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Effect of sex-sorting and cryopreservation on the post-thaw sperm quality of Iberian red deer spermatozoa

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ABSTRACT

This study investigated the effect of sex-sorting and cryopreservation on post-thaw characteristics and fertility of red deer (*Cervus elaphus*) sperm for the first time. Semen was collected by electroejaculation from 10 mature stags during the breeding season, and each ejaculate split into four experimental groups: Bulk sorted spermatozoa, sorted but not sexed (BSS); sorted high purity X-spermatozoa (XSS); sorted high purity Y-spermatozoa (YSS); and, control non-sorted spermatozoa (NS). Following, all samples were frozen over liquid nitrogen. Two straws per stag and sample type were analyzed immediately post-thaw and following a 2-h incubation period at 37 °C. Post-thaw total motility (TM) as assessed by CASA was not different ($P < 0.05$) among NS, BSS and YSS sperm. For XSS, post-thaw TM was lower (39%, $P < 0.05$) than that for NS (54%) or BSS (50%), but similar ($P > 0.05$) to that of YSS (47%) sperm. The percentage of apoptotic spermatozoa as assessed by PI/YO-PRO-1 and flow cytometry analysis, was higher (17%, $P \leq 0.05$) for XSS sperm than NS (12%), BSS (13%) and YSS (14%) sperm. Following incubation there were no differences ($P > 0.05$) in TM or percent apoptosis among treatments. Post-thaw chromatin stability calculated as the DNA fragmentation index (%DFI) was similar among treatments; following incubation % DFI increased in all except YSS, which displayed the lowest value ($P < 0.05$). Artificial insemination of synchronized hinds yielded 44, 52 and 62% delivery rates for YSS, NS and standard frozen-thawed sperm, respectively ($P < 0.05$). Notably, 93 and 55% of fawns born were males for the YSS and NS spermatozoa, respectively ($P < 0.05$). In summary, Y-sorted sperm displayed acceptable post-thaw sperm evaluation parameters and the expected offspring sex ratio. More studies are needed to understand the source of sperm damage that may compromise the fertility of Y-sorted red deer sperm.

1. Introduction

Iberian red deer belong to a deer subspecies that only inhabit the Iberian Peninsula. In recent years, there has been an increased trend towards deer farming in Spain, becoming a viable alternative to raising more conventional livestock species. Generally speaking, these are farms in which the main financial profits rely on antler trophies. Since males have the highest economic value, the possibility of sex selection at breeding would result in considerable cost savings and avoid the massive slaughter of females.

Assisted reproduction technologies have provided the opportunity to choose the offspring sex in domestic livestock species. For instance, with embryo production by MOET (multiple ovulation and embryo transfer) this can be achieved by determining the sex of preimplantation embryos and then selecting those of interest for transfer. However, the low efficiency of this technique derived from discarding embryos of unwanted sex limits its use. Therefore, the only accurate and potentially cost effective method for offspring sex selection is sperm sexing by flow cytometry [1]. Indeed, preselection of spermatozoa based on the different DNA amount between the X and Y chromosomes has become one of the most important reproductive technology advances in livestock species

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[1–3].

The main drawback of this technique is the low fertility yield, which has often been attributed to the low insemination dose used with sex-sorted sperm. However, increasing the number of spermatozoa to numbers comparable to those used with non-sexed sperm did not improve pregnancy rates in heifers [4]. In addition, the flow cytometry procedure is not innocuous given that sperm are exposed to stressors such as fluorescent dyes, high dilution rate, mechanical injury, laser illumination and subsequent passage through an electric field for sex-sorting [2]. Capacitation-like changes have been observed after sorting of ram, bull and boar spermatozoa [5–8] reducing their fertilizing lifespan in vivo [9]. Unlike the capacitation status, however, motility, as well as acrosome and DNA integrity of sorted sperm are usually high because prior to the procedure cells are stained with a food dye that discriminates nonviable cells [10] thus eliminating them from the sorted pool. Nevertheless, some of the negative effects of sorting on sperm quality measures may not be immediately evident, but only after an incubation period [11–13].

Because sex-sorted spermatozoa are usually cryopreserved for logistic reasons, this aggravates any existing cell damage, further impacting on fertility [14]. Cryopreservation alone may lead to capacitation-like changes, motility impairment and oxidative damage [14]. Hence the low levels of antioxidants present in the sperm sorting medium, paired with the high production of reactive oxygen species during the sorting and freeze-thaw processes may further compromise the fertilizing ability of sex-sorted sperm samples.

