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Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics

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ABSTRACT

Density-gradients centrifugation (DGC) and filtration columns (FC) are used to separate deformed or dead sperm, debris, and other cells that may negatively affect the fertilizing capacity of sperm in fresh, chilled and frozen/thawed semen. The present study was conducted to evaluate the suitability of DGC (BoviPure®, Percoll® and Accudenz®) and FC (Sephadex G-15®) sperm selection procedures for fresh-extended and cold-stored ram semen by assessment of post-treatment sperm quality variables. Twenty normospermic ejaculates from ten adult Merino rams were used. Sperm concentration of recovered cells was greater ($P < 0.001$) after BoviPure treatment than other procedures in both fresh and cold semen. With the Sephadex method, there were more desirable values than with use of DGC procedures in several sperm motility variables measured by using the CASA system. In non-refrigerated semen samples, the percentage of progressive sperm motility (%PSM) after Sephadex filtration was greater ($P < 0.05$) than after BoviPure treatment; the straightline velocity (VSL) value after Sephadex filtration was greater ($P < 0.01$) than after Accudenz treatment; the amplitude of lateral head displacement (ALH) after Sephadex and Accudenz treatment was less than non-filtered semen ($P < 0.001$) and after Percoll ($P < 0.01$) and BoviPure ($P < 0.05$) treatments. In cold-stored semen samples, the %PSM after Sephadex filtration was greater than non-filtered ($P < 0.05$) semen and after BoviPure ($P < 0.05$), Percoll ($P < 0.05$) and Accudenz ($P < 0.001$) treatments. It is concluded that Sephadex column filtration can be used to select ram sperm in non-refrigerated and cooled semen, because percentage progressively motile sperm and some other sperm motility characteristics are greater with use of this techniques as compared with use of DGC methods.

1. Introduction

Artificial insemination has an important role in sheep breeding but its use is limited because the poor fertility achieved when stored semen is used for vaginal insemination (Gil et al., 2003). The success of this procedure in sheep is limited by the anatomic characteristics of the ewe's cervix and the short time that ram sperm can be stored as a liquid.

The sperm characteristics and fertilizing capacity of extended ram semen may be improved by applying methods for sperm selection prior to artificial insemination. Dead and non-functional sperm, debris and all dead cells could affect live sperm probably as

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a production of reactive oxygen species (ROS) that accumulates in deleterious concentrations, which have been reported as inductors of apoptosis (Wang et al., 2003). Sperm selection methods are used with the aim of gaining seminal plasma-free suspensions of predominantly motile sperm with reduced presence of abnormal, immature, and dead spermatozoa, epithelial cells, cell debris, lymphocytes, etc. (Valcárcel et al., 1996; Phillips et al., 2012), potentially allowing for an enhanced fertilizing capacity (Graham and Graham, 1990). Sperm selection methods, however, may have adverse "iatrogenic" effects related to mechanical damage due to centrifugation-resuspension procedures, and the promotion of lipid peroxidation during sperm pelleting due to the close vicinity of leukocytes and damaged sperm that may generate reactive oxygen species (ROS)(Sbracia et al., 1996).

With selective washing techniques based on density-gradient centrifugation (DGC), the sperm are selected according to the density, allowing for the isolation of motile and morphologically normal sperm. Currently, there are several commercial solutions available for sperm separation by DGC´s procedures. BoviPure® is an iso-osmotic salt solution containing colloidal silica particles coated with silane used to select bull sperm for use with artificial reproductive techniques (Samardzija et al., 2006). Silane-coated silica media are have also been proposed to be used in a single layer centrifugation (Martinez-Alborcia et al., 2013). Percoll® is a medium composed of colloidal silica particles (15–30 nm in diameter), coated with polyvinylpyrrolidone, and can be used for sperm selection in several mammalian species (Batista et al., 2011). Accudenz® (formerly called Nycodenz) is a non-ionic tri-iodinated derivative of benzoic acid with three aliphatic hydrophilic side chains of high density (2.1 g/ml) due to the presence of a substituted ring, which is linked to hydrophilic groups to enhance water solubility, and has been used in sperm selection of humans (Gellert-Mortimer et al., 1988; Sbracia et al., 1996), and cheetahs (Crosier et al., 2009), among other species. To our knowledge, no studies have been reported on the use of Accudenz® for selecting ram sperm.

