each sample were diluted in 150 µl of Tris containing 20 µl of PI (0.5 mg/ml in PBS) and 5 µl of CFDA (0.46 mg/ml in DMSO). Using DBP 485/20 nm excitation and DBP 580-630 nm emission filters, 200 cells from each sample were examined under an epifluorescence microscope (400X; Carl Zeiss, Göttingen, Germany). Green fluorescence was interpreted as an intact membrane, whereas red indicated a damaged membrane.

For detection of acrosomal integrity, sperm were stained with fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA), as described by Roth *et al*. (1998). Aliquots (5 µl) of semen from each treatment were placed on microscope slides and air-dried. The slides had 20 µl of FITC-PNA working solution (100 µg/ml) spread over them, and were incubated at 4°C in a moisture chamber for 15 to 20 min (in darkness). The slides were then immersed in PBS at 4°C twice and air-dried in darkness. At the time of evaluation, 5 µl of solution containing 4.5 ml of glycerol, 0.5 ml of PBS and 5.0 mg of phenylenediamine was placed on the slide. Then the slide was covered with a coverslip and viewed with epifluorescence (1000X; Carl Zeiss, Göttingen, Germany) using BP 450-490 nm excitation and LP 515 nm emission filters. Among the 200 cells examined, sperm were classified as having an intact acrosome (iAC) when the

acrosome region was stained fluorescent green, and as having a reacted acrosome when the fluorescent green was absent from the head region, or when it was present only in the equatorial region of the sperm head.

Computer assisted sperm analysis (CASA)

Three straws from each treatment (CON, CAT, CON12, and CAT12) were thawed (37°C, 30 sec) and mixed. Immediately and after 1 h of incubation at 37°C, samples were diluted (1:1) in sodium citrate solution at 2.94% (v/v) and sperm motion characteristics were detected by CASA. The latter consisted of an optical phase-contrast microscopy system (Nikon™ H5505, Eclipse 50i, Japan) with stroboscopic illumination, a warming stage (37°C), video camera (Basler Vision Tecnologie™ A312FC, Ahrensburg, Germany), and a personal computer with Sperm Class Analyzer (SCA™) software (Microptics, S.L., version 3.2.0, Barcelona, Spain). A pre-warmed Makler Chamber® (Sefi Medical Instrument, Haifa, Israel) was loaded with 5 µl of diluted sample; at least four nonconsecutive, randomly selected microscopic fields per sample were scanned, assessing at least 400 motile sperm. Events not related to sperm were excluded, and image sequences were saved and subsequently analyzed. Assessment included the following end points: total motility (TM), progressive motility (PM), curvilinear velocity (VCL), progressive velocity (VSL), and path velocity (VAP). Motility end points were measured with the following settings: temperature 37°C; frames acquired, 25; frame rate, 25 seconds; minimal contrast, 75; frame number, 25 per field; sperm velocity that can be analyzed, 0 to 180 µm/sec; and threshold STR, 75%.

Conception rate after laparoscopic artificial insemination

Multiparous mixed-breed ewes (n = 123) raised in an extensive production system with natural light (9.8498 S, 36.1010 W) were used. They had *ad libitum* access to hay and good quality drinking water. Ewes were ranked according to body condition (BC, 1-5) and age (years) and only ewes with BC and age ≥ 2 were then allocated into two treatment groups. To synchronize estrus, intravaginal sponges containing 60 mg medroxyprogesterone acetate (Progespon, Syntex, São Paulo, SP, Brazil) were inserted (day 0), with 50 µg of a prostaglandin F2 alpha analogue (Ciosin, Schering-Plough Coopers, São Paulo, SP, Brazil) given im on day 9. On day 11, sponges were removed and 250 U of equine chorionic gonadotropin (Folligon, Intervet, São Paulo, SP, Brazil) was given im. Between 56 and 60 h after sponge removal, fixed-time laparoscopic artificial insemination (LAI) was done in 116 ewes, allocated into two experimental groups (CON, n = 55; CAT12, n = 61) using a single 0.25 ml straw per insemination (25×10^6 sperm/ewe). Immediately before LAI, the straw was thawed (37°C, 30 sec) and approximately half of the volume of each straw was placed into each uterine horn (maximum 5 min after thawing). The same person, working without knowledge of treatment assignment, did all inseminations. Aiming to evaluate the efficacy of the hormonal protocol to induce ovulation, concurrent with LAI, seven ewes submitted to the same estrus synchronization protocol were selectively exposed to rams (deemed breeding sound) for natural service. At 45 days after breeding or insemination, conception rate was determined by transrectal ultrasonography using a 5.0 MHz linear-array transducer in both LAI and naturally bred ewes (CTS 900V, Siui, China).

