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Theriogenology

Theriogenology xx (2011) xxx

www.theriojournal.com

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

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Received 1 July 2011; received in revised form 3 October 2011; accepted 8 October 2011

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]/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.

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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 D'Alessandro, et al. [11], who tested two types of

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0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2011.10.013

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.

2.4. Spermatozoa evaluation

The assessment of motility parameters was carried out using CASA (ISAS v. 1.1; Proiser, Valencia, Spain). Samples were diluted ($10–20 \times 10^6$ cells/mL) in the same TTF medium with 320 mOsm/kg, and warmed on a 37 °C plate for 5 min. Then, a 5-μL drop was placed into a Makler counting cell chamber (10-μm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at magnification ×10 (negative phase-contrast) in a microscope (Eclipse E400, Nikon) with a warmed stage (38 °C). The standard parameter settings were set at 25 frames/sec, 20 to 90 μm^2 for head area and curvilinear velocity (VCL) > 10 μm/sec to classify a spermatozoon as motile [15]. At least five sequences or 200 spermatozoa were saved and analyzed afterward. Reported parameters were VCL (μm/sec), linearity (LIN, %), and amplitude of lateral head displacement (ALH, μm). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 μm/sec, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 μm/sec, and straightness (STR) > 80% (also provided by the system).

2.5. Sperm viability and acrosome status

Viability and acrosomal status were assessed simultaneously using florescence probes and flow cytometry, according to methods described previously [16]. Briefly, samples were diluted in phosphate-buffered saline (PBS) at 5×10^6 spermatozoa/mL, and incubated for 15 min with 24 μM of propidium iodide (PI) and 1 μg/mL of PNA-FITC (peanut agglutinin). PI stains membrane-damaged spermatozoa red, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (nonviable sperm, intact acrosome), green (viable sperm, damaged acrosome), red and green (nonviable sperm, damaged acrosome), or not stained (viable sperm, intact acrosome). As a caveat, the PNA-FITC stain may have a low percentage of false negatives, because spermatozoa with a completely lost acrosome cannot be stained by PNA-FITC. The percentage of spermatozoa with damaged acrosomes (ACR) was calculated as the sum of viable and nonviable PNA+ spermatozoa.

To evaluate sperm viability, we used the double stain SYBR-14/PI. Sperm samples were diluted with phosphate-buffered saline down to 5×10^6 sperm/mL, and incubated with 24 μM PI and 100 nM SYBR-14. The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to live spermatozoa (green), moribund spermatozoa (red + green), and dead spermatozoa (red).

YO-PRO-1/PI was used distinguish three populations of sperm: sperm nucleus with red fluorescence (PI+, dead), spermatozoa with green nucleus indicating intracellular YO-PRO-1 (increased membrane permeability), and unstained spermatozoa (viable). The diluted sample was stained with 100 nM of YO-PRO-1 and 24 μM of PI, and then incubated at 37 °C for 10 min before being analyzed by flow cytometry. In this analysis, we also calculated the ratio (RATIO) among the proportion of spermatozoa with increased membrane permeability (PI-/YO-PRO-1+) and the proportion of PI-spermatozoa (sum of YO-PRO-1− and YO-PRO-1+).

Evaluation of flow cytometer parameters was carried out using a FACSCalibur flow citometer (Becton Dickinson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Calibration was carried out periodically using standard bead (Calibrites; Becton Dickinson System). Data corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10 000 spermatozoa were recorded for each stain combination.

