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Brown bear sperm double freezing: Effect of elapsed time and use of PureSperm® gradient between freeze–thaw cycles

Manuel Alvarez-Rodríguez [a,b], Mercedes Alvarez [a,b], Elena López-Urueña [a,b], Carmen Martínez-Rodriguez [a,c], Santiago Borragan [d], Luis Anel-López [e], Paulino de Paz [a,c,*], Luis Anel [a,b]

[a] ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain
[b] Animal Reproduction and Obstetrics, University of León, 24071 León, Spain
[c] Molecular Biology (Cell Biology), University of León, 24071 León, Spain
[d] Cabárceno Park, 39627 Cabárceno, Cantabria, Spain
[e] SaBio IREC (CSIC-UCLM-JCCM), Campus Universitario s. n, 02071 Albacete, Spain

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ABSTRACT

The use of sexed spermatozoa has great potential to captive population management in endangered wildlife. The problem is that the sex-sorting facility is a long distance from the semen collection place and to overcome this difficulty two freeze–thaw cycles may be necessary. In this study, effects of refreezing on brown bear electroejaculated spermatozoa were analyzed. We carried out two experiments: (1) to assess the effects of the two freezing–thawing cycles on sperm quality and to analyze three different elapsed times between freezing–thawing cycles (30, 90 and 180 min), and (2) to analyze the use of PureSperm between freezing–thawing cycles to select a more motile and viable sperm subpopulation which better survived first freezing. The motility, viability and undamaged acrosomes were significantly reduced after the second thawing respect to first thawing into each elapsed time group, but the elapsed times did not significantly affect the viability and acrosome status although motility was damaged. Our results with the PureSperm gradient showed higher values of viability in freezability of select sample (pellet) respect to the rest of the groups and it also showed a significant decrease in the number of acrosome damaged. In summary, the double freezing of bear semen selected by gradient centrifugation is qualitatively efficient, and thus could be useful to carry out a sex-sorting of frozen–thawed bear spermatozoa before to send the cryopreserved sample to a biobank. Given the low recovery of spermatozoa after applying a selection gradient, further studies will be needed to increase the recovery rate without damaging of the cell quality.

Introduction

The establishment of brown bear Genome Resource Banks (GRBs) required the development of a specific methodology for freezing brown bear semen [1] allowing the reproductive potential of each male to be maximized. The collection and handling of samples in wildlife is complex, and thus the different technologies must be adapted to these species.

The Cantabrian brown bear (*Ursus arctos*) is the last indigenous brown bear population in the Iberian Peninsula. Due to habitat loss and population fragmentation (approximately 150 individuals restricted to two isolated populations in the North of Spain) it is considered to be at risk of extinction (Real Decreto 439/1990, Regulation of the National Catalogue of Endangered Species). Unbalanced sex ratios can have negative effects on small population of wildlife, and sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for endangered wildlife [3]. The practical problem of this is that the sex-sorting facility is a long distance from the semen collection position and to resolve this problem two repeated freeze–thaw cycles may be necessary. The benefits of the double freezing technique for endangered species conservation are enormous [5,28].

Studies of sperm re-freezing have been carried out in several species (bull [5], [26]; stallion [7,18]; ram [9]; mouse [4] evaluating the motility and viability of sperm following two freeze–thaw cycles. During the time elapsed between the two freezing–thawing cycles, the thawed sample undergoes changes that can affect sperm viability. Previous experiments showed that the fraction of motile sperm after of first freezing also shown to be more resistant to the secondary freezing than the original population. The bases of such cryoresistance has been associated with a high cholesterol content, high levels of unsaturated fatty acids and low level of lipid peroxidation in sperm membranes [13,14]. When motility,

* Corresponding author at: Molecular Biology (Cell Biology), University of León, 24071 León, Spain. Fax: +34 987291322.
E-mail address: ppazc@unileon.es (P. de Paz).

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viability and acrosome integrity of bull spermatozoa were compared, conventional single freezing was significantly superior to the double freezing [26]. Nevertheless, these studies showed that due to improvements in assisted reproductive techniques, the amount of sperm needed for the insemination has decreased and they could freeze straws with less viable cells concentration in the second cycle without detrimental effect in final fertility.

Sperm selection techniques could be a good alternative to minimize the percentage of damaged spermatozoa from the first cycle but these techniques involve a significant decrease in terms of sperm recovery after the gradient selection [17,30]. Previously, our group has studied the effects of different extenders, dilution rates and factors of centrifugation on the recovery of brown bear spermatozoa and semen quality parameters after thawing have been evaluated [23,25].