Another technique of sperm separation is filtration through Sephadex columns. Sephadex® is a dextran gel available in different pore (filtration) sizes (G-10 to G-120). The post-thaw quality of sperm recovered after filtration through Sephadex was assessed to be highly acceptable when Sephadex was used for human (Drobnis et al., 1991), bull (Januskauskas et al., 2005; Lee et al., 2009), buffalo (Ahmad et al., 2003), stallion (Sieme et al., 2003), and boar (Bussalleu et al., 2008) semen, and was promising when used for filtering ram sperm (Landa et al., 1980; Valcárcel et al., 1996). In addition, with use of semen from bulls with lesser fertility for insemination that had been filtered through Sephadex there was an improved 60–90 day non-return rate (Graham and Graham, 1990). The mechanism for the trapping of sperm in Sephadex columns remains unclear. The filtration, however, is believed to be based on the fact that non-viable sperm tend to adhere to the Sephadex matrix to a greater extent than motile and seemingly functional sperm, and the latter are able to cross the filtration barrier without modification of functional characteristics (Bussalleu et al., 2008).

For ram semen, a number of sperm selection methods have been tried, including Sephadex filtration (see above), swim-up methods (García-López et al., 1996), sucrose washing, and Millipore filtration (Marti et al., 2006), as well as Percoll density gradient centrifugation (DGC) (Valcárcel et al., 1996). Even though there have been great advances in DGC procedures for sperm selection with different species (Santiago-Moreno et al., 2014, 2016), these procedures include centrifugal forces that may be harmful, especially for ram sperm (García-López et al., 1996). Hence, it was hypothesized that methods that involve little or no centrifugation, such as Sephadex filtration methods, may have advantages as compared with DGC for use in sperm selection in this species. In the present research, the effectiveness of Sephadex G-15® was compared with different DGC procedures (BoviPure®, Percoll®, Accudenz®) in fresh-extended semen and cold-stored ram semen.

2. Material and methods

Percoll® (Sigma P1644) and Sephadex G-15® (Sigma G15120-50 g) were obtained from Sigma Chemical Co., (St. Louis, Missouri, USA); Accudenz® (AN7050) was obtained from Accurate Chemical and Scientific Corporation (Westbury, New York, USA); and BoviPure® (BP-100) was obtained from Nicadon Laboratory (Nidacon, Mölndal, Sweden). All diluents and media were prepared in the INIA Department of Animal Reproduction Research Laboratory using reagent-grade chemicals purchased from Panreac Chemistry S.A. (Barcelona, Spain) and Sigma Chemical Co.

2.1. Animals, semen collection and initial evaluation

Ten adult Merino rams (2–7 years of age) that were assessed to be clinically healthy were used. All animals were housed at INIA Department of Animal Reproduction (Madrid, Spain, 40°25´N) using the same management conditions for all animals and rams were fed a basal diet that consisted of: grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available *ad libitum*. All animals were handled according to procedures approved by the INIA Ethics Committee, and the research 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.

Twenty semen ejaculates (two ejaculates per ram, collected with a 7 day interval) were collected using an artificial vagina (42–43 °C). The rams were previously trained with an intact oestrus induced-ewe and then an ovariectomized female was used as a "teaser" to stimulate ram sexual behaviors. The volume of each ejaculate was measured in a graduated conical glass tube in millilitres (ml). Briefly, after collection, the semen was diluted 1:1 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). All the materials, including the artificial vagina and glass collecting tube were maintained at 37 °C before collections. This fresh-extended semen samples were transported to the research laboratory immediately after collection at 37 °C and initial motility was evaluated prior to processing. Sperm concentration was determined using a photometer (SDM 1, Minitube, Germany).

