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Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm

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ABSTRACT

The poor fertility of ram semen stored chilled for long periods has encouraged the development of protocols designed to improve the kinetic vigour and cervical barrier-crossing capacity of sperm. The present work evaluated the effect of sperm selection with Sephadex filtration and the supplementation of 2% glycerol (GLY) to extenders based on ultra-heat-treated skimmed milk (UHT) or Tris-Tes-Glucose (TEST) on ram sperm kinetic parameters, plasma membrane integrity, acrosome integrity, mitochondrial function and fertilizing ability, over long chilling times. The results showed that for non-filtered semen, values for progressive sperm motility (%PSM), straight line velocity (VSL, μm/s) and the percentage of sperm with an intact plasma membrane/intact acrosome/a high mitochondrial function index (%IPIAHM) at all times up to 96 h of chilling were higher when the UHT extender ($P < 0.01$) was used compared to TEST extender irrespective of the presence of GLY. When semen was previously filtered with Sephadex, the addition of GLY to the UHT extender improved total motility (%TM), the %PSM and the VSL at 96 h compared to all other treatments ($P < 0.01$). The best results of all were obtained with non-filtered semen and UHT either with or without GLY. Heterologous IVF using zona-intact bovine oocytes was used to assess the fertilizing capacity of non-filtered fresh (FS0), chilled-for-24 h (CS24) or chilled-for-48 h (CS48) ram semen diluted in UHT extender (GLY-free). Heterologous IVF showed that ram sperm, either FS0, CS24 or CS48, were equally capable of penetrating zona pellucida intact bovine oocytes, leading to pronuclear formation and hybrid embryo cleavage (46.3 ± 3.2; 48.8 ± 3.2; and 43.3 ± 3.5, respectively). No differences were seen with respect to fresh sperm in terms of sperm binding, penetration, polyspermy, pronucleus formation or cleavage rates ($P > 0.05$). In conclusion, neither Sephadex filtration nor addition of glycerol provided extra benefits to ram sperm chilled up to 96 h. Chilled, non-filtered sperm extended with UHT without GLY showed better sperm functionality than did similar sperm extended with TEST extenders. Indeed, sperm diluted in UHT extender, maintained fertilizing ability up to 48 h.

1. Introduction

Artificial insemination (AI) is an essential technique to the running of sheep selection programs. Success is limited by the short length of time that ram sperm can be stored as a liquid if it is to maintain its fertility capacity [1]. The majority of sheep that undergo AI do so against a backdrop of hormone-induced oestrus synchronization, which allows the procedure to be performed at desired times [2]. Cervical AI with frozen-thawed sperm is associated with low fertility rates (e.g. 3.8–20.5% or 20.0–22.0% reported by Valente et al. [3] or Masoudi et al. [4], respectively), and has only been employed on a limited scale. Laparoscopic AI with frozen-thawed sperm returns better results [5]. However, a number of technical, handling and animal welfare issues determine that cervical AI using chilled sperm is the most common procedure

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followed for genetic improvement programs in sheep [6].

The survival of ram sperm over extended periods inversely correlates with the cells' metabolic activity and motility, morphological integrity and fertility [7]. Synthetic TEST and Tris-based extenders have both been used to preserve ram sperm under chilled conditions [8–10], but non-synthetic skimmed milk powder-based extender is associated with better survival and fertility when using either chilled [11] or frozen-thawed [12] sperm. The use of ultra-heat-treated "long-life" skimmed milk has been shown a satisfactory extender for use with chilled ram sperm [13]. Egg yolk (EY) has been added to both synthetic-based [14,15] and non-synthetic-based extenders [6] given the protection it affords the plasma membrane against cold shock.

Glycerol (GLY) is the main penetrating cryoprotectant agent used when freezing ram sperm [16], but many of its effects remain unclear. Early studies reported GLY to reduce the quality of fresh and frozen-thawed semen in some species, including sheep [17]. It has also been reported harmful when added to extenders at over 30 °C, as well as at concentrations over 6% or below 2% [12]. Certainly, high concentrations of GLY have a negative effect on sperm membrane integrity [18]. In addition, GLY is osmotically active, changing the water content of sperm cells [19]; any osmotic stress induced could reduce sperm longevity and accelerate sperm capacitation. Only a few studies examining the addition of GLY to extenders for chilling [20,21] have been performed; its effects on sperm functionality, especially kinetic variables and mitochondrial integrity, need to be further investigated. Unlike that reported by Colas [12], Morrier et al. [22] indicated that the addition of 7% GLY did not significantly reduce the motility or viability of ram sperm maintained for 24 h at 5 °C, suggesting this agent might be used in cryopreservation protocols. Reducing the percentage of GLY below 7% under similar chilling conditions might allow its cryoprotective advantages to be maintained while reducing its cytotoxic effects; it could also provide an extra source of energy [23], and promote membrane stabilization [24].

Sperm selection by density-gradient centrifugation (DGC) has been used to select ram sperm prior to *in vitro* fertilization (IVF) [25]. In goats, the advantages of DGC become clear with chilled sperm, with live and motile sperm numbers reported to remain constant for up to 96 h [26]. This suggests that DGC removes sperm with sub-optimal functionality along with most of the apoptotic cells [27]. Galarza et al. [28] who compared different sperm selection procedures, found Sephadex filtration to be even better than DGC, improving both the motility and viability of ram sperm. Considering the aforementioned, it should be expected that the addition of GLY in low concentration to extenders following sperm selection through the use of Sephadex columns, would improve the functionality and fertilizing capacity of ram sperm chilled and stored for long times.

Alongside the basic parameters evaluating sperm fertilizing ability, IVF is a competent indicator providing information on gametes interaction, sperm penetration, pronuclear formation and early embryo development [29]. The fertilizing capacity of frozen-thawed ram semen has been successfully assessed via homologous IVF using either ewe oocytes [30], or heterologous IVF involving either zona-free hamster ova [31], and or zona-intact bovine oocytes [32]. However, chilled ram semen has been successfully used in either homologous [6] or heterologous [33] IVF in limited scale involving only zona-free oocytes. Furthermore, mainly in sheep, the accessibility to homologous adequate oocytes is limited. Therefore, the use of heterologous IVF bypasses this limitation.

The aim of this study was to evaluate the effect of addition of GLY in low concentration to extenders, following sperm selection through the use of Sephadex G-15® columns, on sperm functionality of ram sperm chilled to 5 °C and stored up to 96 h. The fertilizing capacity of the sperm returning the best results was examined by heterologous IVF involving mature zona-intact bovine oocytes.