In deer, few studies have addressed sex-sorting of sperm despite an increased interest by the industry. When Sika deer sperm were sorted prior to insemination of Wapiti hinds, calving rates were close to half of those obtained with non-sorted sperm from the same proven stags [15]. In that study, however, sperm characteristics were not assessed and the reasons for the low fertility were unknown. Another laboratory compared the effect of sex-sorting on DNA fragmentation in cryopreserved sperm from white-tailed deer stags [16]. Interestingly, in this study rates of DNA damage were lower in sorted than non-sorted sperm, although samples were only evaluated immediately after thawing and some detrimental changes might have become evident over time. Therefore, the objective of this study was to assess sperm quality at different steps during the sex-sorting procedure as well as to examine the fertility rates obtained with sex-sorted-frozen-thawed samples from Iberian red deer.

2. Materials and methods

2.1. Reagents and media

Fluorescence probes YO-PRO-1, Hoechst 33342 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and PNA-FITC were acquired from Sigma (Madrid, Spain), and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Fluorescent probe stock solutions were prepared in DMSO (50 μM YO-PRO-1; 1 mM Mitotracker Deep Red) or water (7.5 mM PI; 0.2 mg/mL PNA-FITC; 1 mg/mL acridine orange; 25 mg/mL Hoechst 33342) and stored in the dark at −20 °C, except for Hoechst 33342 (H42) and acridine orange which were stored at 5 °C. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA) or Becton Dickinson (San Jose, CA, USA).

The collecting medium used during sorting was a Tris-Citrate-Glucose (TCG) (pH: 7.3 and pOsm: 380 mOsm/kg) containing: glucose (250 mM), sodium citrate (12 mM), EDTA (1.6 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk at 5% (V/V) penicillin (0.7 mM), and streptomycin (1.14 mM). The ejaculate washing medium was the transport extender with the addition of 2.5% (V/V) egg yolk. The transport medium was a Tris-Citrate-Fructose (TCF) (pH: 7.3 and pOsm: 330 mOsm/kg) containing: Tris (213 mM), citric acid monohydrate (71.83 mM), fructose (55.51 mM), egg yolk at 20% (V/V), penicillin (0.7 mM), and streptomycin (1.14 mM). The working medium for running samples through the flow cytometer was the bovine gamete medium (BGM-3) and was composed of: NaCl (87 mM), KCl (3.1 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.4 mM), NaH_2PO_4 (0.3 mM), HEPES (40 mM), sodium lactate (21.6 mM), sodium pyruvate (1 mM), kanamycin (0.017 mM), phenol red (28.22 mM) and BSA (6 mg/mL) (pH 7.5). Solutions for SCSA (Sperm Chromatin Structure Assay) were prepared as follows [17]: TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4); acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4); and, acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, 6 μg/mL acridine orange pH 6.0). These solutions were kept at 5 °C in the dark.

2.2. Ejaculate collection and sperm sample preparation

One ejaculate per male was obtained from 10 mature stags during the breeding season (mid-September). Animals were housed in a semi-free ranging regime at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in accordance with Spanish law in regards to the care and use of research animals (RD 53/2013) conforming to European Union regulation 2010/63. Semen collection by electroejaculation was carried out as described Martínez-Pastor et al. [18]. Briefly, males were anesthetized with 0.75 mg/kg of Xylazine (Rompun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of feces and the prepucial area was shaved and washed with physiological saline solution. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The electroejaculation regime consisted of a consecutive series of 5 pulses of similar voltage and separated by 5 s. Initially, 1 V was applied that was then progressively increased at the next series until reaching a maximum of 5 V. Semen was collected by fractions in graduated glass tubes. We discarded the fractions with urine contamination, that is, positive to Urea Test Strips (Diagnostic Systems GmbH, Holzheim, Germany). Fractions with total sperm motility under 80% were also discarded.

Semen was diluted 1:3 in TCG 2.5% egg yolk and then centrifuged at 600 × *g* for 5 min. The supernatant was removed and sperm concentration of the pellet was assessed using a hemocytometer (Bürker chamber; Brand Gmbh, Wertheim, Germany), after diluting an aliquot of the sample in a glutaraldehyde solution (5 μL of sample in 500 mL of 2% glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). Then, sperm aliquots were diluted to a concentration of 800 × 10^6 sperm/mL in TCF medium supplemented with 20% (v/v) of egg yolk and transported to the sorting facility (about 8 h at 17 °C). Upon arrival to the laboratory each of the sperm samples was split into two aliquots, for control (non-sorted sperm or NS) and sperm sorting (bulk sorted sperm or BSS; X-enriched sperm or XSS; and, Y-enriched sperm or YSS). Sperm samples for sorting were further diluted to 100 × 10^6 sperm/mL with TCG (0% egg yolk) medium and stained with 2.6 μL of H-42 (Stock solution: