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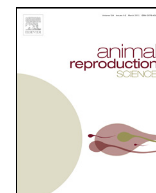

Different extenders in the cryopreservation of bovine epididymal spermatozoa

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ABSTRACT

The objective of this study was to evaluate the effects of two different egg yolk extenders incubated with or without Sperm Talp on the motility and plasma membrane integrity of cryopreserved bovine epididymal spermatozoa after freezing. Twenty-five testicles with epididymides from mature bulls were collected at the abattoir. Epididymal sperm recovery was performed by retrograde flushing using a skim milk-extender (Botu-Semen™). After recovery, sperm were incubated either without or with Sperm Talp and then submitted to centrifugation. For the freezing process, half of the testes were processed with Tris egg yolk extender, and half were processed with Botu-Bov™ egg yolk extender. Samples incubated in Sperm Talp exhibited better results than epididymal spermatozoa that were incubated without Sperm Talp ($p < 0.05$). Both Botu-Bov™ and Tris could be utilised to freeze sperm from the bovine epididymides if the sperm were previously incubated with Sperm Talp. The extenders examined in this work did not differ in their effect on plasma membrane integrity after freezing.

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1. Introduction

Sperm recovery from the cauda epididymis can be advantageous after the unexpected death of animals with genetic merit. This procedure is an important tool for the recovery and use of germplasm after death (Kaabi et al., 2003). However, it is known that the *in vivo* fertility of bovine epididymal sperm tends to be lower than that of ejaculated spermatozoa. Ejaculated sperm differ from epididymal sperm in many factors, including the types of proteins that are bound to the plasma membrane (Lee et al., 1985) and their motion characteristics (Goovaerts et al., 2006).

The recovery of spermatozoa from *cauda* epididymal sperm can be performed by aspiration (Sharma et al., 1997), flotation (Hewitt et al., 2001), retrograde flush (Garde et al., 1994) and a modified retrograde flush technique (Muradás et al., 2006). According to Turri et al. (2012), the retrograde flush technique is better than the flotation method in terms of sperm quality, total motility and the viability of bull epididymal spermatozoa.

The cryopreservation of epididymal spermatozoa allows an efficient and economical use of the genetic material because it can be stored indefinitely (Martins et al., 2007). Papa et al. (2008) and Monteiro et al. (2011) reported the fertility of cryopreserved equine epididymal sperm

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using an egg yolk extender (BotuCrio™, Botupharma, Botucatu, Brazil) and obtained a conception rate higher than previous studies using either fresh or frozen equine semen (Morris et al., 2002). Previous studies also observed the beneficial effect of Sperm Talp exposure on frozen epididymal sperm in horses and cats (Buranaamnuay, 2013; Morris et al., 2002; Papa et al., 2008). However, a cryopreservation protocol that was optimised for the sperm of one species might not be ideal for the sperm of another. Sperm from different species show a diversity in size, shape, and lipid composition, all of which affect cryosurvival (Purdy, 2006).

There is no specific diluent or protocol for the cryopreservation of bovine sperm harvested from the epididymis. Thus, this study was designed to investigate the effects of two different egg yolk extenders used largely in Brazil on the preservation of frozen bovine epididymal sperm incubated with or without Sperm Talp.

2. Materials and methods

Twenty-five testicles with epididymides from 25 sexually mature Nelore bulls (2–3 years old) were collected from a slaughterhouse and transported at 5 °C. In the laboratory, the cauda epididymides were isolated from the testis. The testicular length and width were measured with pachymeter. The testicular length is the distance from the testis caput to the caudal region not including the epididymis. The volume was calculated by applying the formula $2\,[(r^2) \times \pi \times h]$, in which r = width/2, π = 3.14 and h = testicle length (Fields et al., 1979).

The connective tissue was carefully dissected to allow passage of the flushing extender, and the cauda epididymis was straightened. A 200 μL pipette tip was attached to a 10 mL syringe. Sperm harvesting was performed using the modified retrograde flushing technique (Muradás et al., 2006). The cauda epididymis of each bull was flushed using 20 mL of warm (37 °C) skim milk or a glucose-based extender (Botu-Semen™, Botupharma, Botucatu, Brazil), which is used during semen collection. Each flushed fluid sample obtained was divided after collection into four groups for the treatments −ST/BB, −ST/TRIS, +ST/BB, and +ST/TRIS, described below.

