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

D.A. Galarza a, b, M. Ladrón de Guevara a, P. Beltrán-Breña a, M.J. Sánchez-Calabuig c, * D. Rizos b, A. López-Sebastián a, J. Santiago-Moreno a

a Departamento de Reproducción Animal, INIA, 28040, Madrid, Spain
b Facultad de Ciencias Agropecuarias, Universidad de Cuenca, ECO10205, Cuenca, Ecuador
c Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, UCM, Madrid, Spain

A R T I C L E   I N F O
Article history:
Received 16 February 2019
Accepted 25 April 2019
Available online 25 April 2019

Keywords:
Skim milk
TEST
Cold-stored sperm
Filtration

A B S T R A C T
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

* Corresponding author.
E-mail address: m Sanchez26@ucm.es (M.J. Sánchez-Calabuig).
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-hart-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 and frozen semen [26]. The rams were housed at the INIA Department of Animal Reproduction in the same conditions as non-synthetic extenders were made according Gil et al. [20] with some modifications: (1) ultra-hart-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) ultra-HLT plus 2% (v:v) glycerol (GLY-TEST). In addition, two non-synthetic-based extenders were made according Gil et al. [20] with some modifications: (1) ultra-hart-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) ultra-HLT plus 2% (v:v) glycerol (GLY-TEST). 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 osmolality 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 × 10⁸ 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 × 10⁶ 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®, 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, 150 µL of semen diluted in TALP Stock medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH2PO4, 0.50 mM MgCl2.H2O, 1 mM Na-Lactate, 1.97 mM MgCl2.2H2O, 0.50 mM MgCl2.6H2O, 10 mM HEPES sodium, and 25 mM NaHCO3; 320 mOs/mg, pH 7.3) to a concentration of 25 × 10⁶ 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 fluorescence 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 Pradie 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% CO2 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 × 10⁶ sperm/mL.

A heterologous group using ram sperm and 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% CO2 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 above for sperm-zona pellucida binding. The cleavage rate was established: (1) non-filtered sperm extended with TEST-GLY; (2) 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 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 312 ± 312 ng/mL] were 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, Tulsa, 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 at all times. Finally, the %DPDALM value was lower (P < 0.05) with UHT extenders than either TEST extenders at all times. The %DPDALM value was lower (P < 0.05) with either UHT extender than either TEST extender for 24 to 96 h. The %DPDALM 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.

3.2. 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 %IPIAHM value was greater (P < 0.05) with either UHT extender than either TEST extender at all times. Finally, the %IPIAHM value was lower with UHT diluents than using TEST diluents at 96 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.
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. 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, d–e, 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).
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

---

**Fig. 2.** Status of sperm, plasma-, acrosome- and mitochondrial membranes (association of PI/PNA-RTIC/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. %IPAHM, intact plasma membrane/intact acrosome/high mitochondrial function; %IPDAHM, intact plasma membrane/damage acrosome/high mitochondrial function; %DIPAHM, intact plasma membrane/intact acrosome/high 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).
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-washed or classically washed sperm. An increased ALH value during semen filtration therefore seems to facilitate the selection of non-capacitated ram sperm.

To prove the fertilizing capability 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–Citic–Fructose [45] or UHT [2] extenders, respectively. Thus, the IVF values obtained in the present study suggest that ram sperm preserved in UHT extender with Sephadex for over 96 h, irrespective of sperm filtration, maintain in vitro fertilization capacity similar to that of fresh sperm.

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.

Acknowledgements

This work was part of a project that received funding from the European Union’s Horizon 2020 Research and Innovation Programme under grant agreement N°677353. Also, part of this work was funded by the Spanish Ministry of Economy and Competitiveness AGL2015-70140-R. The pre-doctoral funding granted by SENESCYT-Ecuador via the program of PhD scholarships for university teachers 2016, is gratefully acknowledged (Scholarship No. ARSEQ-BEC-008856-2016).

References


