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Post-thaw quality of buck semen samples cooled at 5 °C up to 2 days before cryopreservation☆

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ABSTRACT

This study aimed to assess the influence of cool storage (5 °C) prior cryopreservation over the post-thaw quality of buck semen samples. Semen of six Majorera bucks ($n = 18$ ejaculates) was collected, pooled and diluted in a Tris-yolk extender. Then, diluted semen was divided into six aliquots; the first aliquot (group C) was processed and frozen in liquid nitrogen (final concentration of 400×10^6 spermatozoa/mL, 12% egg-yolk and 4% glycerol). The remaining aliquots (diluted with Tris–glucose, 12% egg yolk) were hold for 1–48 h at 4 °C: R1, the semen was cooled for 1 h; R6, the semen was cooled for 6 h; R12, the semen was cooled for 12 h; R24, the semen was cooled for 24 h and R48, the semen was cooled for 48 h. After each cooling period, a second extender was added to reach a final composition (400×10^6 spermatozoa/mL, 12% egg-yolk and 4% glycerol) similar to group C; finally, semen was packed and frozen in liquid nitrogen. After freezing–thawing, the sperm motility, acrosome integrity and the percentage of abnormal spermatozoa were assessed. No differences ($P > 0.05$) were detected in progressive sperm motility (mean range: 35.4–39.9%) and damage acrosomes percentages (mean: 10.8–15.5%) among the control group and the cooled semen samples (R1, R6, R12, and R24) for up to 24 h; however, R48 samples showed a lower (21.6%, $P < 0.01$) progressive fast spermatozoa percentage and a higher percentage of damage acrosome (38.3%, $P < 0.01$) than those observed in the control group and in R1, R6, R12 and R24 samples. The present study confirmed that buck semen could be preserved at 5 °C for up to 24 h before freezing; however, after 2 days of chilling, semen quality experienced a notable decrease and its utility could be lower.

1. Introduction

In bucks, the motility of spermatozoa from fresh semen decreases quickly unless is diluted to reduce metabolic activity and the temperature is lowered (Leboeuf et al., 2000). Cooling and freezing of caprine semen has become basic tools in assisted reproduction techniques, allowing the preservation of semen samples for short and long periods of time. Semen samples preserved at 4–5 °C kept acceptable values of sperm quality for 24–72 h (Mara et al., 2007; Batista et al., 2011), but it is widely accepted that sperm longevity of bucks decreases markedly after a few days of storing (Roca et al., 1997; Paulenz et al., 2005; Salvador et al., 2006). Frozen caprine samples show limited longevity after thawing and cryopreservation is more complex than chilling (Cabrera et al., 2005; Mara et al., 2007; Dorado et al., 2009), but allows the storage of samples of high genetic value for long periods of time.

Lately, the air transportation of frozen semen samples in liquid nitrogen has become more difficult, because handling of liquid nitrogen is subject to regulation as a hazardous material (Bielanski, 2005). The transport of chilled semen requires less infrastructure (Leboeuf et al.,

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2000); and allow the shipment of semen samples over long distances in a relatively short period of time. In addition, in different geographical areas, liquid nitrogen and the technical support required are relatively expensive and sometimes the availability of the liquid nitrogen is limited (Álamo et al., 2005). Therefore, it would be interesting to try to overcome the limitations associated with the shipping of frozen semen by the use of cooled semen, and then, freeze the chilled semen samples for long-time storage for future use.

Cooling of semen prior to cryopreservation has been assessed in bulls (Foote and Kaproth, 2002), boars (Guthrie and Welch, 2005), stallions (Backman et al., 2004), and dogs (Santana et al., 2013). In rams, different studies have confirmed that semen can be cooled and maintained at 5 °C for up 48 h, without deleterious effects on the post-thaw sperm quality (Purdy, 2006; Purdy et al., 2010). In addition, chilling ram sperm for 24 h before cryopreservation did not influence the fertility rate, compared to semen frozen by a conventional procedure (Purdy et al., 2010); however, cooling of boar semen before freezing reduces prolificacy in pigs (Guthrie and Welch, 2005).

At present, no studies have assessed the effects of holding time at 4–5 °C prior to cryopreservation on post-thaw buck sperm quality. Therefore, the aim of this study was to define the influence of cool storage before freezing on the quality of frozen–thawed semen samples in Majorera bucks.

2. Materials and methods

2.1. Animals and semen collection

Semen was collected from six Majorera bucks, aged between 3 and 6 years. The animals were housed at the experimental farm of the Veterinary Faculty of Las Palmas (Canary Islands, Spain, 28° N, 23° W) and were fed with a mixture of corn, oat, bran and dehydrated alfalfa; vitamin–mineral corrector and water were offered *ad libitum*. During the breeding season (June–December), semen was collected using an artificial vagina, once a week, during three consecutive weeks.