2.6. Insemination procedures

A total 762 adult Churra ewes were used and distributed into three experimental groups (200, 400, and 800×10^6 mL^{-1}) during the breeding season. The 1600×10^6 mL^{-1} treatment showed a clear detrimental effect in the in vitro tests, and it was not included in the fertility trials. The ewe number was estimated through a power analysis, taking into account D'Alessandro et al. [11] results, to detect a difference of at least 13 points in fertility rates, with a statistical power of 0.9 and a significance level of 0.05 (total number of females: 745). These females were subjected to treatment for estrus induction and synchronization using intravaginal sponges with 40 mg fluorogestone acetate for 14 days. Then, the sponges were removed and the ewes were treated with 500 IU of eCG (im). Laparoscopic inseminations were performed by two experienced technicians between 62 and 64 h after the removal of the sponges. The animals, having fasted for the previous 24 h, were placed on a special cradle (IMV) adjusted at an inclined plane (45°). The abdominal area in front was shaved and cleaned. Then, two portals (for vision and manipulation/injection) were inserted by performing a pneumoperitoneum (CO_2). The semen, placed in a special applicator (transcap, IMV), was injected under visual inspection into both uterine horns

Table 1
Post-thawing motility (CASA) for the four sperm concentrations.

Motility variables	Concentration ($\times 10^6$ mL^{-1})			
	200	400	800	1600
TM (%)	65.2 ± 4.5^{a}	63.4 ± 3.5^{a}	58.3 ± 4.2^{a}	41.3 ± 4.6^{b}
PM (%)	39.7 ± 3.5^{a}	35.9 ± 2.4^{ab}	33.0 ± 2.9^{b}	22.2 ± 2.4^{c}
VCL (μm/sec)	120.3 ± 4.0^{ab}	123.7 ± 5.1^{ab}	126.8 ± 4.6^{a}	107.6 ± 3.3^{b}
LIN (%)	63.5 ± 1.6	62.3 ± 1.7	63.0 ± 1.5	59.2 ± 1.2
ALH (μm)	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.1 ± 0.1

Results are shown as mean ± SD. Different superscripts in the same row indicate significant differences among concentrations ($P < 0.05$).
ALH, amplitude of the lateral head movement; CASA, computer-assisted sperm analysis system; LIN, linearity index; PM, progressive motility; TM, total motility; VCL, curvilinear velocity.

(0.12 mL per horn). Sperm concentration was equalized to 200 $\times$ 10^6 mL^{-1} just before insemination, using freezing extender, thus 25 $\times$ 10^6 spermatozoa were applied per horn in the three treatments. Fertility results were noted as lambing rates (percentage of lambing ewes at 137–154 day postinsemination with respect to the total number of inseminated ewes).

2.7. Statistical analysis

Statistical analyses were carried out using the R statistical package, version 2.13.0 (http://www.r-project.org). Data were fitted to linear mixed-effect models (lmer package) by maximizing the log-likelihood (ML method) [17]. Sperm concentration (four levels) was included in the fixed part of the model, whereas male and ejaculate within male were included in the random part of the model. A pairwise comparison among sperm concentrations was performed whenever the effect of sperm concentration was significant, using Tukey contrasts. Fertility results were analyzed by logistic regression. Odd ratios (OR) and 95% confidence intervals (CI) were generated during the logistic regression. Results are given as mean ± SEM.

3. Results

The results of this experiment are shown in the Table 1. The CASA parameters TM, PM, and VCL did not show significant differences between the concentrations 200, 400, and 800 $\times$ 10^6 mL^{-1}, but the highest concentration used in this experiment (1600 $\times$ 10^6 mL^{-1}) yielded significantly lower results (TM and PM: $P < 0.001$ for 200, 400, and 800 vs. 1600; VCL: $P = 0.024$ for 200 vs. 1600, $P = 0.002$ for 400 vs. 1600, and $P < 0.001$ for 800 vs. 1600). Mean values of LIN and ALH were not significantly different among concentrations.

The analysis of physiological parameters using fluorescence probes showed that the highest concentration (1600 $\times$ 10^6 mL^{-1}) yielded significantly lower viability (Figs. 1, 2, and 3) and a higher "apoptotic ratio" (Fig. 3), comparing with the other concentrations. Contrarily, the proportion of damaged acrosomes was little affected by sperm concentration, with no significant differences detected among concentrations (Fig. 1).