Statistical analyses

For *in vitro* tests (fluorescent probes and CASA), the variables used for comparison purposes were media and pre-freezing equilibration times (CON, CAT, CON12, and CAT12), immediately and 1 h after thawing. Percentage data were arc-sine transformed prior to statistical analysis (one-way ANOVA, followed by Tukey's test), although all data are reported as non-transformed means ± SEM. In addition, the same analysis was used to compare, among treatment groups, body condition scores and age of ewes. A Chi-square was used to compare conception rates between treatments (CON and CAT12). For all statistical analyses, results were considered significant when $P < 0.05$.

Results

There was no influence ($P > 0.05$) of treatments on sperm traits evaluated immediately post-thaw (Fig. 1 and 2). Although there was a numerical reduction in almost all end points after 1 h of post-thaw incubation, the addition of catalase resulted in a modest increase in velocity parameters (VCL, VSL and VAP) after 1 h of incubation (Fig. 2). Furthermore, the combination of 12 h pre-freezing equilibration and catalase resulted in inferior damage of the sperm plasma membrane ($P < 0.05$; Fig. 1) and higher total motility 1 h after thawing compared to the control sample ($P < 0.05$; Fig. 2), cryopreserved without antioxidant and pre-freezing equilibration (38.7 *vs*. 25.7% and 46.7 *vs*. 25.0%, respectively). Therefore, samples from CON and CAT12 treatments were used for intrauterine laparoscopic insemination.

After estrus synchronization protocol, all seven ewes that were served by rams became pregnant. The average body condition score and age of inseminated ewes from CON and CAT12 were nearly identical (2.56 *vs*. 2.55 and 3.72 *vs*. 3.84, respectively; $P > 0.05$) and there was no difference on conception rate on day 45 (day 0 = LAI) between CON and CAT12 (27.3 *vs*. 32.8%; $P > 0.05$) after LAI.

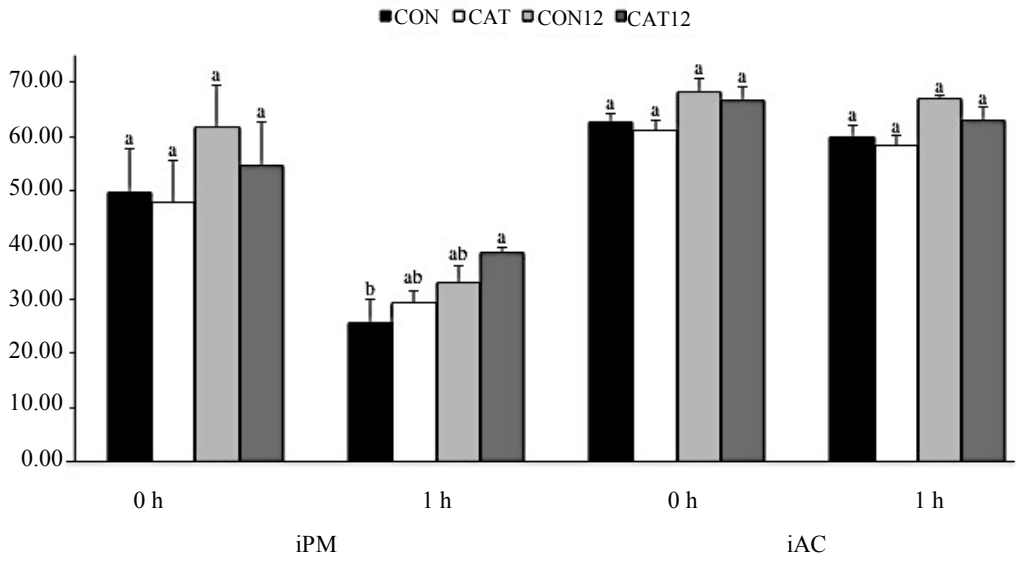

Figure 1. Mean ± SEM percentages of frozen-thawed ram sperm with intact plasma membrane (iPM) and intact acrosome (iAC) cryopreserved in Tris-egg yolk extender with no antioxidant (CON) or supplemented with 20 U/mL of catalase (CAT), and cryopreserved after thermal equilibrium was reached at 5°C; or 12 h after equilibration (CON12 and CAT12, respectively). Evaluations were done 0 and 1 h after thawing.
[a,b]Within an end point (iPM, iAC) and time (0, 1 h), means without a common superscript differed ($P < 0.05$). Data from four pooled semen samples from four rams.