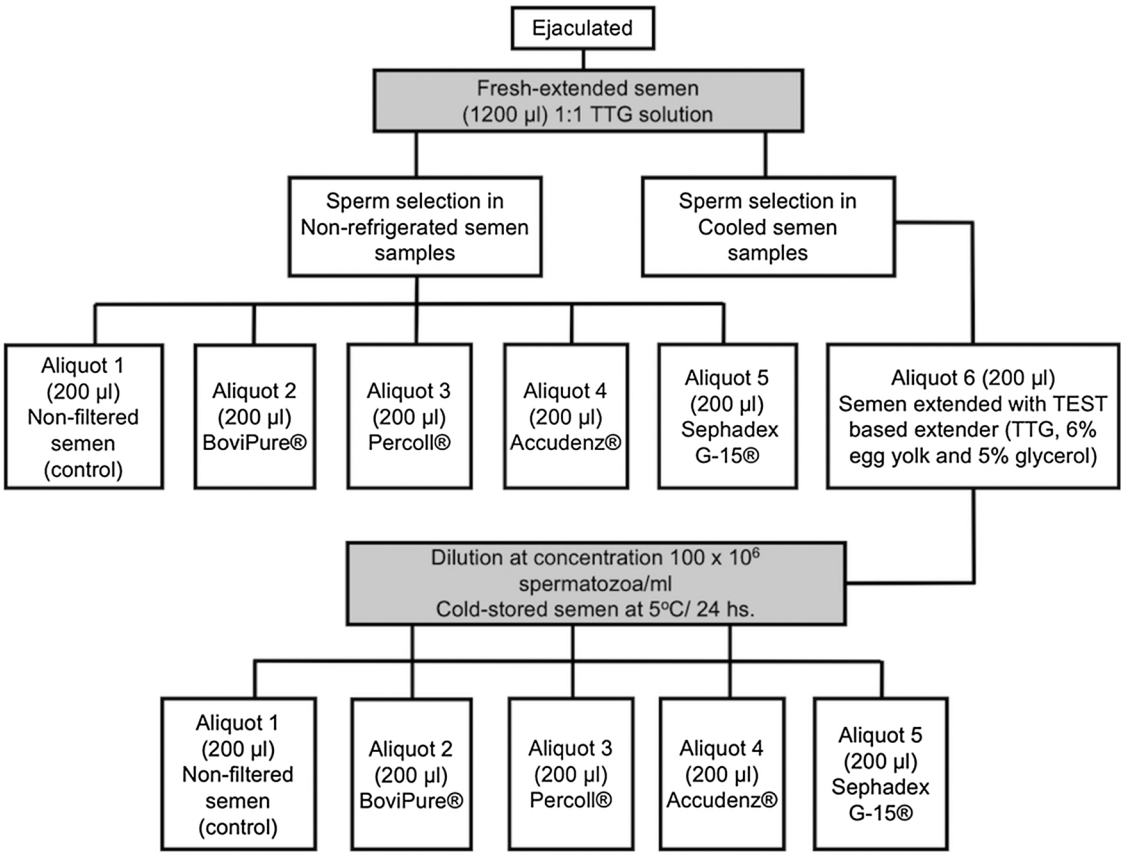

Fig. 1. Experimental design.
TTG solution: Tes, Tris and glucose.

2.2. Experimental design

A total of 1200 µl of fresh-extended semen from each ejaculate was used and divided into six 200 µl-aliquots. The sperm selection procedures were performed with both non-refrigerated and cooled semen samples (Fig. 1).

2.2.1. Sperm selection in non-refrigerated semen samples

Five aliquots of 200 µl each of fresh-extended semen of each ejaculate were maintained and used at room temperature. One aliquot was considered as the control (non-filtered semen samples) and the four other aliquots were used to purify the sperm with BoviPure, Percoll, Accudenz and Sephadex G-15 procedures. These five samples per ejaculate are referred to as "non-refrigerated semen".