2. Materials and methods

All extenders and media were prepared at the Department of Animal Reproduction Research Laboratory (INIA, Madrid, Spain) using reagent-grade chemicals purchased from Panreac Chemistry S.A. (Barcelona, Spain), Sigma Chemical (St. Louis, MO, USA) or Invitrogen (Eugene, OR, USA).

2.1. Extenders

Two synthetic-based extenders for the liquid storage of ram semen were made with TTG solution (210.59 mM Tes, 95,75 mM Tris, 10.09 mM glucose, 0.54 mM streptomycin, and 2.14 mM penicillin; 324 mOsm/kg, pH 7.1) and 6% (v:v) EY: (1) TEST, and (2) TEST plus 2% (v:v) glycerol (TEST-GLY). In addition, two non-synthetic-based extenders were made according Gil et al. [20] with some modifications: (1) ultra-heat-treated (UHT) made from commercial skimmed milk plus antibiotics (100000 IU penicillin sodium and 100 mg dihydrostreptomycin/100 mL) plus 6% (v:v) EY, and (2) UHT plus 2% (v:v) glycerol (UHT-GLY). All extenders were centrifuged for 30 min at 4000×*g* to remove any large particles, and the supernatant filtered through a sterile Minisart® NML Syringe Filter 16555 (pore size 0.45 μm) (Sartorius, Germany). The osmolarity of all extenders was 298–310 mOsm/kg. The pH was adjusted to 7.2. All extenders were stored at −20 °C until use.

2.2. Animals and semen collection

The animals used in this work were eight healthy Merino rams aged 2–7 years. All were handled according to procedures approved by the INIA Ethics Committee, and all work was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. The rams were housed at the INIA Department of Animal Reproduction. All were fed a diet of grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available *ad libitum*.

Using an artificial vagina (pre-warmed at 42–43 °C), a total of 21 semen ejaculates were collected from the experimental animals during the September–December 2017 rutting season. Immediately after collection, each ejaculate was diluted with TTG (1:1 v:v) solution at 37 °C and transported to the laboratory for initial assessment. Those ejaculates with a volume of 0.75–2 mL, a sperm motility value of >70%, a score of >3 on a mass motility scale of 0–5, and a sperm concentration of $>3.5 \times 10^9$ sperm/mL were used in the subsequent experimental work.

2.3. Sperm filtration

Sperm selection by Sephadex filtration columns was performed according to Galarza et al. [28]. For this purpose, each semen ejaculate diluted 1:1 (v:v) with TTG solution was divided into two aliquots. The first aliquot (400 μL) was used for non-filtered semen and then was subdivided and extended at room temperature with each diluent type to a concentration of 800×10^6 sperm/mL. The second aliquot (1200 μL) was filtered through Sephadex columns. The filtered samples were then re-suspended in 400 μL TTG solution at 37 °C, subdivided and diluted with each extender type under same conditions as non-filtered semen samples (temperature and

concentration). All these samples were then slowly cooled to 5 °C and stored for 0, 24, 48, 72 or 96 h. Sperm variables were then assessed.

2.4. Sperm analysis

Sperm motility analysis was assessed using a CASA system (Sperm Class Analyzer, SCA® 1999, v.4.0 software. Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) in accordance with Santiago-Moreno et al. [26]. The sperm samples were loaded into a warmed (37 °C) 20 μm Leja® 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). A minimum of three fields and 200 sperm tracks were evaluated at 100 X for each sample chamber (image acquisition rate 25 frames/s). Values were recorded for the percentage of motile sperm (%SM), percentage of progressive sperm (%PSM), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), and the amplitude of lateral head displacement (ALH, μm).

Plasma and acrosome membrane status and mitochondrial function, were assessed using a triple association of fluorescent probes - propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut *(Arachis hypogaea)* agglutinin (PNA-FITC, Sigma L7381), and Mitotracker Green *FM*® (MITO, Invitrogen M7514) - according to Forero-Gonzalez et al. [34] with some modifications. For this, samples of 150 μL of semen diluted in TALP Stock medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH2PO4 H2O, 1 mM Na-Lactate, 1.97 mM CaCl2 2H2O, 0.50 mM MgCl2 6H2O, 10 mM HEPES sodium, and 25 mM NaHCO3; 320 mOsm/Kg, pH 7.3) to a concentration of 25×10^6 sperm/mL were mixed with 2 μL of PI (2 mg/mL), 2 μL of MITO (1 mM) and 50 μL of PNA-FITC (100 μg/mL) and co-incubated in the dark at 38.5 °C for 8 min (preliminary studies were performed to examine the intensity of staining of each fluorescent probe and its association with different incubation times [3, 5, 8, 10, 12 and 15 min]; data not shown). After incubation, the samples were transferred to a slide, covered with a cover slip, and examined immediately using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (excitation: 450 nm, emission: 490 nm). A total of 200 sperm cells per slide were examined and eight subpopulations of cells quantified, i.e., those showing: (1) intact plasma membrane/intact acrosome/high mitochondrial function (%IPIAHM); (2) intact plasma membrane/intact acrosome/low mitochondrial function (%IPIALM); (3) intact plasma membrane/damaged acrosome/high mitochondrial function (%IPDAHM); (4) intact plasma membrane/damaged acrosome/low mitochondrial function (%IPDALM); (5) damaged plasma membrane/intact acrosome/high mitochondrial function (%DPIAHM); (6) damaged plasma membrane/intact acrosome/low mitochondrial function (%DPIALM); (7) damaged plasma membrane/damaged acrosome/high mitochondrial function (%DPDAHM); and (8) damaged plasma membrane/damaged acrosome/low mitochondrial function (%DPDALM).

Sperm morphological abnormalities (abnormal heads, loose heads, coiled tails, abnormal tails, cytoplasmic droplets) were assessed according to Galarza et al. [28] using 5 μL sperm samples in 100 μL of 2% (v:v) glutaraldehyde solution, and counting 200 sperm cells.

2.5. Heterologous in vitro fertilization (Bos taurus oocytes x Ovis aries sperm)

The fertilizing capacity of non-filtered sperm extended with UHT, GLY-free, stored for 0 (FS0), 24 h (CS24) or 48 h (CS48) was tested by heterologous IVF involving zona-intact bovine oocytes according to Pradieé et al. [35] with some modifications. Briefly, oocyte *in vitro* maturation was performed in 60 μL drops (30 COCs per drop) of maturation medium (TCM-199) supplemented with 10-ng/mL EGF and 10% (v:v) fetal calf serum (FCS) for 24 h at 38.5 °C under an atmosphere of 5% CO_2 in air at maximum humidity. After 24 h, the matured oocytes were washed twice in FERT medium (FERT-TALP media supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 10 μg/mL heparin) and transferred to 30 μL drops of FERT medium (30 COCs per drop).

