Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Effect of two cooling protocols on the post-thaw characteristics of Iberian ibex sperms

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ARTICLE INFO

Keywords:
Ibex
Semen cryopreservation
Cooling protocols
Sperm quality
sperm head morphometry

ABSTRACT

The rate at which lethal intracellular ice forms during sperm cryopreservation is highly dependent on the cooling protocol. The present work compares two cooling protocols for use with Iberian ibex (*Capra pyrenaica*) sperm by assessing the effects on the motility, viability, and size of frozen-thawed sperm cells. Ejaculates, obtained from six adult ibex males via transrectal, ultrasound-guided massage of the accessory sex glands plus electro-ejaculation if necessary, were cooled via either 1) Protocol 1 (decelerating cooling), involving cooling in liquid nitrogen vapor from 5 °C to −35 °C (40 °C/min), from −35 °C to −65 °C (17 °C/min), and then from −65 °C to −85 °C (3 °C/min); or 2) Protocol 2 (accelerating cooling) involving cooling in a biological freezer from 5 °C to −5 °C (4 °C/min), from −5 °C to −110 °C (25 °C/min), and then from −110 °C to −140 °C (35 °C/min). Compared to fresh ejaculates, sperm quality at thawing was found to be reduced by both protocols ($p < .05$), but especially by Protocol 1. Sperm head size was also significantly reduced by both protocols, although the Protocol 1 sperm heads were also significantly smaller than those of Protocol 2 sperms heads ($p < .05$). In fresh sperm samples, clustering analyses revealed two subpopulations of sperms with different morphometric characteristics, SP1 with larger cells, and SP2 with smaller cells. Both cooling protocols caused reduction in the proportion of SP1 cells, and an increase in the proportion of SP2 cells. In conclusion, the decelerating cooling protocol (Protocol 1) caused greater cryodamage to the sperm cells than the accelerating protocol (Protocol 2).

1. Introduction

Sperm cryopreservation is an essential tool in the maintenance of genetic resources for species conservation [3,35]. However, protocols need to be optimized to ensure sperm samples viability and fertility after thawing. The success of sperm cryopreservation depends on the initial quality of the ejaculate (which can usually be improved through by sperm selection methods, such as dextran swim-up or density-gradient centrifugation [7,43]), the freezing protocol followed [9,33], and the diluents used [24,25]. Efforts are being invested in the search for new additives and cryoprotectants, but the optimization of cooling rates, which can influence sperm quality in a species-specific manner, should, not be forgotten. The rate at which lethal intracellular ice forms during sperm cryopreservation is strongly dependent on the cooling protocol. The optimum protocol may differ between species [26]. During cooling, sperms are subjected to large drops in temperature, resulting in cold shock [51]. Not only are they very sensitive to rapid cooling from room temperature to 5 °C [52], ice crystals formed during freezing can injure the cells (causing damage to their DNA, and membrane breakage [22,30]) as can the progressive increase in osmotic pressure caused by continuing ice formation. Following an optimized cooling curve, however, should minimize cryoinjury [32].

In the Iberian ibex (*Capra pyrenaica*), a model species for sperm cryobiology research in wild ruminants, numerous studies have examined the effectiveness of the sperm collection method [39,41,45], cooling times [34], and the use of different diluents and cryoprotectants [10,42,46]. However, no study has determined the effects of different cooling protocols on the functional and morphometric characteristics of ibex sperms. The optimal freezing procedure thus remains to be established.

The success of the cryopreservation of sperm in part depends on obtaining the best compromise between cell dehydration and ice crystal formation - neither of which are good for cells. During cooling −5 and −10 °C, ice crystals begin to form in the medium in which the sperm cells are suspended. The intracellular medium, however, only becomes supercooled, with no crystal formation. This leads to an osmotic

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https://doi.org/10.1016/j.cryobiol.2018.01.003
Received 19 October 2017; Received in revised form 11 January 2018; Accepted 11 January 2018

imbalance between the intra- and extracellular spaces, and the sperms respond by losing water to the external environment. This leads to their dehydration, with cell volumes reduced to levels that compromise structural viability. In addition, the consequent increase in the concentration of intracellular solutes may cause the plasma membrane proteins to denature. As the temperature falls below −10 °C, intracellular ice crystals are produced that damage cell structures and disorganize and displace the proteins of the plasma and acrosomal membranes. Traditional freezing over liquid nitrogen vapor involves an initial very rapid cooling rate followed by a slower rate [40] (decelerating cooling). While this prevents some of the above dehydration, it has the drawback that it encourages rapid ice crystal formation in the cell. The use of an initially slower cooling rate, followed by a gradually faster cooling (accelerating cooling), might allow water more time to exit the cell [6], and despite the damage involved with this, reduce damage caused by ice crystal formation, thus improving their post-thaw quality [36]. The present work examines the effects of two protocols representing each of the above options on the post-thaw quality and morphometry of Iberian ibex sperm.

2. Materials and methods

2.1. Animals and semen collection

Sperm samples were collected over September–January from six 3-7 year-old ibex males maintained in captivity at the INIA Department of Animal Reproduction (Madrid, 40° 25′N). All were fed Visan K-59 (Visan Ind. Zoot. S.A, Madrid, Spain) supplemented with barley grain, barley straw, and dry alfalfa. Water and vitamin/mineral blocks were available *ad libitum*. All handling procedures were approved by the INIA Ethics Committee and were performed in accordance with the Spanish Policy for Animal Protection RD53/2013 which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments. During all manipulations, the eyes were covered with a mask to reduce stress.