2.2.2. Sperm selection in cooled semen samples

The remaining (sixth) aliquot of 200 µl fresh-extended semen of each ejaculate was diluted with TEST based extender (TTG solution, egg yolk 6% (v/v), and glycerol 5% (v/v); 320 mOsm/kg water, pH 7.2) to a concentration of 100×10^6 sperm/ml. These diluted sperm samples were chilled at 5 °C and stored for 24 h, then five aliquots of 200 µl each were taken and processed as described above for non-refrigerated semen samples. One aliquot was considered as the control (non-filtered cooled semen) and the remaining four aliquots were processed with the four sperm selection procedures. These chilled samples are referred to as "cold-stored semen".

2.3. Sperm selection procedures

Sperm selection was performed in fresh-extended and cold-stored semen samples. The entire sperm selection process for both DGC and filtration column (FC) methods were performed at room temperature (i.e. cold stored semen samples were allowed to rewarm to room temperature prior to being used).

2.3.1. BoviPure® DGC

Following the protocol described by Santiago-Moreno et al. (2017), BoviPure® solution was diluted with BoviDilute® solution to obtain BoviPure® Bottom layer medium and BoviPure upper layer of medium, at 80% and 40% concentration, respectively. The

BoviPure DGC columns were prepared in 15-ml Falcon® tubes: equal volumes of BoviPure® Bottom layer- and Top layer medium were successively layered in the tubes, using a total column volume of 1 ml per 333 million sperm present in the 200 µl sperm sample. The fresh-extended or cold-stored semen samples, which had been initially allowed to reach room temperature, were gently layered on top of the BoviPure Top medium. The columns were centrifuged at 300 *g* for 20 min. After centrifugation, the fluid above the sperm pellet was carefully removed. The final pellets were re-suspended, each in 100 µl TEST based extender at 37 °C, and sperm quality variables were evaluated.

2.3.2. Percoll® DGC

The 90% isotonic density solution was prepared by diluting 900 µl commercial Percoll® with 100 µl TAPL10 X medium (992.30 mM NaCl, 248 mM NaHCO3, 99.87 mM HEPES sodium, 0.03 mM KH2PO4 H2O, 0.15 mM MgCl 6H2O, 0.26 mM CaCl 2H2O, and 0.1 mM Na-Lactate); the 60% isotonic density solution was prepared by diluting 335 µl of 90% isotonic density solution and 165 µl of TALP Stock medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH2PO4 H2O, 1 mM Na-Lactate, 1.97 mM CaCl 2H2O, 0.50 mM MgCl 6H2O, 10 mM HEPES sodium, and 25 mM NaHCO3; 320 mOsm/Kg, pH 7.3); and the 30% isotonic density solution was prepared by diluting 165 µl of 90% working density solution and 335 µl of TALP Stock medium (Parrish et al., 1995). The Percoll density gradients were made by layering sequentially 400 µl of 90% Percoll solution, 400 µl of 60% solution, and 400 µl of 30% solution in 15 ml Falcon® tubes. Subsequently, 200 µl of the fresh-extended or cold-stored semen samples, which had been allowed first to reach room temperature, were layered on top of the prepared Percoll layers and then tubes were centrifuged at 600 *g* for 10 min. The pellets were re-suspended in 2.5 ml Sperm TALP medium (TALP Stock medium, 6 mg/ml BSA (Sigma-Aldrich A-9647), 0.11 mg/ml Na Pyruvate, and 5 µl/ml gentamycin; 326 mOsm/Kg, pH 7,6) at 37 °C and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.3.3. Accudenz® DGC