For heterologous IVF, a pool of semen samples from three individual rams was performed in each replicate for all experimental groups (FS0, CS24 and CS48). In addition, for each sperm group, a control was prepared using frozen-thawed (in a water bath at 37 °C for 50 s) sperm from a single Asturian Valley bull (Asturgen, Gijon, Spain) of proven fertility. Semen samples from FS0, CS24 and CS48 groups were maintained at 37 °C for 30 min before sperm selection. Motile sperm from each group were selected by density gradient centrifugation (BoviPure, Nidacon International, Sweden) prior to attempting heterologous IVF. Sperm were diluted in FERT medium and 30 μL of this suspension was added to each fertilization drop obtaining a final concentration of 1×10^6 sperm/mL.

A heterologous group using ram sperm, a homologous control group using bovine sperm and a parthenogenetic control group were tested in each IVF replicate. A total of fifteen IVF replicates were performed in the three chilling time point groups: FS0 (n = 5), CS24 (n = 5), and CS48 (n = 5). Gametes were co-incubated at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

Sperm-oocyte interaction was assessed by sperm-zona pellucida binding assay at 2.5 h post-insemination (hpi). For that purpose, oocytes were vortexed for 3 min, fixed and stained with Hoechst 33342 to count the number of sperm that remained bound to the zona pellucida by a Nikon Eclipse E200 epifluorescence microscope (UV-2E/C, excitation: 340–380 nm, emission: 435–485 nm). Sperm penetration and polyspermy was evaluated at 12 hpi, while pronucleus formation was evaluated at 18, 20, 22, 24 and 26 hpi for heterologous and at 18 h for homologous IVF. For this, presumptive zygotes were treated and examined as explained above for sperm-zona pellucida binding. The cleavage rate was evaluated at 48 hpi in all groups.

2.6. Experimental design

The effects of the different extenders, cold storage time, and sperm selection on different sperm variables and sperm fertilization ability were examined. Eight experimental groups (samples of 300 μL at a concentration of 800×10^6 sperm/mL) of sperm were established: (1) non-filtered sperm extended with TEST; (2) non-filtered sperm extended with TEST-GLY; (3) non-filtered sperm extended with UHT; (4) non-filtered sperm extended with UHT-GLY; (5) filtered sperm extended with TEST; (6) filtered sperm extended with TEST-GLY; (7) filtered sperm extended with UHT; and (8) filtered sperm extended with UHT-GLY. All groups were maintained in chilled conditions and sperm parameters were assessed at 0, 24, 48, 72 or 96 h. The freshly collected semen samples (n = 21) extended simply with TTG [1:1 (v:v), at concentration of $2178.5 \pm 221.5 \times 10^6$ sperm/mL] provided controls.

The fertilizing capacity of non-filtered sperm extended with UHT without GLY (selected based on the optimal sperm quality parameters measured in the previous analysis), stored for 0, 24 and 48 h, was tested by heterologous IVF with zona intact bovine oocytes. Homologous fertilization and parthenogenesis were used as control. A total of 3840 cumulus oocyte complexes (COCs) obtained from ovaries from heifers and cows at slaughter, in 15 individual

replicates were used for heterologous [FS0 (n = 707), CS24 (n = 832) or CS48 (n = 611)] and homologous IVF (n = 1356), or parthenogenesis (non-fertilized oocytes, n = 334). Sperm zona binding (2.5 hpi), penetration and polyspermy (12 hpi) pronuclei formation (18, 20, 22, 24 and 26 hpi) and cleavage rate (48 hpi) were evaluated in all groups.

2.7. Statistical analysis

Data were expressed as means ± S.E.M. Values for sperm variables showing a skewed distribution (as determined by the Shapiro-Wilks test) were arcsine- (for percentages values) or Log10- (for numeric values) transformed. Factorial ANOVA and Bonferroni post hoc multiple comparison tests were used to examine the effects of the interactions between extender type with and without GLY, Sephadex filtration, and cold storage up to 96 h, on sperm kinetic variables, plasma membrane and acrosome status, mitochondrial function, and morphological integrity. For heterologous IVF, the percentage of bound sperm, oocyte penetration, polyspermy, male pronucleus formation and the cleavage rate were analyzed by parametric one-way ANOVA and Tukey's post hoc multiple comparison tests. All calculations were made using Statistica for Windows v.12.0 software (StatSoft, Tusla, OK, USA).

3. Results

The interaction *Sephadex filtration x extender type x chilling time* had no significant effect on the sperm variables examined. However, the interaction *Sephadex filtration x extender type* did have an effect on %PSM ($P < 0.001$) and AHL ($P < 0.001$), and the interaction *Sephadex filtration x chilling time* significantly influenced VCL ($P < 0.05$) and VSL ($P < 0.01$). It should be noted that the results for ALH obtained with the non-filtered sperm and TEST extenders (with or without GLY) were better than those obtained for filtered sperm (see Fig. 1).

3.1. Effect of extenders and storage time on non-filtered semen

The motility values (%SM and %PSM) of sperm diluted with either UHT extender were greater ($P < 0.01$) than those recorded for either TEST extender (i.e., with or without GLY) up to 96 h of cold storage. However, when diluted with either UHT extender (i.e., with or without GLY), a significant reduction ($P < 0.05$) was seen in %PSM and VSL between 48 and 72 h of storage (Fig. 1). The VSL value was greater ($P < 0.01$) with either UHT extender than either TEST extender up to 72 h. The ALH value was lower ($P < 0.001$) in the samples diluted with either UHT extender (either type) than either TEST extender at all times. The addition of GLY to either the TEST or UHT extender had no significant effect ($P > 0.05$) on these kinetic variables compared to the same extenders without GLY.

Similarly, the %IPIAHM value was greater ($P < 0.05$) with either UHT extender than either TEST extender at all times. However, the values fell with increasing storage time for all extenders. No differences were seen in the %IPDAHM, %DPIAHM and %DPDALM values at 96 h between the UHT and TEST extenders with or without GLY, despite both the UHT extenders returning high values at 24 h. The %DPDAHM value was lower ($P < 0.05$) when either UHT extender was used compared to either TEST extender at all times (Fig. 2).

No differences were recorded between any of the extenders in terms of morphological abnormalities (percentage of abnormal heads, loose heads, abnormal tails, cytoplasmic droplets or total abnormalities) at any storage time. At 24 h, however, the percentage of sperms with coiled tails was higher ($P < 0.05$) with the UHT-GLY extender than with any other.