Sperm was collected by transrectal ultrasound-guided massage of the accessory sex glands (TUMASG), and electroejaculation if required. For this, the animals were anaesthetized using intramuscular detomidine (138 mg/kg; Domosedan, Pfizer Inc., Amboise, France), ketamine hydrochloride (1.3 mg/kg; Imalgene-1000, Rhône Mérieux, Lyon, France) and tiletamine-zolazepam (1.3 mg/kg; Zoletil-100, Virbac España S.A., Barcelona, Spain). Isoflurane (Isobavet, Intervet Schering Plough Animal Health, Madrid, Spain) was used to maintain anesthesia [44]. With the anaesthetized animals in the lateral recumbent position, the hair was clipped from around the penis, the surrounding area cleaned, and the penis held protruded by wrapping a gauze behind the glans. This prevents the collapse of the urethra during ejaculation. The protruded penis was cleaned with a Tris/citric acid/glucose sperm-washing solution (345 mOsm, pH 6.8) [11].

Ultrasonic examination of the bulbourethral glands, seminal vesicles, and ampulla of the vas deferens was performed using real-time transrectal ultrasonography employing a 7.5 MHz linear array probe (Prosound 2, Aloka CO., LTD, Tokyo, Japan) to ensure that the animal could provide a sperm sample. For TUMASG, an ultrasonographic probe was placed on the ampulla of the vas deferens, and a back-and-forth motion applied to favor sperm expulsion. This was alternated with vigorous massage of the bulbourethral glands, while maintaining pressure on the symphysis pubis and moving the fingertip in a caudal direction at all times. The penile, perineal and pelvic parts of the urethra were simultaneously massaged to push the ejaculatory fluid through the urethra into a funnel-topped 3 mL glass collection tube. The procedure was repeated several times until ejaculation.

If the animal did not ejaculate in 15–20 min of applying TUMASG, electrical stimuli (0.1 or 0.2 mA lasting 5 s) were provided using a Lane Pulsator IIIZ electroejaculator (Lane Manufacturing Inc., Denver, CO., USA) (with intermittent breaks for TUMASG) following the method of Santiago-Moreno et al. 2013 [40]. All the males ejaculated only with TUMASG or applying 1–3 electric pulses at most. Ejaculates with sperm motility less than 50% were discarded. A total of 18 ejaculates, 3 ejaculates per ibex, were collected.

After ejaculation, ultrasound scanning of the vas deferens ampullae was performed to verify emptying. After sperm collection, anesthesia was reversed with intravenous and intramuscular yohimbine hydrochloride (Sigma-Aldrich, Germany) (0.7 mg/kg - half of the dose intravenous and half intramuscular).

2.2. Evaluation of fresh sperm quality

The volume of each ejaculate was measured using a micropipette (Gilson, Villiers Le Bel, France), and total sperm concentrations calculated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility, viability, acrosome integrity and morphometry were assessed for each sample to determine *in vitro* sperm quality. Sperm motility was evaluated by subjective [11] and objective tests by computer-aided sperm analysis system (CASA) using Sperm-Class Analyzer® v.4.0 software (Microptic S.L., Barcelona, Spain) [40]. Previously sperm motility assay, the samples were diluted over the range 1:60 to 1:100 (% v/v) as required in a Tris–citric acid–glucose washing medium (345 mOsm, pH 6.8). Sperm viability and acrosomal status were analyzed simultaneously by fluorescence microscopy using a fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) [48]. Four subpopulations of sperms can be distinguished in this way: live sperms with intact acrosome, live sperms with damaged acrosome, dead sperms with an intact acrosome, and dead sperms with a damaged acrosome. Sperm head morphometric analysis was performed automatically using Sperm-Class Analyzer® v.5.3.0.1 software (Microptic S.L., Barcelona, Spain): frosted microscopic slides were prepared for each sample, placing 5 mL of semen at the end of the slide and dragging the drop across with another. Smears were air-dried and stained using a Diff-Quick® staining technique (Medion Diagnostics AG, Düdingen, Switzerland) [17]. The morphometric dimensions for sperm head parameters [length (L), width (W), area (A), perimeter (P)] were acquired for 100 images.

2.3. Sperm selection by Capripure® density gradient centrifugation

Before freezing, all sperm samples were subjected to sperm selection using Capripure® density gradient centrifugation as reported by Santiago-Moreno et al., 2014 [43]. Sperm motility, viability, acrosome integrity and morphometry were then again measured as above.

2.4. Freezing procedures

The sperm pellets obtained in the above centrifugation were divided into two aliquots and suspended in an extender composed of Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM plus 6% egg yolk (v/v) and 5% glycerol (v/v) (TCG-ey-GLY) to a final concentration of 100×10^6 sperm/mL. Both samples were then placed in a beaker with 30 mL of water at room temperature, and maintained at this temperature for 5 min before transfer to a refrigerator at 5 °C where they were maintained for a further 3 h: 1 h of refrigeration time and 2 h of equilibration time. Aliquots of these samples were then loaded into 0.25 mL French straws (IMV, L'Aigle, France) and subjected to one of two freezing protocols:

Protocol 1: Half the straws were frozen by holding them in nitrogen vapor 5 cm above liquid nitrogen for 10 min, and then plunging them into the latter, providing the following decelerating cooling rate: from 5 °C to −35 °C at 40 °C/min, from −35 °C to −65 °C at 17 °C/min, from −65 °C to −85 °C at 3 °C/min, and then transfer into liquid nitrogen to cool to −196 °C. The procedure was standardized to ensure such cooling and checked using a Ventix® K/J/T thermometer (Ventix, China) equipped with a probe resistant to freezing.