The proportions of the subpopulations obtained from the SYBR-14/PI and YO-PRO-1/PI stains varied among concentrations, as shown in Figures 2 and 3. The viable subpopulation according to both stains showed its highest value for 400 $\times$ 10^6 mL^{-1}, with 800 and 1600 $\times$ 10^6 mL^{-1} significantly lower, especially the latter. Interestingly, while the percentages of dead (PI+) spermatozoa showed little changes among concentrations (being significantly higher for 1600 $\times$ 10^6

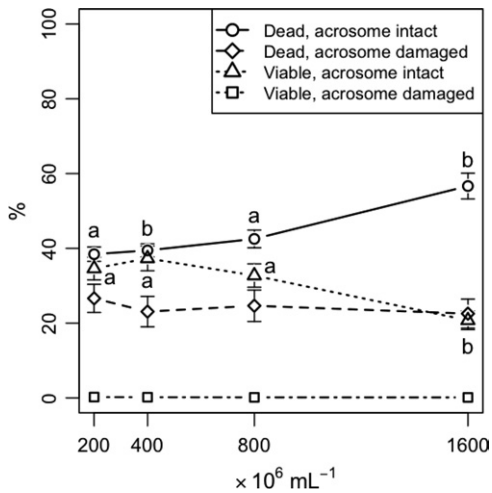

Fig. 1. Mean ± SEM of four sperm populations obtained from PNA-FICT/PI (propidium iodide) (Invitrogen; Barcelona, Spain) stain for each of the four concentrations tested, analyzed after thawing: dead with intact acrosome (PI+/PNA−); dead with damaged acrosome (PI+/PNA+); viable with inctact acrosome (PI−/PNA−); viable with damaged acrosome (PI−/PNA). Different letters indicate differences between sperm concentrations for each sperm population ($P < 0.05$).

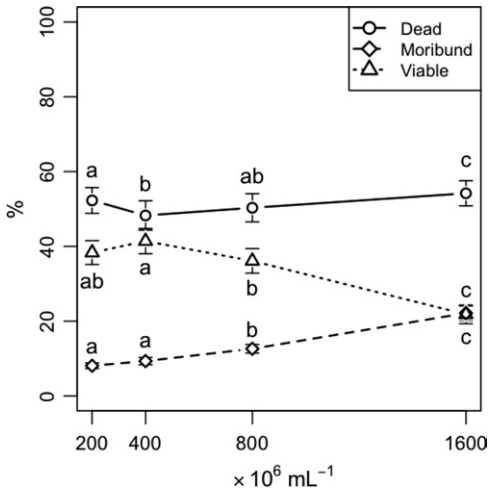

Fig. 2. Mean ± SEM of the three sperm populations obtained from the SYBR-14/propidium iodide (PI) (Invitrogen; Barcelona, Spain) stain for each of the four sperm concentrations: viable (SYBR-14+/PI−), moribund (SYBR-14+/PI+), and dead spermatozoa (SYBR-14−/PI+). Different letters indicate differences between sperm concentrations for each sperm population ($P < 0.05$).

mL^{-1}), the percentages of moribund (SYBR-14+/PI+) and spermatozoa with increased membrane permeability (YO-PRO-1+/PI−) increased significantly when