3.2. Effect of extenders and storage time on filtered semen

The values for %SM and %PSM and VSL were higher ($P < 0.01$) with the UHT-GLY extender than with any other extender after 96 h of cold storage - although these values dropped between 48 h and 72 h ($P < 0.05$). The ALH value was lower ($P < 0.001$) in samples diluted with either UHT extender than either TEST extender (Fig. 1).

The %IPIAHM value was greater ($P < 0.05$) with either UHT extender than either TEST extender from 24 to 96 h. The %DPDAHM value was lower ($P < 0.05$) with either UHT extender than either TEST extender at all times. Finally, the %DPDALM value was lower with UHT diluents than using TEST diluents at 96 h ($P < 0.05$).

The percentage of abnormal heads was higher ($P < 0.05$) with the UHT extender than with UHT-GLY at 48 h of storage. The percentage of coiled tails at 0 h was greater ($P < 0.05$) with UHT-GLY than with TEST-GLY; from 24 to 48 h the value was higher with UHT-GLY than any other extender; and at 72 h the values were higher with UHT diluents than TEST diluents. In addition, the percentage of coiled tails increased ($P < 0.05$) at 96 h of storage with the TEST-GLY and UHT (without GLY) extenders.

3.3. Heterologous in vitro fertilization

Results of heterologous IVF are depicted in Table 1. Heterologous IVF showed that ram sperm, either fresh or cold-stored (24 or 48 h), were equally capable of penetrating bovine oocytes, leading to pronuclear formation and hybrid embryo cleavage. No differences were seen in sperm binding, penetration, polyspermy, pronuclear formation or cleavage rate between fresh semen and semen chilled for 24 h or 48 h ($P > 0.05$).

As expected, homologous IVF was associated with higher percentages of penetration compared to heterologous IVF for the FS0 sperm ($P < 0.01$). Polyspermy was higher in the CS24 heterologous IVF group than in the CS24 and CS48 homologous IVF group ($P < 0.05$). As expected, and for all groups, homologous IVF group returned a higher percentage of pronucleus formation at 18 hpi than did heterologous IVF ($P < 0.001$). Nevertheless, no differences were seen in pro-nuclei formation between the heterologous IVF groups at different time points (18–26 hpi) ($P > 0.05$). Similarly, for all groups, the cleavage rate was higher ($P < 0.001$) in homologous than heterologous IVF.

4. Discussion

The results showed that chilling non-filtered ram semen diluted with UHT extender without GLY preserves sperm motility, plasma membrane, acrosome integrity, mitochondrial function and morphological integrity, better than TEST extenders of either type at all times, maintaining a fertilizing capacity similar to that of fresh semen for up to 48 h of cold storage. Interestingly, the results also showed that neither sperm selection via the use of Sephadex columns, nor the addition of GLY, improves the functionality of stored sperm.

The use of GLY in semen refrigeration has returned variable results across different studies. Low GLY concentrations have been successfully used with ram sperm diluted with skimmed milk-based extenders [20], and with goat sperm diluted with a Tris-fructose-citric acid-soybean lecithin-based extender [18]. The present results indicate that 2% GLY provided no extra benefit to unfiltered ram sperm cold-stored for up to 96 h; however, when the semen sample was previously filtered with Sephadex, GLY improved total motility, the percentage of sperm showing progressive motility, and the VSL values. These findings suggest that Sephadex filtration might select sperm cells subpopulations capable of metabolizing GLY, rendering it an extra source of energy