An iso-osmotic Accudenz® solution was prepared according to the manufacturer's directions. The Accudenz Stock medium was prepared by mixing 2.76 g Accudenz® (density = 1.15 g/ml) with a buffer solution (7.5 mg/ml NaCl dissolved in a solution of 100 ml of 5 mM Tris-HCl (pH 7.5), 3 mM KCl, and 0.3 mM CaNa2 EDTA). A series of isotonic Accudenz media were prepared with 35%, 40%, 50%, 65%, 70%, 80% and 100% of the Accudenz stock medium mixed with the appropriate amount of TALP stock medium. Centrifugation times and centrifugal (*g*) forces were used according to Gellert-Mortimer et al. (1988) and Sbracia et al. (1996) to test which of these combinations resulted in greater sperm motility after semen processing (subjective sperm motility was used as a reference sperm quality variable). These working conditions were established in previous experiments (data not shown). The discontinuous gradients that gave the optimal motile sperm selection consisted of 400 µl of 100%, 400 µl of 65%, 800 µl of 50%, and 400 µl of 35% Accudenz media layered on top of each other in 15-ml Falcon® tubes. The fresh-extended and cold-stored semen samples, which had been allowed first to reach room temperature, were gently placed on top of the top layer (35% solution) and the tubes were centrifuged at 300 *g* for 12 min. The pellets were re-suspended in 2.5 ml of sperm TALP medium at 37 °C and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.3.4. Sephadex® filtration column

A Sephadex suspension was prepared by hydrating Sephadex G-15® for at least 24 h in sodium citrate 3% (v/v), according to Valcárcel et al. (1996). Filtration columns of three cm height were prepared in a 2.5-ml glass chromatography syringes (Sigma-2099820,998. Hamilton® syringe, 1000 series GASTIGHT®, PTFE luer lock. 1002 TL L, volume 2.5 mL), with needle size 22 G, L 51 mm-2 in (Sigma-21746. Hamilton® needles). The columns were prepared immediately before filtration and kept in a vertical position at room temperature. The fresh-extended and cold-stored semen samples, which had been initially been allowed to reach room temperature, were placed on top of the Sephadex columns and filtered for 15 min. The filtration process was achieved by lightly pressing the syringe plunger. Subsequently, the filtrate liquid was diluted in 2.5 ml of sperm TALP medium at room temperature and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.4. Sperm analysis

The sperm concentration post-selection was estimated using a Neubauer chamber (Marienfeld, Lauda- Königshofen, Germany). The motility analysis was objectively assessed using a CASA system (Sperm Class Analyzer, SCA® 1999, v.4.0, software. Microptic S.L., Barcelona, Spain) coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast). 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). The following sperm kinetic variables were assessed, as previously described by Dorado et al. (2007): percentage motile sperm (%SM), percentage progressive sperm (%PSM), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), wobble (%WOB), linearity (%LIN) straightness (%STR), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, µm). Sperm viability and acrosomal membrane status were analyzed by fluorescence microscopy (counting 200 cells), using a Nikon Eclipse E200 epifluorescence microscope (D-FL epifluorescence, C-SHG1 super high-pressure mercury power supply; Nikon Instruments Inc., New York, USA), using propidium iodide (PI, Sigma P-4170) and fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC, Sigma L7381), as previously described by Santiago-Moreno et al. (2014).

The FITC-PNA/PI fluorescence test provides percentages of four sub-categories: sperm with intact plasma and acrosome membranes (%PIAI), with intact plasma membrane and damaged acrosome (%PIAD); with damaged plasma membrane and intact acrosome (%PDAI); and with damaged plasma and acrosome membranes (%PDAD). Sperm morphological abnormalities were evaluated placing a 5 µl of the semen samples in 100 µl of glutaraldehyde 2% (v/v) solution. A drop of 5 µl of this mixture was examined using a phase contrast microscope at magnification 400×. The morphology of 200 sperm was assessed to score the percentage of abnormal heads, loose normal heads, mid-piece defects, abnormal tails and coiled tails and cytoplasmic droplets (Frank, 1950).