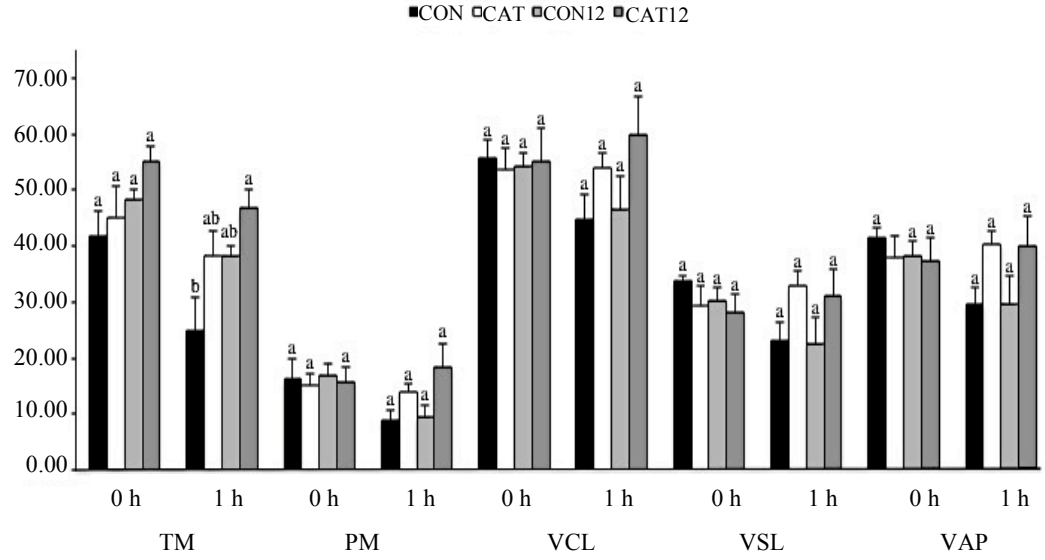

Figure 2. Mean ± SEM of total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), progressive velocity (VSL, µm/sec), and path velocity (VAP, µm/sec) of frozen-thawed ram sperm cryopreserved in Tris-egg yolk extender with no antioxidant (CON) or supplemented with 20 U/ml of catalase (CAT), and cryopreserved after thermal equilibrium was reached at 5°C; or 12 h after equilibration (CON12 and CAT12, respectively). Evaluations were done 0 and 1 h after thawing.
[a,b]Within an end point (TM, PM, VCL, VSL, VAP) and time (0, 1 h), means without a common superscript differed ($P < 0.05$). Data from four pooled semen samples from four rams.

Discussion

The post-thaw semen quality in the present study has similar motility, higher percentage of viable cells and lower percentage of intact acrosome when compared with data reported by Bucak *et al*. (2008), who tested other antioxidants besides catalase, although Bucak *et al*. (2008) did not use CASA or fluorescence technique to evaluate sperm endpoints. When compared to the present study, Câmara *et al*. (2011b) reported

similar percentage of sperm with intact acrosome, higher values of sperm motility and VCL, and lower percentage of viable sperm after using a very similar experimental design. We inferred that, in this case, these differences could be related to both individual and seasonal influence on sperm quality and freezability (Simplício *et al*., 1982; D'Alessandro and Martemucci, 2003; Frazão Sobrinho *et al*., 2014), since both experiments were conducted using different breeds and year season.