Density gradient centrifugation has been widely used to select motile sperm subpopulations and to remove bacterial contamination [26]. PureSperm® (Nidacom, Gothenburg, Sweden) is a product designed for the density gradient centrifugation that selects viable and morphologically intact human spermatozoa in Assisted Reproduction Technology [29]. PureSperm has been previously tested for brown bear sperm and it is suitable for improving spermatozoa qualitative characteristics in fresh and post-thawed semen [24]. In summary, the double freezing of selected spermatozoa by density gradient can increase possibilities in the management of cryopreserved spermatozoa from a Genome Resource Banks.

Thus, the two main objectives of this study were: (a) to assess the effects of the two freezing–thawing cycles on sperm quality and to analyze three different elapsed times between freezing–thawing cycles (30, 90 and 180 min), and (b) to assess the efficacy of using of PureSperm between freezing–thawing cycles to select the sperm subpopulations which better survived first freezing.

Materials and methods

Reagents and animal regulation

All the products were obtained from Sigma (Madrid, Spain), except the Equex STM Paste (Minitüb, Tiefenbach, Germany).

Animals and sample collection

Semen samples from 18 sexually mature male brown bears were obtained by electroejaculation during the breeding season (late April to early July). The animals were housed in a half freedom regime in Cabárceno Park (Cantabria, Spain; 43°21′ N, 3°50′ W, altitude: 142 m), and fed with a diet based on chicken meat, bread and fruits. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to 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). All electroejaculation were performed under general anaesthesia, and all efforts were made to minimize suffering.

The males were immobilized by teleanaesthesia, using 750 mg of zolazepam HCl + tiletamine HCl (Zoletil100®; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin®, Orion Pharma Animal Health, Finland, 10 mg/mL). After immobilization, the males were weighed and monitored (pulse, oxygen peripheric saturation, ECG and breathing). Prior to electroejaculation, the prepucial area was shaved and washed with physiological saline serum, and the rectum was emptied of faeces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average).

To prevent urine contamination or low cellular concentration, the ejaculates were collected as isolated fractions in 15 mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™ , Berlin, Germany) and the pH value was determined (CG 837 pH meter; Schott Instruments, Main, Germany). For each fraction, subjective motility was assessed with a phase contrast microscope (100×) and urospermia evaluated using urea test strips, (DiaSys Ecoline® GmbH, Holzheim, Germany). Fractions with a reduced concentration (<200 × 10^6 - cell/mL), low motility (<50%) or contaminated urine (>80 mg urea/dL) were rejected. All valid fractions of the same electroejaculation were mixed and constituted one ejaculate. The selected samples were centrifuged at 600*g* for 6 min and the pellet was processed according to the experimental design in each assay.

Cryopreservation protocol

The cryopreservation process was carried out following our standard protocol ([2] modified by [10]). The tubes with the 1:1 extended samples (3% glycerol) were put in glasses containing 100 mL of water at room temperature and transferred to a refrigerator, where they were slowly cooled down to 5 °C (−0.25 °C/min). Then, the samples were diluted with the same volume of each extender containing 9% glycerol, to achieve the final glycerol concentration of 6%. Final sperm concentration (100 × 10^6 spermatozoa/mL) was achieved by adding the appropriate volume of each extender to the 6% glycerol. After packaging into 0.25-mL plastic straws and equilibrating for 1 h at 5 °C, the samples were frozen in a programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of two weeks. The straws were thawed by dropping them in water at 65 °C for 6 s.

Experimental design

Experiment 1. Re-freezing effects (30 min). Effects of elapsed time between freezing/thawing cycles on thawed sample (30, 90 and 180 min)

For this experiment, we used 20 ejaculates from 18 males. After thawing, the sperm sample (~1.2 mL of final volume) was divided into three aliquots each of which was maintained at room temperature (~22 °C) for a different time (30, 90 and 180 min). After of the different elapsed times, each sample was processed for a second cryopreservation applying the same procedure as in the first freezing–thawing cycle (see Section Cryopreservation protocol). The samples were evaluated at four times during treatment: (1) Before the first freezing cycle (P1), (2) After first thawing (T1), (3) After the time elapsed at room temperature before the second freezing cycle (P2) and (4) After second thawing (T2).

Experiment 2. Use of PureSperm® between freezing/thawing cycles

PureSperm® gradient was used for selecting 8 brown bear semen samples following the protocol used by our group [24]. Eight straws were thawed and each was divided into four aliquots which were subjected to the following treatments. Control sample (CTL) was stored at room temperature without any experimental process. Centrifuged Control sample (CTL_W) was subjected to the centrifugation process (600*g*/20 min) to evaluate its possible detrimental effect on sperm samples. Pellet sample (PS_P) was the pellet obtained after the semen was treated using PureSperm® density