Small Ruminant Research 93 (2010) 206–209

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Short communication

Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen

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ARTICLE INFO

Article history:
Received 29 January 2010
Received in revised form 27 April 2010
Accepted 4 May 2010
Available online 9 June 2010

Keywords:
Extender
Frozen semen
Ram

ABSTRACT

This study tested trehalose and low-density lipoprotein (LDL) as cryoprotectants in extenders for frozen ram semen. In the first experiment, the extenders were Tris, with 20% egg yolk (E1-1); E1-1 with 5% glycerol (E1-2); E1-1 with 100 mM trehalose (E1-3); and E1-1 with 100 mM trehalose and 5% glycerol (E1-4). Sperm motility and membrane integrity of the E1-2, E1-3 and E1-4 extenders were greater than for E1-1 ($P < 0.05$), but acrosome integrity following cryopreservation did not differ. In the second experiment, the extenders were Tris, with 20% egg yolk and 100 mM trehalose (E2-1); Tris with 8% LDL and 5% glycerol (E2-2); Tris with 8% LDL and 100 mM trehalose (E2-3); and Tris with 8% LDL, 100 mM trehalose and 5% glycerol (E2-4). Sperm membrane integrity was lowest for the E2-1 extender ($P < 0.05$), but similar for extenders including LDL. Sperm motility post-thawing was highest for E2-2 and E2-3 extenders ($P < 0.05$), but acrosome integrity did not differ. Thus, extenders including trehalose and LDL as cryoprotectants recorded a post-thawing ram sperm quality similar to that achieved when using conventional cryoprotectants.

1. Introduction

In sheep, the use of artificial insemination with frozen semen is still inefficient. The ewe's cervical anatomy limits the insertion of conventional catheters (Kershaw et al., 2005) and the variation between rams used as semen donors also contribute to this inefficiency (Paulenz et al., 2004). Additionally, post-thawing sperm motility and membrane integrity, due to either the occurrence of cold shock and osmotic stress during freezing (Salamon and Maxwell, 2000; Watson, 2000), or premature capacitation due to lipid peroxidation may be reduced (Bailey et al., 2000; Aisen et al., 2005). These functional disabilities relate to the high content of long-chain polyunsaturated fatty acids in the ram sperm membrane, which increases its sensitivity to oxidative damage (Watson, 1995). Egg yolk currently is the most common used non-penetrating cryoprotectant. It is included in extenders to stabilize the sperm membrane (Salamon and Maxwell, 2000), despite containing substances that may interfere with the cell metabolism, thus reducing sperm motility (Moussa et al., 2002). Glycerol, on the other hand, is the most commonly utilized penetrating cryoprotectant in extenders for frozen semen, but it is potentially cytotoxic (Holt, 2000; Watson, 2000). Thus, the search for alternative cryoprotectant solutions for frozen ram semen is justified.

Trehalose may be used as a cryoprotectant (Molinia et al., 1994; Sánchez-Partida et al., 1998), as it promotes cell dehydration, which reduces the negative effects of water flow through the sperm membrane during freezing (Yildiz et al., 2000) and the formation of ice crystals (Aisen et al., 2002, 2005). Trehalose also interacts with the membrane phospholipids and proteins, providing the membrane more flexibility against cryo-injuries (Aisen et al., 2002; Bucak et al., 2007). The low-density lipoprotein (LDL) present in the egg yolk has been effectively used as a non-penetrating cryoprotectant in extenders for semen of bulls (Moussa et al., 2002), boars (Jiang et al., 2007) and dogs (Varela Junior et

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doi:10.1016/j.smallrumres.2010.05.003

Table 1
The effect of trehalose and glycerol in extenders for frozen ram semen on post-thawing sperm motility, membrane integrity and acrosome integrity.