It is well known that cryopreservation causes physical and chemical stresses to sperm membranes, along with oxidative stress, which can reduce sperm viability and fertilizing capability (Watson, 2000), being the sperm damage due to the release of endogenous reactive oxygen species the major cause of reduction in sperm motility and conception rate associated with semen cryopreservation (Bucak *at al*., 2008). However, the benefits of catalase are not concentration-dependent and excessive concentrations can have a deleterious effect on ram sperm (Maxwell and Stojanov, 1996). We inferred that the catalase/sperm ratio used in the present study (20 U/100 × 10^6 sperm) was appropriate for cryopreservation of ram semen, as it yielded some apparent (albeit not significant) improvements in velocity parameters (VCL, VSL, and VAP) of thawed ram sperm samples after 1 h incubation, probably due to a biochemical equilibrium between ROS generation and scavenging, supporting physiological aspects of sperm cell metabolism.

The combination of pre-freezing equilibration plus the benefits of catalase addition significantly improved (compared to the control group) percentages of motile sperm and those with an intact plasma membrane after 1 h of incubation post-thaw. We inferred that the better plasma membrane integrity in CAT12 was due to fewer ultrastructural changes previously reported during ram sperm cryopreservation (Silva *et al*., 2013), especially considering the sensitivity of ram sperm to cold-shock, apparently due to the cholesterol-phospholipid ratio (Tapia *et al*., 2012). Results similar to those in the present study were reported for bovine semen (Anzar *et al*., 2011), with beneficial effects of pre-freezing equilibration on plasma and acrosomal membranes of sperm. These authors stated that during prolonged refrigeration, sperm can probably adapt to the low temperature and enabled egg yolk lipoproteins to more effectively exert their protective effects. However, it is noteworthy that prolonged pre-freezing equilibration did not consistently improve *in vitro* or *in vivo* aspects of sperm quality. In this regard, Purdy *et al*. (2010) reported no improvement with 24 h of pre-freezing equilibration at 5°C on motion parameters or conception rate of ram semen.

Thus, LAI was performed to determine whether the beneficial effects of catalase and pre-freezing equilibration time observed *in vitro* were also manifested as improved in *in vivo* fertility, since there are many studies reporting *in vitro* effects of antioxidants during ram semen cryopreservation but with no data regarding its influence on *in vivo* fertility (Bucak *et al*., 2008; Maia *et al*., 2010; Câmara *et al*., 2011a, b; Silva *et al*., 2011, 2012, 2013; Mata-Campuzano *et al*., 2012, 2014, 2015). The conception rate of seven ewes subjected to hand-mated natural breeding confirmed the efficacy of the hormonal protocol to synchronize estrous and ovulation. For LAI, a reduced number of sperm (25 × 10^6 sperm/ewe) were inseminated compared to other reports (Purdy *et al*., 2010; Alvarez *et al*., 2012; Richardson *et al*., 2012; Del Olmo *et al*., 2013) and the number of motile sperm can directly interfere on the conception rate after LAI (Maxwell, 1986; Eppleston and Maxwell, 1995), which could explain the low conception rate obtained in both groups inseminated. In that regard, our intention was to avoid using a large number of sperm that could potentially mask real differences in sperm quality and function (Eppleston and Maxwell, 1995; Amann and Waberski, 2014) due to compensable sperm defects (Saacke, 2008).

Sperm evaluation immediately after thawing is considered unable to detect sub lethal damage, such as that reported in ionic channels and transmembrane proteins (Tapia *et al*., 2012), which can affect *in vivo* conception rates due to alterations in the mechanisms involved in the passage of sperm through the uterotubal junction or perhaps affect the interaction between sperm and oviduct cells (Druart, 2012). Del Olmo *et al*. (2013) reported a positive correlation among *in vivo* conception rate of thawed ram sperm and kinematic parameters (VCL, VAP, and BCF) after 2 h incubation; moreover, sperm plasma membrane integrity had a greater association with conception rate than sperm motility end points (Rodriguez-Martinez, 2013a). However, the benefits detected in this study on motility and membrane integrity *in vitro* after 1 h storage post-thaw were not able to significantly increase the conception rate in CAT12 compared to CON, reinforcing that although routine sperm evaluation tests (viability, motility, concentration, sperm morphology) are useful indicators, they cannot adequately predict potential conception rate (Tsakmakidis, 2010), due to the existence of a complex modulation to maintain semen quality and fertility (Rodriguez-Martinez, 2003; Mata-Campuzano *et al*., 2015).

In conclusion, the combination of exogenous catalase added to extender medium and a 12 h pre-freezing equilibration at 5°C significantly reduced ram sperm cryodamage but did not improve the conception rate of ewes that were laparoscopically timed-inseminated.

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