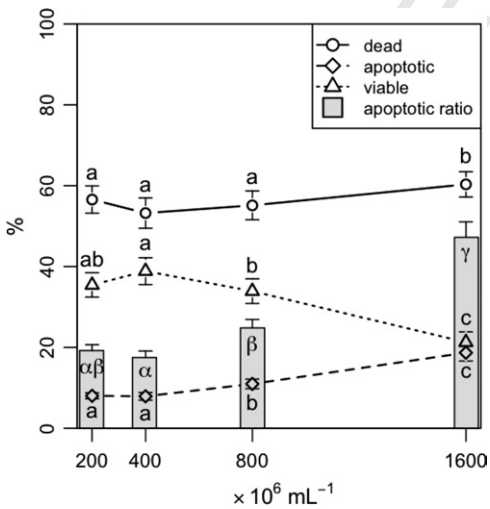

Fig. 3. Mean ± SEM of the sperm populations obtained from the YO-PRO-1/propidium iodide (PI) (Invitrogen; Barcelona, Spain) stain for each of the four sperm concentrations: viable (YO-PRO-1−/PI−), increased membrane permeability ("apoptotic", YO-PRO-1+/PI−), and dead spermatozoa (YO-PRO-1+/PI+). The "apoptotic ratio" (YO-PRO-1+/total PI-spermatozoa) is shown as columns. Different letters indicate differences between sperm concentrations for each sperm population, latin letters for YO-PRO-1/PI subpopulations and greek letters for the "apoptotic ratio" ($P < 0.05$).

Table 2
Fertility of ewes after intrauterine insemination with semen doses frozen at different sperm concentrations (lambing rates).

Concentration ($\times 10^6$ mL^{-1})	Lambing rate (%)	200 vs. higher concentration	
		Odds ratio (95% CI)	P value*
200	154/268[a] (57.5)	Referent†	—
400	135/248[a] (54.4)	0.88 (0.62–1.25)	0.489
800	112/246[b] (45.5)	0.62 (0.44–0.88)	0.007

Insemination dose was fixed at 25×10^6 spermatozoa per uterine horn in all cases. Odds ratios are given taking 200×10^6 mL^{-1} as the reference group. Fertility values with different superscripts differ $P < 0.05$ (χ^2 test).
CI, confidence interval.
* Wald χ^2 for sperm concentration from logistic regression model.
† Reference group for odds ratio.

freezing at 800×10^6 mL^{-1} (200×10^6 mL^{-1}, $P = 0.048$; 400×10^6 mL^{-1}, $P = 0.036$) and 1600×10^6 mL^{-1} ($P < 0.001$ comparing with the other three concentrations).

The fertility results after intrauterine insemination (lambing rates) are reported in Table 2. Fertility was affected by the sperm concentration used for freezing the semen doses. It was significantly higher when the ewes were inseminated with doses frozen at 200 and 400×10^6 mL^{-1} (yielding 57.5 and 54.4% fertility, respectively), comparing with 800×10^6 mL^{-1} (45.5%; $P < 0.05$). Females inseminated with 800×10^6 mL^{-1} doses were 0.62 times less likely of becoming pregnant than those inseminated with 200×10^6 mL^{-1} doses (odds ratio, 95% CI, 0.44–0.88; $P = 0.007$).

4. Discussion

Semen cryopreservation induces a series of structural and biochemical changes in spermatozoa, thus reducing the integrity of the membrane [18,19], mobility [13,14], and fertilizing capacity [20,21]. Many factors influence the survival and functionality of the frozen/thawed spermatozoa, but sperm concentration has been little explored. The optimization of semen doses and the utilization of sorting technologies has driven interest toward freezing using low sperm concentrations [22,23]. However, freezing at high concentrations might be interesting in some cases, and it could be necessary to increase the absolute number of fertile spermatozoa post-thawing while still managing a small volume. Utilization of very high concentrations is usual in fish species, and it does not seem to be detrimental for the cryopreservation of spermatozoa [24]. However,

increasing sperm concentration for freezing could have undesirable consequences in mammals, exceeding the possible advantages of this approach.