2.2. Semen assessment

Immediately after collection, the ejaculates were placed into a water bath (36–37 °C) and aliquots were taken to assess the semen quality. Semen volume was recorded directly in the graduated tube. Sperm concentration was estimated with a spectrophotometer (Spermacue®, Minitüb Ibérica, Tarragona, Spain) calibrated for buck semen. Sperm motility was defined with a CASA system (Sperm Vision Lite®, Minitüb Ibérica, Tarragona, Spain). The semen variables included in the analysis were the percentages of total motile spermatozoa, progressive fast spermatozoa (straightness > 80% and path velocity > 60 μm/s), locally motile spermatozoa and non-motile-sperm cells. Straightness measured the departure of the cell path from a straight line and path velocity was defined as the average velocity of the smoothened cell path (μm/s). Acrosome integrity and the percentage of spermatozoa with abnormal morphology were determined using the Spermac® stain (Batista et al., 2011). A minimum of 100 spermatozoa per slide were assessed under a phase-contrast microscope at 1000× magnification. Only ejaculates with a sperm concentration >2000 × 10^6 spermatozoa/mL, a progressive fast spermatozoa >60%, an acrosome-intact spermatozoa >85% and abnormal sperm morphology <20% were included in the present study.

2.3. Semen processing, chilling and semen freezing

After individual evaluation, the ejaculates were pooled and the pooled semen was diluted in washing solution (250 mM Tris, 28 mM glucose, 104 mM citric acid) at 37 °C and centrifuged twice (700 × *g*, 15 min) to eliminate the seminal plasma. Then, the sperm pellet was diluted in a Tris-yolk extender (250 mM Tris, 28 mM glucose, 104 mM citric acid, 12% egg yolk, 0.05% streptomycin, 500 UI penicillin/mL and distilled water to 100 ml) to reach a final concentration of 800 × 10^6 spermatozoa/mL. At this point, semen samples were divided into six aliquots (2 ml/aliquot). The first aliquot (aliquot C, control, 1/6 of the pooled semen) was frozen in liquid nitrogen using a conventional protocol; briefly, 10 min after the first dilution with the Tris-yolk extender, a second diluent (250 mM Tris, 28 mM glucose, 104 mM citric acid, 12% egg yolk, 8% glycerol, 0.05% streptomycin, 500 UI penicillin/mL and distilled water to 100 ml) was added at room temperature (20 °C) to result a final concentration of 400 × 10^6 spermatozoa/mL, 12% egg-yolk and 4% glycerol. Then, the semen was packed into 0.5-ml plastic straws and the straws were chilled from room temperature to 5 °C over 2 h (into a cooler), and were then kept for 2 h more at 5 °C (equilibration period). Finally, the straws were placed horizontally on a rack situated 4 cm above the surface of liquid nitrogen for 15 min and then were plunged into and stored in the liquid nitrogen.

The remaining five aliquots (diluted with Tris–glucose, 12% egg yolk, 800 × 10^6 spermatozoa/mL) were placed into glass vials at room temperature. Then, these five aliquots were cooled at 5 °C for different amounts of time: R1, the semen was cooled for 1 h; R6, the semen was cooled for 6 h; R12, the semen was cooled for 12 h; R24, the semen was cooled for 24 h and R48, the semen was cooled for 48 h. After each chilling period, a second extender was added (Tris–glucose, 12% egg yolk, 8% glycerol) to reach a final composition (400 × 10^6 spermatozoa/mL, 12% egg-yolk and 4% glycerol) similar to aliquot C. Ten minutes after the second dilution, sperm motility was defined using aliquots of each sperm sample. Finally, semen was packed into 0.5-ml plastic straws and frozen in liquid nitrogen, in a similar way as that described for the aliquot C.

2.4. Post-thawing assessment

Thawing was carried out approximately 15 days after freezing; frozen straws were thawed into a water bath at 37 °C for 30 s, and then the semen was poured into a glass tube containing 1 ml of PBS at 37 °C. Sperm motility and the percentages of abnormal sperm cells and acrosome integrity was assessed after warming the semen samples (37 °C) for 5 min; in addition, sperm motility was also valued at 1 and 2 h after thawing. The post-thaw sperm quality was assessed in 20 straws from each of the six experimental groups in three replicates.

2.5. Statistical analysis

The results are presented as mean ± standard error of the mean. Fresh semen data were analyzed using analysis of variance (ANOVA) for repeated measures of SPSS 10.0 (SPSS Inc., Chicago, IL, USA). In addition, an ANOVA was also performed using a general linear model including the effects of the cooling time before freezing (0, 1, 6, 12, 24 and 48 h) and the preservation time (0, 1 and 2) for sperm motility; and the effects of the cooling time before freezing for the percentages of spermatozoa with damaged acrosomes and sperm cells with abnormal morphology. When significant differences were present, the means were compared using the Tukey test. The level of significance was established at $P < 0.05$.

3. Results

The percentages of progressive fast spermatozoa, abnormal spermatozoa and non-damaged acrosomes in fresh semen were very similar ($P > 0.05$) among individual bucks; the only observed differences were in ejaculate volume (male 4 *vs* male 1, $P < 0.05$) and sperm concentration (male 1 *vs* male 4, $P < 0.05$). After pooling, the percentages of progressive fast spermatozoa, normal spermatozoa and non-damaged acrosomes were higher than 60%, 85% and 85%, respectively. Table 1 shows the sperm quality of