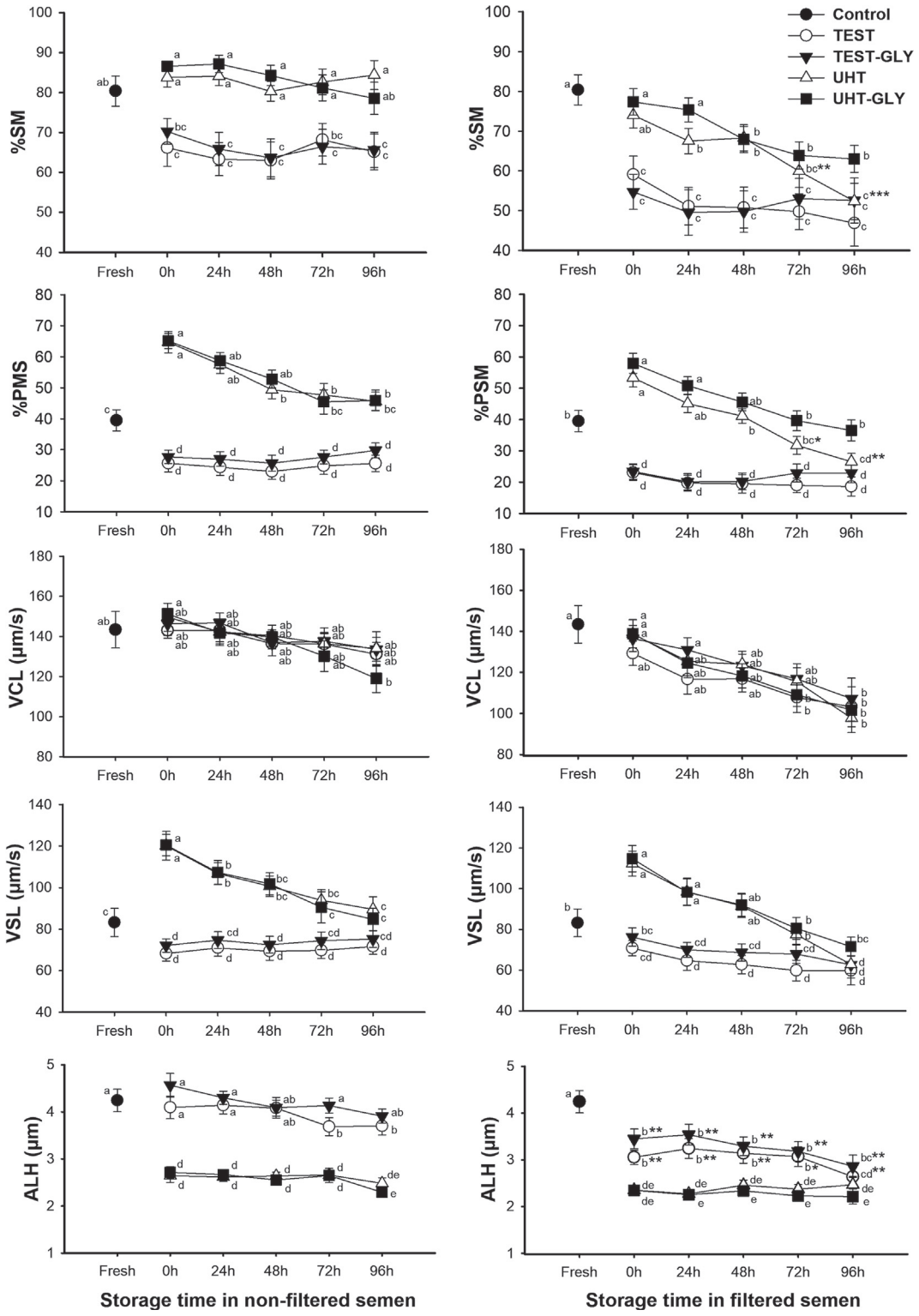

Fig. 1. Kinetic variables of non-filtered and filtered ram semen samples, diluted with different extenders - Control, TEST, TEST-GLY, UHT, UHT-GLY - and cold-stored at 5 °C. %SM, sperm motility; %PSM, progressive sperm motility; VCL, curvilinear velocity; VSL, straight line velocity; and ALH, amplitude of lateral head displacement. Different letters in superscript (a–e) at each evaluation time indicate significant differences between values for extenders and cold storage times ($P < 0.05$ for a – b, and b - c; $P < 0.01$ for a – c, c – d, and c - e; and $P < 0.001$ for a – d, a – e, b – d, and b - e). * Significant reduction between non-filtered and filtered semen samples at the same evaluation-time and for the same extender (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

[23]. A similar improvement in VCL and in the fertility rate was reported for bull sperm when using 7% GLY [21].

Glycerol has been indicated to accelerate the acrosome reaction in ram sperm stored at 5 °C [36,37] which might reduce the reproductive lifespan of cells [38]. However, the triple association of fluorescent probes showed a lack of difference in %IPDAHM at

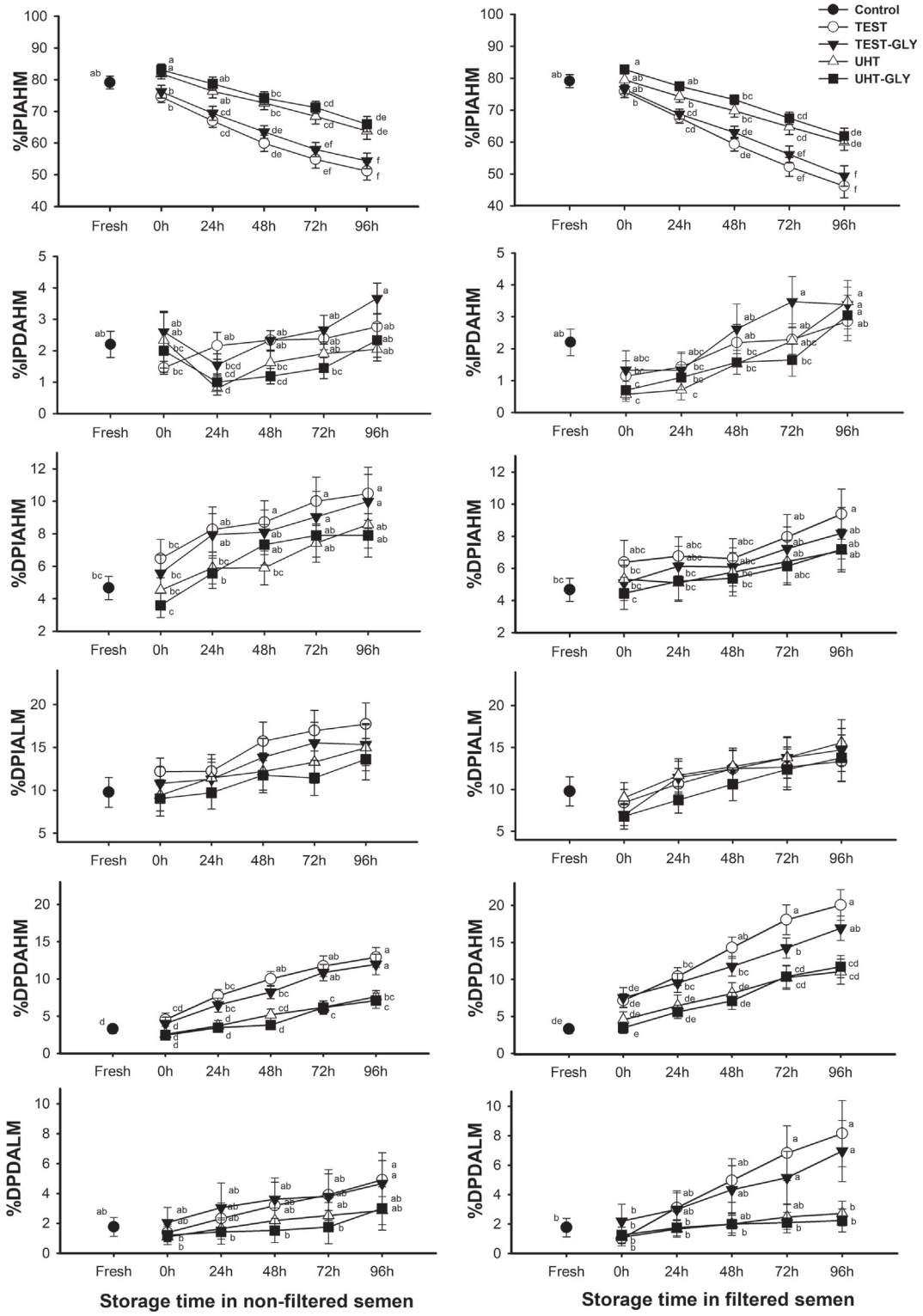

Fig. 2. Status of sperm, plasma-, acrosome- and mitochondrial membranes (association of PI/PNA-FITC/Mitotracker probes) in non-filtered and filtered ram semen samples, diluted with different extenders - Control, TEST, TEST-GLY, UHT, UHT-GLY - and cold-stored at 5 °C. %IPIAHM, intact plasma membrane/intact acrosome/high mitochondrial function; %IPDAHM, intact plasma membrane/damage acrosome/high mitochondrial function; %DPIAHM, intact plasma membrane/intact acrosome/high mitochondrial function; %DPIALM, damaged plasma membrane/intact acrosome/low mitochondrial function; %DPDAHM, damaged plasma membrane/damage acrosome/high mitochondrial function; %DPDALM, damaged plasma membrane/damage acrosome/low mitochondrial function. Different letters in superscript (a–f) at each evaluation time indicate significant differences between values for extenders and cold storage times ($P < 0.05$ for a - b, b - c, c - d, d - e, and e – f; $P < 0.01$ for a - c, b - d, and c - f; and $P < 0.001$ for a - d, a – e, a - f, b – e, b – f, and c - f).