2.5. Statistical analysis

The results are presented as mean ± SEM. The sperm variable values were normally distributed as determined by Shapiro-Wilk´s test. These percentage variables (kinetic variables of CASA analysis, plasma and acrosome membrane integrity and morphological abnormalities), therefore, were arcsine transformed and the non-percentages variables (sperm concentration of cells recovered, velocities, BCF and ALH) were log-transformed before analysis. One-way ANOVA and Bonferroni's multiple comparisons test was used to examine the effect of sperm selection procedures in the non-refrigerated and cooled semen samples on sperm kinetic variables, plasma and acrosomal membrane integrity and sperm concentration post-selection. In addition, data were compared for non-refrigerated vs cooled semen samples in each sperm selection methods by one-way ANOVA. For the assessment of sperm morphological abnormalities, a factorial analysis of 2 × 5 was used that included semen type (fresh-extended and cold-stored semen samples) and sperm selection method (non-filtered semen and filtered semen with BoviPure, Percoll, Accudenz and Sephadex). A multifactorial ANOVA and Bonferroni´s test was used to assess the interaction between factors and the effect of the sperm selection procedures.

All calculations were made using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA).

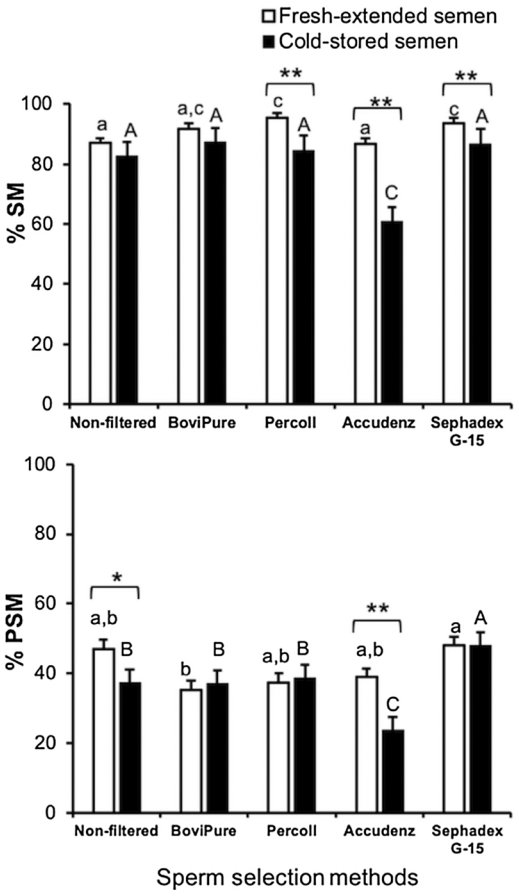

Fig. 2. Sperm motility (%SM) and progressive sperm motility (%SPM) in non-filtered samples and after sperm selection using different procedures with fresh-extended and cold-stored semen.

[a,b,c]Different superscripts for the fresh – extended semen data indicate differences between selection procedures ([a,b]$P < 0.05$ and [a,c]$P < 0.001$).

[A,B,C]Different superscripts for the cold – stored semen data indicate differences between selection procedures ([A,B]$P < 0.05$; [B,C]$P < 0.01$, and [A,C]$P < 0.001$).

**Asterisks indicate differences between values for fresh-extended and cold-stored semen within each selection procedure (*$P < 0.05$, **$P < 0.001$).

