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Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen

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ABSTRACT

The objective of this study was to evaluate different bovine and canine commercial semen extenders for cryopreservation of brown bear ejaculates and the effect of semen collection directly into extender on sperm agglutination. Semen samples were obtained by electro-ejaculation from 13 adult males. In experiment 1, eleven ejaculates from eight bears were used to evaluate Bioxcell and Andromed as extenders, whereas in experiment 2, nine ejaculates from six bears were used to evaluate Triladyl canine, CaniPro, and Extender 2 as extenders. An extender specifically developed for brown bears (Test-Tris-fructose-egg yolk-glycerol, TTF-ULE/bear) served as a control extender in both experiments. After thawing, total and progressive sperm motility and sperm viability were greater ($P < 0.05$) for TTF-ULE/bear and Andromed extenders than for Bioxcell in experiment 1 and greater ($P < 0.05$) for TTF-ULE/bear extender than for Triladyl Canine, CaniPro, and Extender 2 in experiment 2. In experiment 3, addition of handling extender (TTF-H) to the semen collection tube for eight ejaculates from seven bears resulted in less ($P < 0.05$) sperm agglutination in fresh samples (score 0.5 ± 0.2 vs. 1.8 ± 0.4 in diluted and control samples, respectively) with no effect on pre-freeze and post-thawing semen quality. In conclusion, TTF-ULE/bear is the most suitable extender for brown bear semen cryopreservation, but comparable results can be obtained with the commercial extender Andromed. In addition, collection of ejaculates directly in TTF-H extender decreases sperm agglutination in fresh samples.

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1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is considered at risk of extinction (Real Decreto 439/1990, regulation of the National Catalog of Endangered Species), and the availability to cryopreserve sperm could help preserve biodiversity in this wild species [1,2]. The success of semen cryopreservation depends on several factors, including the initial quality of the semen sample, cryopreservation protocol, and freezing extenders used [3,4]. It is fundamental to develop specific extender and cryopreservation protocols adapted to the characteristics of a particular species [5]. Our group has conducted studies in brown bear to develop optimal sperm cryopreservation technique, including developing a specific semen extender [6],

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evaluating different freezing and glycerol rates [7], timing of glycerol addition [8], and testing different centrifugation protocols [9–11]. Although a bear-specific extender has been successfully used for cryopreservation of sperm [7], assessing the suitability of commercial extenders designed for other species could provide a practical solution for unexpected situations when the specific extenders may not be available.

To optimize semen cryopreservation protocols, other particularities of brown bear ejaculates must also be taken into account. Sperm agglutination is a problem that occurs frequently with brown bear ejaculates [5,6,12], and this phenomenon can interfere with semen assessment and pre-freezing handling and possibly affect freezability [5]. In brown bear, high or very high level of agglutination is observed in 32% of the ejaculates [6]. Thus, on the basis of the fact that agglutination may disappear in boar semen after diluting in extender [13], dilution during ejaculate collection might be used as a strategy to improve the initial quality of seminal samples in brown bears.

The objective of the present study was to evaluate different bovine and canine commercial semen extenders for cryopreservation of brown bear ejaculates. In addition, the effect of semen collection directly into extender on sperm agglutination was also evaluated.

2. Materials and methods

2.1. Animals and ejaculate collection

All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010), and all procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. This study was performed at the Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m). The animals were housed in a half-freedom regime and fed chicken meat, bread, and fruits.

Ejaculates from 13 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July). The males were immobilized using 750 mg of zolazepam HCl and tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin, Orion Pharma Animal Health, Finland, 10 mg/mL), concurrently in the same dart. After immobilization, the males were weighed and monitored (pulse, oxygen saturation and breathing) using a portable veterinary monitor and were transported to a collection room (18 °C–24 °C). Before electroejaculation, the prepucial area was shaved and washed with physiological saline, and the rectum was emptied of feces. 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) using a transrectal probe 320 mm in length and 26 mm in diameter. Electric stimuli were given until ejaculation (10 V and 250 mA, on average) [14]. The ejaculates were collected in graduated glass tubes protected in a double-walled conservation tube filled with water at 30 °C. In experiment 3, each ejaculate was collected in a funnel and a piping system in the form of an “inverted Y” distributing the sample into two tubes with approximately equal volume. The collection of ejaculate was performed as isolated fractions to prevent urine contamination or low sperm concentration. The sample tubes were transferred to the laboratory (22 °C) for analysis and processing. Fractions of reduced concentration ($<200 \times 10^6$ sperm/mL), low motility (<50%), or contaminated urine (>80 mg urea/dL; DiaSysEcoline GmbH, Holzheim, Germany) were discarded. All valid fractions from the same electroejaculation were mixed to constitute a single ejaculate.

2.2. Semen extenders and cryopreservation

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

The Test-Tris-fructose (TTF) base extender was prepared from a N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid solution (300 mOsm/kg) and Tris solution (300 mOsm/kg) mixed to pH 7.1, with 4% final volume of D-fructose solution (300 mOsm/kg). The TTF base solution was double filtered through a cellulose acetate membrane (0.22 μm pore), supplemented with 20% egg yolk and centrifuged (3000× *g*, 30 minutes), discarding the sediment. Then, 6% glycerol (final concentration) and 2% EDTA and 1% Equex Paste (Minitüb, Tiefenbach, Germany) were added. The extender was supplemented with 0.302 mg/mL penicillin G sodium salt and 0.625 mg/mL dihydrostreptomycin sesquisulfate; this constituted the control extender (TTF-egg yolk-glycerol [ULE]/bear [6,7]). The agglutination handling extender (TTF-H) was prepared from the TTF base extender supplemented with 1% egg yolk.

The commercial bovine extenders used in this study included Bioxcell (IMV, L`AigleCedex, France) and Andromed (Minitüb). The commercial canine extenders included Triladyl canine (Minitüb), CaniPro (Minitüb), and Extender 2 [15]. CaniPro was supplied as two separate fractions, one with and one without glycerol; a mixture of equal parts of the two fractions was used in this study. All other extenders were supplied as a single fraction containing glycerol.

Ejaculates were centrifuged at 600× *g* for 6 minutes, the supernatant was removed, and the pellet was divided into aliquots according to the number of treatments. Each aliquot was diluted (1:1, vol/vol) with semen extenders at room temperature; accordingly, glycerol concentration was 3% to 3.5% at this stage. Tubes with diluted semen were placed in beakers containing 100 mL of water at room temperature and transferred to a refrigerator, where they were slowly cooled to 5 °C during approximately 75 minutes. Once at 5 °C, the samples were again diluted 1:1 (vol/vol) with the respective extender to which additional glycerolated extender was added to obtain the final concentration recommended by manufacturer after dilution: TTF-ULE/bear = 6%, Bioxcell = 6.4%, Andromed = 7%, Triladyl canine = 7%, CaniPro = 6%, and Extender 2 = 6%. Finally, the samples were diluted again with the respective original extender to adjust the cell concentration to 100×10^6 sperm/mL. After equilibration at 5 °C for 1 hour, the semen

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