Our study follows D'Alessandro et al. [11]. Recapitulating from the introduction, these authors evaluated the survival of sheep semen frozen at several concentrations (50, 100, 200, 400, 500, and 800 $\times$ 10^6 mL^{-1}) and in two extenders (egg yolk-based, Tris-FY; and milk-based, milk-LY). Irrespective of the extender, they found that the overall performance (motility, viability, and acrosomal status) decreased when freezing at 800 $\times$ 10^6 mL^{-1}. Nevertheless, results were not entirely conclusive, with a high variability among concentrations. Subjective motility was lower in the 800 $\times$ 10^6 mL^{-1} samples. In our results, the progressive motility was significantly different among 200 and 800 $\times$ 10^6 mL^{-1}, although total motility and the kinematic parameters remained similar. Viability also decreased in that study for 800 $\times$ 10^6 mL^{-1}, although they reported low viability for several of the lower concentrations too. Interestingly, in our results 400 $\times$ 10^6 mL^{-1} yielded a viability higher than 800 $\times$ 10^6 mL^{-1}, as assessed using PI/SYBR-14 or PI/YO-PRO-1, while 200 $\times$ 10^6 mL^{-1} stayed in between, not being significantly different from 800 $\times$ 10^6 mL^{-1}.

Other studies have detected an effect of high concentrations on cryopreservation yields in other species. Nascimento et al. [13] compared the motility, viability, and mitochondrial activity of stallion spermatozoa frozen at 100, 200, and 400 $\times$ 10^6 mL^{-1}. They found highest motilities at 200 $\times$ 10^6 mL^{-1}, followed by 400 $\times$ 10^6 mL^{-1}, and the lowest values at 800 $\times$ 10^6 mL^{-1}. Similarly, Crockett et al. [25] found higher post thaw progressive motility in cooled samples and after cryopreservation at concentrations of 50 and 250 $\times$ 10^6 mL^{-1} (25% and 23%, respectively) than in samples at a concentration of 500 $\times$ 10^6 mL^{-1} (17%). Peña and Linde-Forsberg [14] obtained diverging results when testing 50, 100, 200, and 400 $\times$ 10^6 mL^{-1}. Whereas viability was higher at lower concentrations, there were no differences on progressive motility and, after incubating the samples for several hours, 400 $\times$ 10^6 mL^{-1} yielded both the highest progressive motility and viability. Therefore, the spermatozoa of some species, such as the horse, seems to be sensitive to high concentrations while freezing, whereas others, dog and possibly ovine, seem to be resilient and even being better cryopreserved at moderately high concentrations.

The causes of this decrease are still little known, but could be multifactorial and intertwined: excess of free radicals, modification of the sperm metabolism, changes in the media due to catabolism products, physical changes during the freezing/thawing, etc. Moreover, acrosomal enzymes and toxic products released from damaged spermatozoa (e.g., free radicals) might contribute to the destabilization of membranes and other structures in live spermatozoa, and it could have a larger effect at higher sperm concentration [26,27]. We aimed at exaggerating any detrimental effects using the 1600 $\times$ 10^6 mL^{-1} concentration. Indeed, the proportion of spermatozoa with damaged membranes (loss of viability) decreased clearly in that treatment, explaining at least in part the concomitant loss of motility. In equine spermatozoa, Crockett et al. [25] found not only a lower motility, but also a higher percentage of sperm with damaged membranes in the 500 $\times$ 10^6 mL^{-1} doses (45%) than in the 50 $\times$ 10^6 mL^{-1} doses (60%). Interestingly, we could not detect an increase of acrosomal damage, even in the 1600 $\times$ 10^6 mL^{-1} samples. Nevertheless, we must take into account that the absolute quantity of enzymes and other molecules released from damaged acrosomes (and, in general, from dead spermatozoa) increase with sperm concentration. Therefore, at the same proportion of damaged acrosomes, samples with higher sperm concentration would have a higher concentration of these potentially harmful substances, which might explain, at least in part, the lower sperm quality.

The YO-PRO-1 stain has the ability to label spermatozoa that have an increasing membrane permeability that not necessarily implies a loss of continuity [28,29], resembling some phenomena occurring in apoptotic somatic cells. We detected that the proportion of spermatozoa showing these early membrane changes increased with sperm concentration. This increase was more evident when the presence of the "apoptotic" population was expressed as an "apoptotic ratio". This is an important variable, because these subtle membrane changes could introduce a higher vulnerability of AQ:6 inseminated spermatozoa to the oviductal environment and a lower ability to attach to the oviductal epithelium [30]. Therefore, samples frozen at 800 or 1600 $\times$ 10^6 mL^{-1} not only had a lower proportion of viable spermatozoa, but a higher proportion of potentially nonfunctional spermatozoa [29].