After recovery, the flushed fluid samples were incubated without (−ST) or with (+ST) modified Tyrode's albumin lactate pyruvate (TALP) sperm medium (99 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.35 mM NaH_2PO_4, 10 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 21.6 mM Na-lactate, 1.1 mg/mL Na-pyruvate, 6 mg/mL BSA and 1 μg/mL gentamycin) at 37 °C for 10 min.

To cryopreserve the epididymal sperm, the samples were centrifuged at 600 × *g* for 10 min. The supernatant was removed, and the pellet was re-suspended either in Tris-glucose-citrate-glycerol solution plus 20% egg yolk (Tris egg yolk extender) or Botu-Bov™ egg yolk extender (Botupharma, Botucatu, Brazil) at a final concentration of 60 million total spermatozoa per mL. The basic composition of Botu-Bov™ is sugars, amino acids, buffers, surfactant OEP (*orvus es paste*) and egg yolk (Albert et al., 2004). Tris and Botu-Bov™ medium were assessed in this work because they are extensively used in Brazil for the cryopreservation of bovine semen. Then, semen samples were packed into 0.5 mL straws, maintained at 5 °C for 4 h in a commercial refrigerator (Minitub™, Porto Alegre, Brazil) and subsequently frozen in liquid nitrogen vapour 6 cm above the surface of liquid nitrogen for 20 min. The straws were immersed in liquid nitrogen and stored at −196 °C until analysis. Semen samples were thawed at 46 °C for 20 s (Dell'aqua et al., 2001) for evaluation.

Semen analysis was performed immediately after sperm recovery, following incubation at 37 °C for 10 min, after centrifugation following dilution in both freezing extenders and post-thaw. Five fields per sample were selected to evaluate motility parameters by Computer-Assisted Semen Analysis CASA (HTM-VOS 12, Hamilton Thorne Research, USA). The CASA setup was pre-adjusted for bovine sperm analysis (number of frames: 30; minimum contrast: 50 pixels; minimum cell size: 6 pixels; contrast to static cells: 30; straightness: 60%; average path velocity cutoff: 30 μm/s; minimum average path velocity: 40 μm/s; straight-line velocity cutoff: 20 μm/s; cell intensity: 80; static head size: 0.23–1.91; static head intensity: 0.56–1.20; static elongation: 8–92; magnification: 1.89×; temperature: 37 °C).

Plasma membrane integrity (PMI) was evaluated at two different timepoints, after sperm recovery and post-thaw, to ensure that the extenders would protect the PMI after cold shock stress. The PMI was analysed at 400× magnification using the fluorescent probes carboxyfluorescein diacetate and propidium iodide (CFDA/PI) as described by Harrison and Vickers (Harrison and Vickers, 1990). For a quantitative assessment of plasma membrane integrity, 200 cells spermatozoa were counted, and spermatozoa that fluoresced green throughout their length after staining with carboxyfluorescein diacetate were classified as being intact, whereas those with heads that fluoresced red after staining with propidium iodide were classified as damaged.

All of the variables were tested for normality. The data for the samples after incubation were examined with the Mann–Whitney test, a nonparametric test. Data before and after freezing were analysed by ANOVA followed by the Tukey test to compare the treatments. The significance level was set to 0.05 (SAS Institute Inc., Cary, NC).

3. Results

The mean and standard deviation values for length, width and total testicular volume were 11.02 ± 1.23 cm, 5.63 ± 0.72 cm and 548.00 cm^3, respectively. The mean values and the standard error of the mean for the sperm parameters immediately after recovery were as follows: total motility (TM) 48.92 ± 3.55%; progressive motility (PM) 22.88 ± 1.81%; straight line velocity (VSL) 61.72 ± 2.32 μm/s; curvilinear velocity (VCL) 156.96 ± 7.10 μm/s; and rapid sperm (RAP) 44.52 ± 3.58%. The plasma membrane integrity (PMI) was 61.56 ± 2.19%.

Table 1 presents the sperm parameters evaluated 10 min after incubation at 37 °C without (−ST) or with (+ST) Sperm Talp. The epididymal spermatozoa incubated with Sperm Talp demonstrated better motility.

After incubation, the samples were centrifuged at 600 × *g* for 10 min and re-suspended in Tris or

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