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Tolerance of brown bear spermatozoa to conditions of pre-freezing cooling rate and equilibration time

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ABSTRACT

Specific protocols for the cryopreservation of endangered Cantabrian brown bear spermatozoa are critical to create a genetic resource bank. The aim of this study was to assess the effect of cooling rates and equilibration time before freezing on post-thawed brown bear spermatozoa quality. Electroejaculates from 11 mature bears were extended to 100×10^6 spermatozoa/mL in a TES–Tris–Fructose–based extender, cryopreserved following performance of the respective cooling/equilibration protocol each sample was assigned to, and stored at −196 °C for further assessment. Before freezing, after thawing, and after 1 hour's incubation post-thawing at 37 °C (thermal stress test), the quality of the samples was assessed for motility by computer-assisted semen analysis, and for viability (SYBR-14/propidium iodide), acrosomal status (peanut agglutinin–fluorescein isothiocyanate /propidium iodide), and sperm chromatin stability (SCSA) by flow cytometry. In experiment 1, three cooling rates (0.25 °C/min, 1 °C/min, and 4 °C/min) to 5 °C were assessed. After thawing, total motility (%TM) was higher and percentage of damaged acrosomes (%dACR) was lower ($P < 0.05$) for 0.25 °C/min than for 4 °C/min. The thermal stress test data indicated equally poor quality ($P < 0.05$) for the 4 °C/min cooled samples in viability (%VIAB), %dACR, %TM, and progressive motility (%PM). In experiment 2, the effect of a pre-freezing equilibration period at 5 °C for 1 hour (cooling at 0.25 °C/min) was evaluated. Samples kept at 5 °C for 1 hour showed higher ($P < 0.05$) values than the nonequilibrated ones for both thawing (%dACR) and thermal stress test (%VIAB, %TM, and %PM). In experiment 3, samples stored without cooling and equilibration (direct freezing) were compared with the samples cooled at 0.25 °C/min and equilibrated for 1 hour (control freezing). Using thermal stress test, we observed that direct freezing causes damage in viability, acrosomal status, and motility of spermatozoa compared with the control group ($P < 0.05$). In conclusion, our results suggest that slow cooling rates to 5 °C and at least 1 hour equilibration time are necessary for the effective cryopreservation of brown bear sperm.

1. Introduction

The development of a specific protocol for sperm cryopreservation adapted to endangered species (i.e., Cantabrian brown bear (*Ursus arctos*) in Spain [1]) is essential to

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create a genetic resource bank. Diverse factors influence the quality of sperm after freezing–thawing. Studies of bear sperm cryopreservation have mainly been focused on the collection method and extenders (black Japanese bear [2–5]; Hokkaido's brown bear [6]) and handling pre-freezing (black Japanese bear [4,7]; giant panda [8]). In brown bear, our research group evaluated, among other aspects of sperm cryopreservation, glycerol addition timing [9], glycerol concentration and freezing rates [10], and a post-thawing incubation (thermal stress test) [11,12].

The specific adaptation of sperm cryopreservation protocols in endangered species aims for a simplification of procedures and a reduction in times, desirable in fieldwork conditions, to achieve the best seminal quality post-thawing. In this sense, a high cooling rate, the removal of equilibration times or omission of cooling, and equilibration periods should be evaluated.

Spermatozoa cooling can result in a damage known as cold-shock, which results in a loss in viability in a number of spermatozoa and decreased fertilizing capacity [13]. In domestic cat, rapid cooling of spermatozoa to 5 °C induced significant acrosomal damage, but slow cooling maintained a high proportion of spermatozoa with intact acrosomes and motility remained unaffected [14]. In a more recent study, Hermansson and Axnér [15] concluded that cat spermatozoa were tolerant to cold-shock at the rates usually applied. Not all species respond in the same way to cold-shock; spermatozoa from ungulate species seem to be particularly sensitive, whereas those of rabbit, dog, bird, and human show a higher resistance [16].

Previous studies of mammal spermatozoa have analyzed cooling rate effects in sperm quality and very different results can be seen. Some authors yielded more scores with slow cooling rate (<0.5 °C/min): dog [17], Bactrian camel [18], bull [19], and stallion [20]. Other researchers showed better results with rapid cooling (≈4 °C/min): red deer [21,22]. However, in some studies no differences have been found between tested cooling rates: cat [15]. High variability in cold-shock resistance is observed among species. Baudi et al. [23] investigated the effect of cooling rates in sperm from two species of the genus *Leopardus* (ocelot: *Leopardus pardalis*, and tigrina: *Leopardus tigrinus*). They found that the quality of ocelot spermatozoa achieved higher scores with 0.7 °C/min cooling rate, whereas for tigrina sperm this rate was 0.16 °C/min.

Another basic aspect of cryopreservation protocols concerns the pre-freezing equilibration period; reduction or omission of this step would simplify the cryopreservation protocol enormously. The exposure time of spermatozoa to a cryoprotectant before freezing influences post-thawing quality [24]. Equilibration times that are too short prevent the permeation of sufficient amounts glycerol through the spermatozoal membrane to achieve protective concentrations. However, excess exposure could be toxic [25]. The effects of equilibration times on semen cryopreservation remain controversial, and the duration of equilibrium required for satisfactory results in post-thaw sperm quality varies among species: bull, 0.5 hours [26] and 4 hours [27]; Mediterranean buffalo, >5 hours [28]; boar, 2–3 hours [29]; dog, >3 hours [30]. In brown bear spermatozoa, only one study of the equilibration period has been reported [4]. The authors found no difference in post-thawing sperm quality between a treatment with 1 hour cooling plus 1 hour equilibration, and another with 3 hours cooling without equilibration.

In wild species such as buffalo, sperm collection could be carried out in the field where this has little to do with the ideal conditions of a laboratory [24]. Due to this premise, it is necessary to simplify the cryopreservation protocol and even omit the cooling and equilibration steps (direct freezing). Kundu et al. [31] directly froze goat epididymal spermatozoa at different cooling rates by a four-step cooling technique without an equilibration period and observed a marked decrease in motility.

The aim of this work was to shorten and/or simplify the semen handling in the field, and for this we evaluated the following: (1) different cooling rates to 5 °C, (2) the influence of equilibration time, and (3) the effect of direct freezing in the cryopreservation of brown bear ejaculates.

2. Materials and methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise stated.

2.2. Animals and sample collection

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation RD1201/2005, which conforms to the European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03–02/2010).

Sperm samples from 11 sexually mature male (≥6 years old) of brown bear (*Ursus arctos*) were obtained by electroejaculation in two or three sessions, with an interval of at least 3 weeks, during the breeding season (end of April to early July) over two consecutive years. Animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m) and fed with a diet based on chicken meat, bread, and fruits.

The males were immobilized by teleanesthesia, using 750 mg zolazepam HCl + tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg medetomidine (Zalopin, Orion Pharma Animal Health, Finland, 10 mg/mL). After immobilization, the bears were weighed and monitored during anesthesia (pulse, saturation of peripheral oxygen, and breathing). Before electroejaculation, the pubic region was cleaned, the penis washed with sterile physiological saline, and the rectum emptied of feces. The bladder was catheterized during semen collection. Electroejaculation was carried out with a PT Electronics1 electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long, with a diameter of 26 mm. Electric stimuli were applied until ejaculation (6–10 V and 250–300 mA, on average). The ejaculates were collected in a graduated glass tube.