Indeed, the fertility obtained after intrauterine artificial insemination was significantly lower in ewes inseminated with 800 $\times$ 10^6 mL^{-1} doses. D'Alessandro et al. [11] observed that increasing the prefreezing sperm concentration to 800 $\times$ 10^6 mL^{-1} negatively affected the proportion of pregnant ewes, but their results were not significant. Moreover, inseminations

were performed without equalizing sperm numbers among different sperm concentrations, and the excess of spermatozoa when using the doses with higher concentration could compensate in part for the lack of quality. In our study, we used a larger number of females per insemination group, and utilized the same number of spermatozoa per insemination, irrespectively of the dose concentration. We have achieved an overall fertility above 50%, similarly to previous studies with laparoscopic insemination [3,4]. The best results for fertility were achieved by 200 and 400 $\times$ 10^6 mL^{-1}, related to the highest results for motility, plasma membrane integrity, and permeability. The 800 $\times$ 10^6 mL^{-1} doses obtained an odds ratio of 0.62 with respect to 200 $\times$ 10^6 mL^{-1} doses. That is, the odds for an insemination with an 800 $\times$ 10^6 mL^{-1} dose to result in a pregnancy are 0.62 times lower than for an insemination with a 200 $\times$ 10^6 mL^{-1} dose.

It is true that preinsemination extension might have penalized the 800 $\times$ 10^6 mL^{-1} doses. However, we think that our trial is realistic, because dilution would occur in vivo after routine insemination, penalizing these spermatozoa anyway. Moreover, extension sensitivity might be due to sublethal membrane damage. Given that samples frozen at 400 $\times$ 10^6 mL^{-1} yielded fertility results similar to the 200 $\times$ 10^6 mL^{-1} doses, we can conclude that these samples were not affected by extension, remarking that detrimental effects occur when samples are frozen at higher concentrations.

In conclusion, increasing sperm concentration in the sperm doses (at least, above 400 $\times$ 10^6 mL^{-1}) affects adversely the post-thawing quality and fertility of ram semen. Sperm quality was slightly affected at 800 $\times$ 10^6 mL^{-1}, but membrane changes (proportion of spermatozoa with apoptotic features) indicated further detrimental effects upon use of the doses in the field. Actually, freezing at those sperm densities affected negatively the fertility of the samples.

One of the purposes of freezing at high concentrations is to increase the number of spermatozoa available in the insemination dose, attempting to improve the odds for achieving a pregnancy. Moreover, this increase of inseminated spermatozoa would compensate for some quality decrease when freezing at high concentrations. Nevertheless, taking into account the results of D'Alessandro et al. [11], it seems that this hypothesis may not be correct, because their insemination results tended to decrease with the 800 $\times$ 10^6 mL^{-1} doses, even though they inseminated with a higher number of spermatozoa (not at a fixed number, like in our case). Therefore, trying to improve AI results in sheep by freezing and inseminating at a higher sperm concentration might not compensate the excess spermatozoa used. Testing this hypothesis should be an objective in future studies. Nevertheless, it is still open to further research if there is any advantage freezing at 400 $\times$ 10^6 mL^{-1} (or higher) and inseminating with the full dose. Because freezing at 200 and 400 $\times$ 10^6 mL^{-1} have yielded similar performance for in vitro quality and in the fertility trial, it is reasonable to think that ram semen could be frozen at least at that concentration, thus increasing the odds of pregnancy.

Acknowledgments

This work was supported in part by INIA (RZ2010-00005-00-00). Felipe Martínez-Pastor was supported by the Ramón y Cajal program (RYC-2008-02560, MICINN, Spain). The authors thank Manuel Alvarez, María Nicolas, Susana Gomes, and Elena López for their help in the acquisition and analysis of the samples.

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