96 h when any of the four types of extender were used.

Sperm selection via Sephadex filtration improves the quality of both normospermic and asthenospermic ram sperm [39]. We previously reported that filtration with Sephadex columns improves the kinetic characteristics of fresh and cold-stored ram semen outside of the reproductive season [28]. The fact that the

Table 1
Rates of penetration, polyspermy, pronucleus formation and cleavage after homologous and heterologous co-incubation involving fresh ram sperm (FS0), ram sperm cold-stored for 24 h (CS24), and ram sperm cold-stored for 48 h (CS48), at different times post-insemination (h). Values are expressed as (mean ± SEM) of five replicates (total number of oocytes or presumptive zygotes examined = 3840).

Ram semen	Groups	Bound sperm	Penetration (%)	Polyspermy (%)	Pronuclear formation (%)					Cleavage rate (%)
		2.5 h	12 h	12 h	18 h	20 h	22 h	24 h	26 h	48 h
FS0	Ho-IVF (n = 477)	64 (0.25 ± 0.1)	54 (44.4 ± 6.8)^{a}	54 (5.6 ± 3.1)^{ab}	52 (67.3 ± 5.8)^{a}					253 (78.3 ± 2.6)^{a}
	He-IVF (n = 707)	61 (0.31 ± 0.1)	56 (12.5 ± 4.5)^{b}	57 (7.0 ± 3.4)^{ab}	54 (35.2 ± 5.6)^{b}	53 (32.1 ± 6.5)	49 (34.7 ± 6.9)	58 (48.3 ± 6.6)	69 (44.9 ± 6.0)	177 (46.3 ± 3.2)^{b}
	Parthenogenic (n = 86)									86 (7.0 ± 2.3)^{c}
CS24	Ho-IVF (n = 534)	75 (0.29 ± 0.1)	78 (42.3 ± 5.6)^{a}	78 (3.8 ± 2.2)^{b}	48 (72.1 ± 4.5)^{a}					255 (78.4 ± 2.6)^{a}
	He-IFV (n = 832)	72 (0.22 ± 0.0)	70 (30.0 ± 5.5)^{ab}	70 (11.4 ± 3.4)^{a}	73 (37.0 ± 5.7)^{b}	72 (29.2 ± 5.4)	66 (37.9 ± 6.0)	78 (42.3 ± 5.6)	81 (45.7 ± 5.6)	252 (48.8 ± 3.2)^{b}
	Parthenogenic (n = 109)									109 (4.9 ± 2.0)^{c}
CS48	Ho-IVF (n = 345)	65 (0.32 ± 0.1)	62 (40.3 ± 6.3)^{a}	62 (1.6 ± 1.6)^{b}	54 (63.0 ± 6.0)^{a}					102 (78.4 ± 3.3)^{a}
	He-IFV (n = 611)	59 (0.29 ± 0.1)	57 (29.8 ± 6.1)^{ab}	57 (7.0 ± 3.4)^{ab}	63 (27.0 ± 5.6)^{b}	63 (34.9 ± 6.1)	58 (46.6 ± 6.6)	52 (44.2 ± 7.0)	59 (47.5 ± 6.6)	143 (43.3 ± 3.5)^{b}
	Parthenogenic (n = 139)									139 (4.3 ± 1.2)^{c}

FS0, fresh semen; CS24, semen cold-stored for 24 h; CS48, cold-stored up to 48 h; Ho-IVF, Homologous *in vitro* fertilization; He-IVF, Heterologous *in vitro* fertilization. $^{a-c}$ Different superscripts in the same column indicate significant differences. $^{a-b;\ b-c}$ $P < 0.05$; $^{a-c}$ $P < 0.001$.

ejaculates in the present study were collected during the rutting season, and were already of good quality, might explain why Sephadex filtration had no effect; it may be that such filtration is only worthwhile outside of the rutting season. Nevertheless, if barriers such as that imposed by cervical mucus are to be overcome, and the oocyte cumulus cell layers are to be penetrated, sperm ALH values need to be appropriate [40], and for the filtered semen diluted with either the TEST or UHT extender (either with or without GLY), the ALH values obtained were lower than for the non-filtered semen. This agrees with previous studies on goat semen [26], in which sperm selection by density gradient centrifugation determined a lower ALH value than that achieved with non-washed or classically washed sperm. An increased ALH value greatly raises the number of hyperactive sperm cells undergoing capacitation; Sephadex filtration therefore seems to facilitate the selection of non-capacitated ram sperm.

To prove the fertilizing capacity of ram sperm fresh or chilled-diluted in UHT extender we used *in vitro* fertilization. However, as mentioned before, the accessibility to homologous adequate oocytes (i.e: from adult ewes) may be limited, as it was in our case. Therefore, the use of heterologous IVF with bovine oocytes was used as a tool to bypass this limitation. Heterologous IVF using ram sperm and bovine oocytes has been described as an effective method for predicting the fertilization capacity of frozen-thawed [25] or chilled [33] ram sperm. To the best of our knowledge this is the first report of the fertilizing capacity of cold-stored ram semen using heterologous IVF and zona-intact bovine oocytes, providing specific information regarding sperm binding, penetration, polyspermy, pronuclei formation (e.i. 18 to 26 hpi) and cleavage rate. The present study revealed the ability of both fresh and chilled sperm to equally bind zona-intact bovine oocytes, penetrate and fertilize them. A similar oocyte-sperm interaction was found between heterologous groups and its control groups. Controversially, previous studies evidenced a greater interaction between zona-intact bovine oocyte and bottlenose dolphin sperm [41,42]. Furthermore, it has been suggested that sperm interaction and penetration could be improved by low levels of sperm DNA fragmentation along with a stable chromatin, as demonstrated in heterologous IVF using Iberian ibex sperm and zona-intact bovine oocytes [35].

We demonstrated that fresh (FS0) or chilled (CS24 or CS48) sperm were able to penetrate zona-intact bovine oocytes leading to pronuclear formation. This indicates that ram sperm were capable to undergo acrosome reaction and bovine oocytes were capable to recognize ram sperm, that is in agreement with a previous study suggesting that the acrosome lytic system is conserved among mammals [43]. The influence of chilling for 48 h on fertilizing capacity was examined since, at this time, the fertility rate is strongly reduced when chilled sperm is used for AI [7]; indeed it is usually recommended that semen be used within 12 h [44]. Interestingly, the present results showed no differences in terms of sperm binding, penetration, polyspermy, pronucleus formation or cleavage rate in heterologous IVF, between sperm (diluted using the UHT extender without GLY) chilled for 24 h or 48 h. The present data indicate that ram sperm refrigerated in a UHT medium for 48 h maintain an *in vivo* fertilizing capacity similar to that of fresh sperm.

On the other hand, previous studies have reported *in vivo* fertility rates of 29.0–39.0% or 47.0–49.0% in ewes after cervical AI using ram chilled sperm diluted with Tris–Citric–Fructose [45] or UHT [2] extenders, respectively. Thus, the IVF values obtained in the present study suggest that ram sperm preserved in UHT extender up to 48 h, maintain its fertilizing ability. In addition, our findings provide useful information for future cervical AI trials in sheep.

5. Conclusion

In conclusion, sperm filtration with Sephadex within the rutting season did not improve sperm quality during chilled storage. Neither did the addition of 2% GLY, provide any benefits for non-filtered semen. The UHT extender with or without GLY returned better sperm quality results than the TEST extenders with or without GLY for storage at 5 °C over 96 h, irrespective of sperm filtration. Long-term chilled sperm with intact plasma-, acrosome- and mitochondrial membranes should have enough kinetic vigour to cross the cervical barrier following AI if an UHT diluent is used. Moreover, ram sperm stored chilled for up to 48 h is able to penetrate and fertilize zona-intact oocytes under *in vitro*

conditions.

