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Preliminary study on effects of bovine frozen semen storage using a liquid nitrogen-independent method on the quality of post-thaw spermatozoa

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ABSTRACT

Frozen semen of eight bulls was used to assess effects of storage temperature and length of storage time on frozen-thawed bovine sperm quality. In experiment 1, 25 straws of frozen semen of each bull were allocated to 3 groups. The control was still maintained in liquid nitrogen (LN_2). The rest were abruptly moved from LN_2 to −80 °C and −30 °C mechanical freezers, respectively. After thawing, it was found that the sperm motility, vitality and membrane integrity were comparable ($P > 0.05$) between the control and the −80 °C samples and were significantly inferior ($P < 0.001$) in the −30 °C samples, irrespective of storage time (1-day, 1-week and 1-month). In experiment 2, two out of the three parts (16–18 straws) of frozen semen of each bull were rapidly removed from LN_2 and further kept in the freezer (−80 °C). One day before being thawed, half of the samples in the freezer were promptly put back to LN_2. The results showed that the frozen-thawed sperm quality was not significantly affected ($P > 0.05$) both by storage temperature (−196 °C, −80 °C and −80 & −196 °C) and storage time [day-2, day-8 (1-week) and day-31 (1-month)]. At the same storage times, the quality measures at different temperatures were not significantly different from one another ($P > 0.05$). In conclusion, a −80 °C mechanical freezer was as effective as LN_2 in preserving *in vitro* quality of frozen-thawed bovine spermatozoa throughout 1-month of storage. When required for use, frozen semen stored in the freezer could be thawed immediately or transferred to the LN_2 tank for thawing elsewhere.

1. Introduction

At present, although a number of bovine semen cryopreservation protocols have been proposed, almost all of these protocols include the same basic steps beginning with semen collection followed by dilution with semen extenders, cooling, packaging, freezing and ultimately storage at −196 °C in liquid nitrogen (LN_2) until thawing (Chaudhari and Mshelia, 2002). Frozen semen stored in LN_2 offers several distinct advantages over diluted chilled semen and even fresh collected semen (Vishwanath and Shannon, 2000). The most prominent one, if proper storage and handling of frozen semen are conducted, is the infinite longevity of the frozen spermatozoa; this permits the use of such spermatozoa anywhere for years afterwards (Eriksson, 2000). Nevertheless, a reliable supply of LN_2 is not guaranteed and for this reason, storage of frozen semen without using LN_2 might be an option for livestock producers especially the dairy farmers, which approximately 95%

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of the total artificial insemination is performed with frozen semen (Thibier and Wagner, 2002).

Several studies have aimed to avoid the use of LN_2 in the freezing and/or storage of semen. For instance, it has been reported that an ultra-low temperature freezer (approximately −150 °C) was successfully used to freeze and store goat semen for up to one year; the quality of the post-thaw spermatozoa evaluated both *in vitro* and *in vivo* did not differ significantly between the samples having been frozen and stored using LN_2 and the freezer (Medrano et al., 2002; Batista et al., 2009). Similarly, trials conducted in dogs have indicated that semen frozen in LN_2 or in the freezer (−152 °C) and then stored in the freezer for as long as one year had the post-thaw sperm quality comparable with semen frozen and stored in LN_2, using traditional methods (Alamo et al., 2005; Batista et al., 2006).

The influence of storage temperature on human sperm characteristics has also been investigated, but in this species results are inconsistent with some studies showing storage of semen at −196 °C in LN_2 resulting in better sperm quality compared to storage at −70 °C (Trummer et al., 1998) and at −90 °C (Fjallbrant and Ackerman, 1969). By contrast, Bunge et al. (1954) and Sherman (1954) reported that cryopreservation of spermatozoa at −70 °C and −78 °C was superior to −196 °C in preservation of the post-thaw sperm motility. Parkes (1945) and Rahana et al. (2011) also reported that sperm cryopreservation at −196 °C offered no advantage over storage at −79 °C and −85 °C.

Due to the conflicting results, the present study was undertaken to evaluate the effects of storage temperature of bovine frozen semen on the quality of frozen-thawed spermatozoa. Three storage temperatures (−196 °C, −80 °C and −30 °C) and three storage times (1–2-day, 1-week and 1-month) were tested.

2. Materials and methods

2.1. Reagents

All reagents included in this study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless indicated otherwise.

2.2. Semen samples

Four hundred doses of frozen semen taken from eight Holstein-Friesian bulls (50 doses/bull) were obtained from the dairy farm named "Farm Chokchai®" in Thailand. Semen cryopreservation was achieved using the modified cryopreservation protocol originally described by Anzar et al. (2011). Briefly, raw semen with a minimum of 65% individual progressive motility was extended at room temperature (approximately 27 °C) in a Tris-egg yolk extender (Buranaamnuay et al., 2015). The diluted samples (approximately 120×10^6 spermatozoa/mL) were gradually cooled (0.5 °C/min) and maintained at 4 °C for 4 h. The processed samples were loaded in ministraws (0.25 mL; Minitub GmbH, Tiefenbach, Germany), frozen with a traditional vapor freezing method in a styrofoam box and then stored in the LN_2 tank.

Irrespective of storage temperature, semen straw was thawed in warm (37 °C) water for 30 s. The thawed semen was transferred to a clean plastic tube and diluted (1:1, v/v) with pre-warmed Tyrode albumin-lactate-pyruvate (TALP) medium (Parrish et al., 1988). The diluted thawed semen was incubated at 37 °C during evaluation of the sperm quality.

2.3. Sperm quality evaluations

The following sperm characteristics were assessed after 15 min (0 h) and 3 h of 37 °C incubation.

2.3.1. Sperm motility

Individual progressive motility of spermatozoa was assessed visually by a skilled technician in a blinded fashion, using bright field microscopy (Helmut Hund GmbH, Wetzlar-Nauborn, Germany) at 400× magnification (Chenoweth 2002). To determine the percentage of spermatozoa having progressive linear motion, a 5 μL volume of the semen was placed on a warm glass-slide and covered with a 18 × 18 mm coverslip; 4–5 microscopic fields were observed. For each sample, the procedure was repeated twice and a mean value was recorded.

2.3.2. Sperm vitality

The one step eosin-nigrosin staining technique (Bjorndahl et al., 2003) was partially adjusted for an examination of the vitality of bull spermatozoa. The staining solution contained 0.6% (w/v) eosin Y and 5% (w/v) nigrosin dissolved in double distilled water. A small amount of the solution was mixed with a similar volume (30 μL) of the sample. After 30 s of mixing, 10 μL of the stained sample was taken onto a clean slide and smeared. Drying a stained smear was done quickly by placing the glass slide on a warming (37 °C) plate. For each slide, 300 total spermatozoa were observed at a magnification of 400× with a bright field microscope. Spermatozoa with unstained bright-head and with pink- or red-head were classified as live and dead cells, respectively.

2.3.3. Sperm plasma membrane integrity

The hypo-osmotic swelling test (HOST) was used as an additional vitality test to study the semi-permeability of the intact cell membrane. An influx of the hypo-osmotic fluid results in an expansion of cell volume which, in spermatozoa, can easily be observed at the sperm tail (Drevius and Eriksson, 1966). The HOST protocol designed for boar spermatozoa (Perez-Llano et al., 2001) was partly modified and described below.

In a 1.5-mL microtube (Eppendorf®, Sigma-Aldrich), one part of the sperm sample was mixed with four parts of hypo- (75 mOsm/kg) or iso- (300 mOsm/kg) osmotic fluids and incubated at 37 °C for 15 min. The osmotic fluids were prepared with fructose, Na-citrate and distilled water; their final osmolalities were measured with freezing point depression (Osmomat 030-3P, Gonotec GmbH, Berlin, Germany). Fifty microliters of the hypo/iso-osmotic solution plus 5% formaldehyde (Merck, Darmstadt, Germany) was added to the incubated sperm mixtures to stop the