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Sperm concentration at freezing affects post-thaw quality and fertility of ram semen

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Abstract

We have investigated the effect of sperm concentration in the freezing doses 200, 400, 800, and 1600 × 10^6 mL^{-1} on the post-thaw quality and fertility of ram semen. Semen was collected from seven adult Churra rams by artificial vagina during the breeding season. The semen was diluted in an extender (TES-Tris-fructose, 20% egg yolk, and 4% glycerol), to a final concentration of 200, 400, 800, or 1600 × 10^6 mL^{-1} and frozen. Doses were analyzed post-thawing for motility (computer-assisted sperm analysis system [CASA]), viability, and acrosomal status (fluorescence probes propidium iodide [PI]/peanut agglutinin conjugated with fluorescein thiocyanate (PNA-FITC), SYBR-14/PI [Invitrogen; Barcelona, Spain] and YO-PRO-1/PI [Invitrogen; Barcelona, Spain]). Total motility and velocity were lower for 1600 × 10^6 mL^{-1} doses, while progressive motility and viability were lower both for 800 and 1600 × 10^6 mL^{-1}. The proportion of viable spermatozoa showing increased membrane permeability (YO-PRO-1+) rose in 800 and 1200 × 10^6 mL^{-1}. Intrauterine inseminations were performed with the 200, 400, and 800 × 10^6 mL^{-1} doses at a fixed sperm number (25 × 10^6 per uterine horn) in synchronized ewes. Fertility (lambing rate) was similar for semen frozen at 200 (57.5%) or 400 × 10^6 mL^{-1} (54.4%), whereas it was significantly lower for 800 × 10^6 mL^{-1} (45.5%). In conclusion, increasing sperm concentration in cryopreserved semen, at least at 800 × 10^6 mL^{-1} and more, adversely affects the postthawing quality and fertility of ram semen.

Keywords: Ram; Sperm cryopreservation; Sperm concentration; Sperm quality; Fertility

1. Introduction

The efficiency of the cryopreservation of ram semen must be improved before widespread application of artificial insemination (AI) in sheep. Acceptable results have been achieved so far using frozen/thawed semen [1–4], but its general use is restricted due to the need of using intrauterine insemination by laparoscopy. Otherwise, AI with frozen semen yields variable and often low fertility results, if applied by vaginal-cervical insemination [4–10]. Another disadvantage of vaginal AI is the high number of spermatozoa required per insemination (100–400 × 10^6 spermatozoa/dose), whereas laparoscopic AI requires lower sperm numbers (25–50 × 10^6 spermatozoa/dose) [4,11,12].

In fact, the effect of sperm dose in the cryopreservation of ram semen has been little explored. To our knowledge, the only study was performed by

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D'Alessandro, et al. [11], who tested two types of diluents (milk-lactose-egg yolk and Tris-fructose-egg yolk), freezing at six different sperm concentrations (50, 100, 200, 400, 500, and 800 $\times$ 10^6 mL^{-1}). They found a variable sperm quality among 50 and 500 $\times$ 10^6 mL^{-1}, but freezing at 800 $\times$ 10^6 mL^{-1} clearly lowered it. They also performed laparoscopic intrauterine insemination with thawed semen, but they did not achieve significant figures. That study showed that freezing ram spermatozoa at concentrations much higher than those used as standard (800 $\times$ 10^6 mL^{-1}) could be detrimental. However, these authors did not reach to definitive conclusions, possibly due to the lack of power in their analyses and to the presence of confounding factors. Although their results suggest a negative effect of increasing sperm concentrations, that trend was not clear. Several studies in different species support this hypothesis. Nascimento et al. [13] evaluated stallion semen doses frozen at different concentrations: 100, 200, and 400 $\times$ 10^6 mL^{-1}, in 0.5-mL and 0.25-mL straws. Those authors found that sperm motility decreased with sperm concentration. Similarly, Peña and Linde-Forsberg [14], evaluated the effect of freezing dog semen at four different sperm concentrations (50, 100, 200, and 400 $\times$ 10^6 mL^{-1}), in 0.5-mL straws finding sperm motility and viability after thawing was significantly lower in samples frozen at 400 $\times$ 10^6 mL^{-1}.

Increasing the sperm concentration might improve vaginal AI in sheep, by allowing more spermatozoa per dose. Paradoxically, this increase could drive to the opposite effect, if high sperm concentration at freezing would decrease sperm quality. Therefore, we aim at confirming and improving the findings of D'Alessandro et al [11]. It is important to confirm and enhance these findings, to improve sheep AI. Thus, the objective of this study is to assess the post-thawing sperm quality and fertility of ram semen frozen in different concentrations (200, 400, 800, and 1600 $\times$ 10^6 mL^{-1}) with a possible practical use for AI in sheep. In this study we have tried to avoid confounding factors—equalizing the number of spermatozoa inseminated—and we have used sensitive techniques (computer-assisted sperm analysis system [CASA] and flow cytometry), to reach more definitive conclusions, and the fertility study was carried out using sheep groups large enough to attain a high statistical power.

2. Materials and methods

2.1. Reagents

Reagents were obtained from Sigma (Madrid, Spain), except fluorescence probes SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1, which were acquired from Invitrogen (Barcelona, Spain).

2.2. Animals and sperm collection

We used seven adult males (2–9 years old) of the Churra breed, of proven fertility and trained for semen collection by artificial vagina. Ejaculates were collected by artificial vagina at 40 °C (Minitüb, Tiefenbach, Germany), and the tubes were maintained at 35 °C during the initial evaluation of semen quality. The volume was estimated using the graduation marks of the collection tube. Mass motility was assessed by microscopy (warming stage at 37 °C, magnification ×40; score: 0–5; Labophot 2, Nikon, Tokyo, Japan), and the sperm concentration was assessed by the photocolorimetric method at 540 nm (Spectronic 20, Baush and Lomb, Madrid, Spain), on a specific calibrated scale. Only ejaculates of good quality were used and frozen (volume: $\geq$ 0.5 mL; mass motility: $\geq$ 4; sperm concentration: $\geq$ 3000 $\times$ 10^6 mL^{-1}).

The seven males yielded 18 good-quality ejaculates, which were divided into four aliquots and frozen at four different sperm concentrations (200, 400, 800, and 1600 $\times$ 10^6 mL^{-1}), obtaining a total of 679 straws. Semen collection was performed from September to November (within the breeding season, which spans from July to December). Four males yielded three good-quality ejaculates, whereas the remaining three yielded two good-quality ejaculates.

2.3. Cryopreservation protocol

Semen was diluted with the same volume (1:1) of freezing extender. The freezing extender was of our own design (UL) [3], consisting of a TTF medium (TES-Tris-fructose, 320 mOsm/kg, pH 7.2) supplemented with 10% egg yolk and 4% glycerol. The sample was then refrigerated in a cold room at 5 °C for an average of 2 h, until the samples reached a temperature of 5 °C. At that point, the sample was divided among four tubes, to which more extender was added to obtain a concentration of 1600, 800, 400, or 200 $\times$ 10^6 sperm/mL. Samples were packed into 0.25-mL plastic straws and equilibrated for 1 h at 5 °C. Then, the straws were frozen using a programmable biofreezer (Kryo 10 Series III; Planer Plc, Sunbury-On-Thames, UK) using a rate of −20 K/min down to −100 °C. The straws were kept in liquid nitrogen containers and stored for a minimum of 2 mo until analysis. Thawing was carried out in a water bath at 65 °C for 6 sec. Sperm quality parameters were evaluated immediately after thawing.