Extender[A]	Motility (%)(Mean ± SE)	Membrane integrity (%)(Mean ± SE)	Acrosome integrity (%)(Mean ± SE)
E1-1	23.7 ± 2.7[b]	16.4 ± 2.6[b]	49.2 ± 3.5
E1-2	54.2 ± 2.7[a]	37.2 ± 2.6[a]	55.4 ± 3.5
E1-3	49.2 ± 2.7[a]	36.5 ± 2.6[a]	65.3 ± 3.5
E1-4	43.7 ± 2.7[a]	35.1 ± 2.6[a]	56.2 ± 3.5

[a,b]Means in columns having different superscripts differ significantly ($P < 0.05$).
[A] E1-1: Tris + 20% egg yolk; E1-2: Tris + 20% egg yolk + 5% glycerol; E1-3: Tris + 20% egg yolk + 100 mM trehalose; E1-4: Tris + 20% egg yolk + 100 mM trehalose + 5% glycerol.

al., 2009), but it has not been tested for ram semen. The LDL generally binds to the seminal plasma proteins, preventing interaction with the sperm membrane (Bergeron and Manjunath, 2006) and the outflow of phospholipids and cholesterol from the membrane (Manjunath and Thérien, 2002; Bergeron et al., 2004). Thus improving its resistance against cold shock. In this study the objective was to test the efficiency of trehalose and LDL as cryoprotectants on the post-thawing quality of ram semen.

2. Materials and methods

Two experiments were conducted, each using different rams as semen donors. The rams were 24–36 months of age old and kept under semi-extensive conditions at the Universidade Federal de Pelotas. In the first experiment (EXP1), four rams from the Crioulo breed were used, with eight ejaculates collected per ram ($n = 32$), during a 4-week period (July–August; winter). In the second experiment (EXP2), five other rams from the Crioulo breed were used, with eight ejaculates collected per ram ($n = 40$), during a 4-week period (September–October; spring). All semen collections were done using an artificial vagina. The tested extenders in the two experiments: 2.7 g Tris (Sigma Chemical Company, St. Louis, MO, USA); 1.25 g glucose (Synth, Diadema, SP, Brazil); 100 mg streptomycin (S 9137) and 2000 IU penicillin (P 4687), both obtained from the Sigma Chemical Company.

In EXP1, four extenders were tested: Tris including 20% egg yolk (E1-1); E1-1 including 5% glycerol (Synth, Diadema, SP, Brazil) (E1-2); E1-1 including 100 mM trehalose (Sigma Chemical Company) (E1-3); and E1-1 including 100 mM trehalose and 5% glycerol (E1-4). One of the extenders tested in EXP1 was selected to be used as a control in EXP2. As E1-2, E1-3 and E1-4 extenders provided similar results in EXP1, E1-3 was chosen, as it included trehalose and did not include glycerol. The choice of the trehalose concentration was based on the reports of Bucak et al. (2007), which were corroborated by unpublished data from this research team that showed superior post-thaw sperm quality with 100 mM trehalose compared with 50 mM trehalose.

In EXP2, the following four extenders were tested: Tris including 20% egg yolk and 100 mM trehalose (E2-1); Tris including 8% LDL and 5% glycerol (E2-2); Tris including 8% LDL and 100 mM trehalose (E2-3); and Tris including 8% LDL, 100 mM trehalose and 5% glycerol (E2-4).

After dilution, semen samples were incubated in water in a thermal box for 2 h at 5 °C. The glycerol-based extenders were stabilized for 20 min, after glycerol inclusion at 5 °C and the procedures for the preparation of the extenders including LDL and the use of LDL at an 8% level were done as described by Moussa et al. (2002) and Varela Junior et al. (2009). Egg yolks were placed on filter paper for removal of traces of the white in the vitelline membrane and broken into a beaker cooled with ice. The LDL-rich fraction of the yolk was obtained by plasma fractionation after two centrifugations at 10,000 × *g*, for 45 min, at 5 °C.

Semen samples were stored in 0.25 mL straws containing 2×10^8 spermatozoa per straw. For freezing, straws were placed horizontally, 5 cm above the level of the liquid nitrogen, for 15 min to freeze in the vapor. Straws were then subsequently stored in liquid nitrogen. Straws were thawed at 37 °C, for 20 s in a water bath and the semen was placed in a conical tube containing 3 mL 3% sodium citrate 3% (Synth, Diadema, SP, Brazil), previously warmed to 37 °C.

All evaluations regarding the sperm quality were conducted by the same trained technician and all recorded values represented the average of three evaluations per sample. Sperm motility was evaluated immediately after collection, and again after thawing, using a phase contrast microscopy (200× magnification) on a 10 μL semen sample. Sperm membrane integrity was evaluated using carboxyfluorescin diacetate and propidium iodide (Harrison and Vickers, 1990), using an epifluorescent microscope (Olympus BX 51, America Inc., Sapporo, Japan), under 400× magnification (filter wave length of 525 nm). Two hundred cells were counted per semen smear and classified as either intact (green fluorescence) or not intact (red fluorescence).

Post-thawing acrosome integrity was evaluated as described by Kawamoto et al. (1999). 20 μL lectin pisum sativum (Sigma Chemical Company) was added to the semen samples at a concentration of 20 mg/mL. On each microscope slide, 200 sperm cells were counted with an epifluorescent microscope (Olympus BX 51, America Inc.) at 1000× magnification (a filter wave length of 450–490 nm and emission of 520 nm). Sperm cells were classified as intact (acrosome with green fluorescence) or not intact (sperm heads with red fluorescence, or no evidence of green fluorescence).

In both experiments, an analysis of variance with repeated measures was used to test the effects of the extenders, semen collections, extender per collection interaction and individual effects of rams nested within the effect of extenders – on semen quality parameters, with comparisons of the means performed using the Tukey-test. As none of the response variables was normally distributed, all were submitted to transformations: arctan transformation for sperm motility; arcsine transformation for sperm membrane integrity; and logarithmic transformation for acrosome integrity. For interpretation, all results were expressed in the original scale. All analyses were done using Statistix® (2003).

3. Results

In EXP1, the mean post-thaw sperm motility was 42.7 ± 18.6%, mean sperm membrane integrity 31.3 ± 14.6% and mean acrosome integrity 56.5 ± 22.7%.

Table 2
The effect of glycerol, trehalose and low-density lipoprotein (LDL) in extenders for frozen ram semen, on post-thawing sperm motility, membrane integrity and acrosome integrity.

Extender[A]	Motility (%)(Mean ± SE)	Membrane integrity (%)(Mean ± SE)	Acrosome integrity (%)(Mean ± SE)
E2-1	31.2 ± 2.1[b]	14.9 ± 1.8[b]	36.0 ± 3.4
E2-2	43.5 ± 2.1[a]	22.9 ± 1.8[a]	38.5 ± 3.4
E2-3	41.7 ± 2.1[a]	24.9 ± 1.8[a]	36.7 ± 3.4
E2-4	32.5 ± 2.1[b]	19.3 ± 1.8[ab]	38.3 ± 3.4

[a,b]Means in columns having different superscripts differ significantly ($P < 0.05$).
[A] E2-1: Tris + 20% egg yolk + 100 mM trehalose; E2-2: Tris + 8% LDL + 5% glycerol; E2-3: Tris + 8% LDL + 100 mM trehalose; E2-4: Tris +8% LDL + 100 mM trehalose + 5% glycerol.

Post-thawing sperm motility and membrane integrity did not differ for the E1-2, E1-3 and E1-4 extenders (Table 1), but both parameters were lowest for E1-1 ($P < 0.05$). The extenders presented similar post-thawing acrosome integrity (Table 1).

In EXP2, the mean post-thaw sperm motility, membrane integrity and acrosome integrity were $37.3 \pm 12.7\%$, $20.5 \pm 9.6\%$ and $37.4 \pm 19.2\%$, respectively, and acrosome integrity did not differ between extenders (Table 2). A greater post-thaw sperm motility ($P < 0.05$) was recorded for the E2-2 and E2-3 extenders, compared to E2-1 and E2-4 (Table 2). Post-thaw sperm membrane integrity was inferior ($P < 0.05$) for the E2-1 compared to E2-2 and E2-3 extenders (Table 2), with no differences between extenders including LDL.

4. Discussion

Post-thaw sperm quality was preserved in the E2-3 extender, which included trehalose and LDL, at levels similar to those obtained with traditional cryoprotectants, such as egg yolk and glycerol. In EXP2, the inclusion of LDL likely explains the greater sperm motility and membrane integrity recorded for E2-2 and E2-3, than for the control extender, including egg yolk – which presented the best results in EXP1. The similar results observed in EXP1, for the four egg yolk-based extenders, and in EXP2, for E2-2 and E2-3, indicate that the replacement of glycerol by trehalose does not impair post-thawing sperm quality. Thus the use of trehalose represents an alternative to glycerol, which is potentially cytotoxic (Holt, 2000; Watson, 2000). In EXP2, the inclusion of LDL in E2-2 may have attenuated glycerol's cytotoxic effect, but the inclusion of LDL, trehalose and glycerol together in E2-4 did not benefit sperm membrane integrity and resulted in sperm motility being inferior following cryopreservation in both E2-2 and E2-3 – that included LDL, but only one of the other two cryoprotectants. That may have been due to osmotic stress caused by the high intracellular concentration of salts and other solutes, as a function of cell dehydration (Woelders et al., 1997). Therefore, it would seem feasible to freeze ram sperm with extenders that replace egg yolk with LDL and do not include a typical penetrating cryoprotectant, as the inclusion of either trehalose or glycerol resulted in a similar post-thawing sperm quality.

The inclusion of trehalose and other sugars as cryoprotectants in extenders for frozen semen can benefit post-thawing sperm viability (Yildiz et al., 2000; Matsuoka et al., 2006). Post-thaw sperm quality can be similar for extenders including either trehalose or glycerol (Moura et al., 1995) and the inclusion of trehalose on glycerol-based extenders has no effect on post-thawing sperm motility (Sánchez-Partida et al., 1998). However, the inclusion of trehalose in extenders without glycerol may improve sperm motility (Molinia et al., 1994). Trehalose has also been used as a cryoprotectant for semen of bulls (Chen et al., 1993) and mice (Storey et al., 1998). The cryoprotective effect of trehalose can be attributed to its anti-oxidant activity (Aisen et al., 2005). Trehalose also promotes cell dehydration and influences the crystallization pattern of the solute channels present in portions of unfrozen water of the extender (Nicolajsen and Hvidt, 1994), contributing to the reduction in the formation of ice crystals (Aisen et al., 2002, 2005). Thus, trehalose can be used in hypertonic extenders. Sperm's osmotic regulation at low temperatures may impair motility and membrane integrity, due to structural damages in the lipid chain of the membrane (Meyers, 2005). In ideal cooling protocols, dehydration in hypertonic media allows sperm to support negative temperatures for long periods of time (Woelders et al., 1997), as likely occurred in EXP1 – in which sperm motility and membrane integrity for E1-3 were at least 20% greater than for the control extender. Additionally, in EXP1, sperm motility and membrane integrity were apparently greater than in EXP2. Such discrepancies may be due to the use of distinct rams as semen donors, or due to the distinct periods in which these studies were conducted: EXP1 was in July, during the winter; EXP2 was conducted in September and October, during spring in the Southern hemisphere.

The cryoprotectant effect of LDL can be attributed to its capacity for stabilizing the sperm membrane, thus preventing the efflux of phospholipids (Thérien et al., 1999) and cholesterol (Bergeron et al., 2004). As lipids present in the LDL are not integrated into the membrane, they form a physical barrier that protects the membrane against cold shock (Manjunath and Thérien, 2002). Also, as LDL sequesters seminal plasma proteins, such proteins do not act in the sperm membrane during cryopreservation (Bergeron and Manjunath, 2006). The replacement of egg yolk by low-density lipoprotein (LDL), also benefits the post-thawing sperm viability by excluding egg yolk components (e.g., minerals and granules) that adhere to the membrane and have a negative effect on sperm motility and respiration (Pace and Graham, 1974). LDL supports the action of seminal plasma proteins and the efflux of cholesterol and phospholipids (Thérien et al., 1999). The concentration of LDL used in the present study (8%) has also been used for semen of bulls (Moussa et al., 2002) and dogs (Varela Junior et al., 2009). The beneficial effects of LDL for post-thawing viability have also been reported in swine sperm (Jiang et al., 2007).

5. Conclusions

Extenders including 100 mM trehalose and 8% LDL can preserve post-thawing sperm motility and membrane integrity of frozen ram sperm as efficiently as extenders including traditional cryoprotectants, such as egg yolk and glycerol.

Acknowledgement

Thanks are due to the CAPES foundation for the financial support of the first author.

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