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References

[1] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92. https://doi.org/10.1016/S0378-4320(00)00099-3.
[2] Olivera-Muzante J, Fierro S, Gil J. Conception rates in ewes after AI with ram semen preserved in milk-egg yolk extenders supplemented with glycerol. Reprod Domest Anim 2011;46:508–12. https://doi.org/10.1111/j.1439-0531.2010.01698.x.
[3] Valente SS, Pereira RM, Baptista MC, Marques CC, Vasques MI, Pereira MVCS, et al. In vitro and in vivo fertility of ram semen cryopreserved in different extenders. Anim Reprod Sci 2010;117:74–7. https://doi.org/10.1016/j.anireprosci.2009.04.007.
[4] Masoudi R, Zare Shahneh A, Towhidi A, Kohram H, Akbarisharif A, Sharafi M. Fertility response of artificial insemination methods in sheep with fresh and frozen-thawed semen. Cryobiology 2017;74:77–80. https://doi.org/10.1016/J.CRYOBIOL.2016.11.012.
[5] Fukui Y, Kohno H, Okabe K, Katsuki S, Yoshizawa M, Togari T, et al. Factors affecting the fertility of ewes after intrauterine insemination with frozen-thawed semen during the non-breeding season. J Reprod Dev 2010;56:460–6. https://doi.org/10.1262/jrd.10-015T.
[6] O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans ACO, et al. Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology 2010;73:541–9. https://doi.org/10.1016/j.theriogenology.2009.10.009.
[7] Maxwell WMC, Salomon S. Liquid storage of ram semen: a review. Reprod Fertil Dev 1993;5:613–38. https://doi.org/10.1071/RD9930613.
[8] Paulenz H, Söderquist L, Pérez-Pé R, Andersen Berg K. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology 2002;57:823–36. https://doi.org/10.1016/S0093-691X(01)00683-5.
[9] Kulaksiz R, Çebi Ç, Akçay E. The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4 °C. Turk J Vet Anim Sci 2012;36:177–82. https://doi.org/10.3906/vet-1103-11.
[10] Quan GB, Wu GQ, Wang YJ, Li DJ, Ma Y, Hong QH. Effects of the Tris, Tes, or skim milk based extender on in vitro parameters of ram spermatozoa during liquid storage. Small Rumin Res 2016;134:14–21. https://doi.org/10.1016/j.smallrumres.2015.11.008.
[11] Paulenz H, Söderquist L, Ådnøy T, Fossen OH, Berg KA. Effect of milk- and TRIS-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid semen. Theriogenology 2003;60:759–66. https://doi.org/10.1016/S0093-691X(03)00048-7.
[12] Colas G. Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J Reprod Fertil 1975;42:277–85. https://doi.org/10.1530/jrf.0.0420277.
[13] Salamon S, Maxwell WMC. Storage of ram semen. Anim Reprod Sci 2000;62:77–111. https://doi.org/10.1016/S0378-4320(00)00155-X.
[14] Hollinshead FK, O'Brien JK, Gillan L, Meyers M, Maxwell WMC, Evans G. Liquid storage of flow cytometrically sorted ram spermatozoa. Theriogenology 2004;62:587–605. https://doi.org/10.1016/j.theriogenology.2003.11.020.
[15] Kasimanickam R, Kasimanickam V, Tibary A, Pelzer K. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. Small Rumin Res 2011;99:208–13. https://doi.org/10.1016/j.smallrumres.2011.03.057.
[16] Salamon S, Maxwell WMC. Frozen storage of ram semen I . Processing freezing, thawing and fertility after cervical insemination. Anim Reprod Sci 1995;37:185–249. https://doi.org/10.1016/0378-4320(94)01327-I.
[17] Watson PF, Martin ICA. Effects of egg yolk, glycerol and the freezing rate on the viability and acrosomal structures of frozen ram spermatozoa. Aust J Biol Sci 1975;28:153–9. https://doi.org/10.1071/BI9750153.
[18] Yotov S. Effect of TFC-based extenders with soybean lecithin and/or low concentration of glycerol on the quality of goat chilled-stored semen. Int J Curr Microbiol Appl Sci 2015;4:752–61.
[19] Sztein JM, Noble K, Farley JS, Mobraaten LE. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology 2001;42:28–39. https://doi.org/10.1006/cryo.2001.2300.
[20] Gil J, Fierro S, Bentancur O, Olivera-Muzante J. Chilled storage of ram semen improves with the addition of egg yolk and glycerol to milk-based extenders. Reprod Domest Anim 2011;46:503–7. https://doi.org/10.1111/j.1439-0531.2010.01697.x.
[21] Papa PM, Maziero RD, Guasti PN, Junqueira CR, Freitas-Dell'Aqua CP, Papa FO, et al. Effect of glycerol on the viability and fertility of cooled bovine semen. Theriogenology 2015;83:107–13. https://doi.org/10.1016/j.theriogenology.2014.08.009.
[22] Morrier A, Castonguay F, Bailey JL. Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. Can J Anim Sci 2002;82:347–56. https://doi.org/10.4141/A01-045.
[23] Mohri H, Masaki K. Glycerokinase and its possible role in Glycerol metabolism of bull spermatozoa. J Reprod Fertil 1967;14:179–94. https://doi.org/10.1530/jrf.0.0140179.
[24] Kundu CN, Chakraborty J, Dutta P, Bhattacharyya D, Ghosh A, Majumder GC. Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. Cryobiology 2000;40:117–25. https://doi.org/10.1006/cryo.2000.2230.
[25] García-Álvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos MR, Esteso MC, Pérez-Guzmán MD, et al. Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. Theriogenology 2009;71:643–50. https://doi.org/10.1016/j.theriogenology.2008.09.036.
[26] Santiago-Moreno J, Esteso MC, Castaño C, Toledano-Díaz A, Delgadillo JA, López-Sebastián A. Seminal plasma removal by density-gradient centrifugation is superior for goat sperm preservation compared with classical sperm washing. Anim Reprod Sci 2017;181:141–50. https://doi.org/10.1016/j.anireprosci.2017.04.002.
[27] Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. Fertil Steril 2009;91:632–8. https://doi.org/10.1016/j.fertnstert.2007.11.068.
[28] Galarza DA, López-sebastián A, Woelders H, Blesbois E, Santiago-Moreno J. Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics. Anim Reprod Sci 2018;192:261–70. https://doi.org/10.1016/j.anireprosci.2018.03.022.
[29] Brahmkshtri BP, Edwin MJ, John MC, Nainar AM, Krishnan AR. Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim Reprod Sci 1999;54:159–68. https://doi.org/10.1016/S0378-4320(98)00108-0.
[30] Papadopoulos S, Hanrahan JP, Donovan A, Duffy P, Boland MP, Lonergan P. In vitro fertilization as a predictor of fertility from cervical insemination of sheep. Theriogenology 2005;63:150–9. https://doi.org/10.1016/j.theriogenology.2004.04.015.
[31] Choudhry T, Berger T, Dally M. In vitro fertility evaluation of cryopreserved Ram semen and its correlation with relative in vitro fertility. Theriogenology 1995;43:1195–2000. https://doi.org/10.1016/0093-691X(95)00091-L.
[32] Ledesma A, Manes J, Ríos G, Aller J, Cesari A, Alberio R, et al. Effect of seminal plasma on post-thaw quality and functionality of corriedale ram sperm obtained by electroejaculation and artificial vagina. Reprod Domest Anim 2015;50:386–92. https://doi.org/10.1111/rda.12500.
[33] Makarevich AV, Spalekova E, Olexikova L, Lukac N, Kubovicova E, Hegedusova Z. Functional characteristics of ram cooling-stored spermatozoa under the influence of epidermal growth factor. Gen Physiol Biophys 2011;30:S36–43. https://doi.org/10.4149/gpb_2011_SI1_36.
[34] Forero-Gonzalez RA, Celeghini ECC, Raphael CF, Andrade AFC, Bressan FF, Arruda RP. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia 2012;44:154–9. https://doi.org/10.1111/j.1439-0272.2010.01154.x.
[35] Pradieé J, Sánchez-Calabuig MJ, Castaño C, O'Brien E, Esteso MC, Beltrán-Breña P, et al. Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (Capra pyrenaica). Theriogenology 2018;108:314–20. https://doi.org/10.1016/j.theriogenology.2017.11.021.
[36] Slavík T. Effect of glycerol on the penetrating ability of fresh ram spermatozoa with zone-free hamster eggs. J Reprod Fertil 1987;79:99–103. https://doi.org/10.1530/jrf.0.0790099.
[37] Abdelhakeamp AA, Graham EF, Vazquez IA. Studies on the presence and absence of glycerol in unfrozen and frozen ram semen: fertility trials and the effect of dilution methods on freezing ram semen in the absence of glycerol. Cryobiology 1991;49:43–9. https://doi.org/10.1016/0011-2240(91)90006-A.
[38] Ellington JE, Samper JC, Jones AE, Oliver SA, Burnett KM, Wright RW. In vitro interactions of cryopreserved stallion spermatozoa and oviduct (uterine tube) epithelial cells or their secretory products. Anim Reprod Sci 1999;56:51–65. https://doi.org/10.1097/WNO.0b013e31828657d6.
[39] Valcárcel A, de las Heras MA, Moses DF, Perez LJ, Baldassarre H. Comparison between Sephadex G-10 and Percoll for preparation of normospermic, asthenospermic and frozen/thawed ram semen. Anim Reprod Sci 1996;41:215–24. https://doi.org/10.1016/0378-4320(95)01453-5.
[40] Aitken R, Sutton M, Warner P, Richardson W. Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. J Reprod Fertil 1985;73:441–9. https://doi.org/10.1530/jrf.0.0730441.
[41] Sánchez-Calabuig MJ, García-Vázquez FA, Laguna-Barraza R, Barros-García C, García-Parraga D, Rizos D, et al. Bottlenose dolphin (Tursiops truncatus) spermatozoa: collection, cryopreservation, and heterologous in vitro

fertilization. JoVE 2017. https://doi.org/10.3791/55237.
[42] Sánchez-Calabuig MJ, de la Fuente J, Laguna-Barraza R, Beltrán-Breña P, Martínez-Nevado E, Johnston SD, et al. Heterologous murine and bovine IVF using bottlenose dolphin (Tursiops truncatus) spermatozoa. Theriogenology 2015;84:983–94. https://doi.org/10.1016/j.theriogenology.2015.06.001.
[43] Adham IM, Kremling H, Nieter S, Zimmermann S, Hummel M, Schroeter U, et al. The structures of the bovine and porcine proacrosin genes and their conservation among mammals. Biol Chem Hoppe Seyler 1996;377:261–5.
[44] Paulenz H, Ådnøy T, Fossen OH, Söderquist L. Effect on field fertility of addition of gelatine, different dilution rates and storage times of cooled ram semen after vaginal insemination. Reprod Domest Anim 2010;45:706–10. https://doi.org/10.1111/j.1439-0531.2008.01338.x.
[45] Álvarez M, Chamorro CA, Kaabi M, Anel-López L, Boixo JC, Anel E, et al. Design and "in vivo" evaluation of two adapted catheters for intrauterine transcervical insemination in sheep. Anim Reprod Sci 2012;131:153–9. https://doi.org/10.1016/J.ANIREPROSCI.2012.03.001.