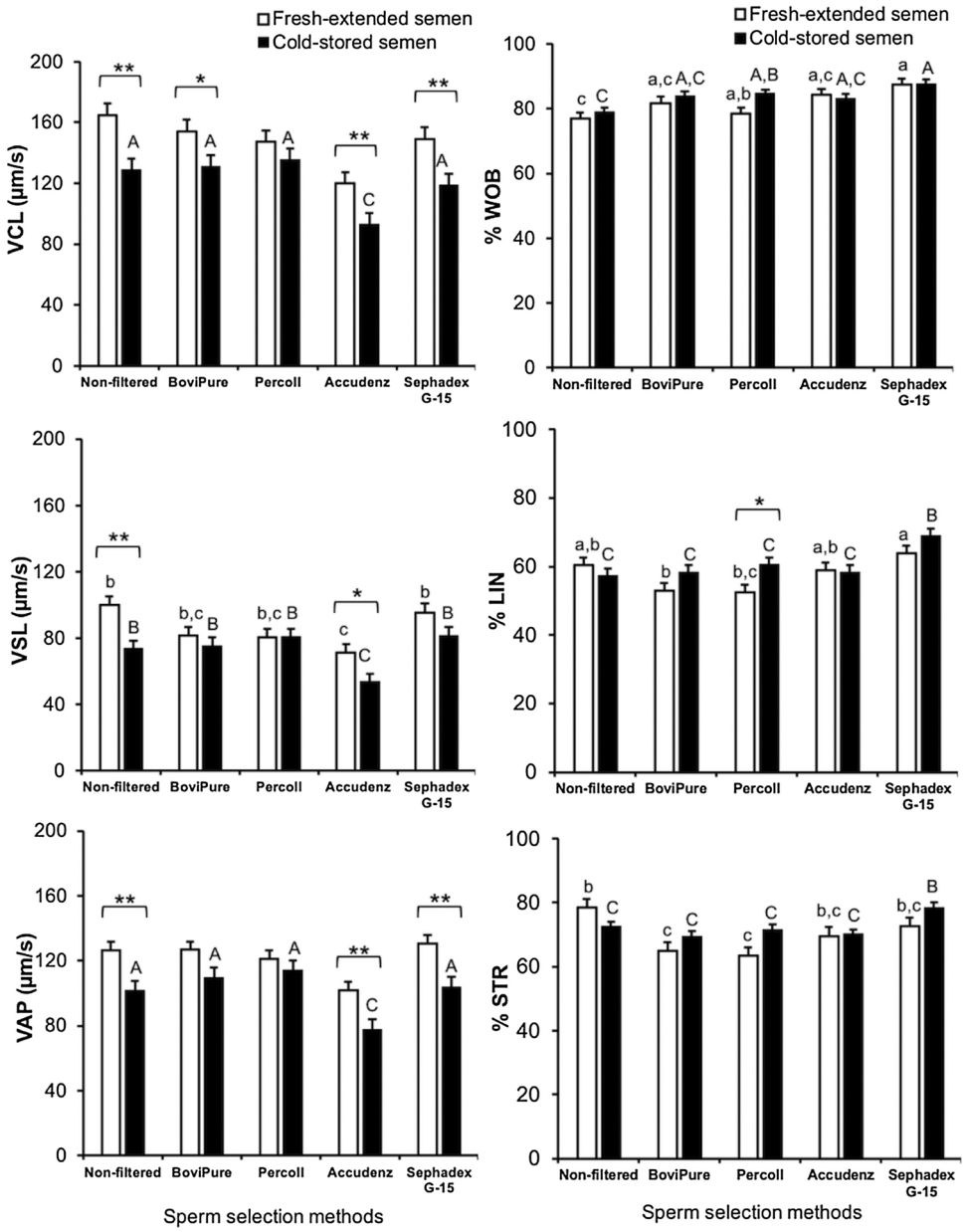

Fig. 3. Sperm motility characteristics (curvilinear velocity: VCL; straight-line velocity: VSL; average path velocity: VAP; linearity: LIN; straightness: STR; Wobble: WOB) for values of non-filtered samples and after sperm selection with different procedures, in fresh-extended and cold-stored semen.
[a,b,c]Different superscripts associated with values for fresh − extended semen indicate differences between selection procedures ([a,b]$P < 0.05$, [b,c]$P < 0.01$ and [a,c]$P < 0.001$).
[A,B,C]Different superscripts associateD with values for cold − stored semen indicate differences with use of the different selection procedures ([B,C]$P < 0.01$, and [A,C]$P < 0.001$).
**Asterisks indicate differences in values between fresh-extended and cold-stored semen for each selection procedure (*$P < 0.05$, **$P < 0.001$).

3. Results

Differences in sperm motility variables between sperm selection methods, and between fresh-extended and cold-stored samples are depicted in Figs. 2–4. Data for membrane and acrosome integrity, and morphological abnormalities are included in Tables 1 and 2.

3.1. Sperm selection in non-refrigerated semen samples

Sperm concentration (x 10^6 sperm/ml) in non-filtered fresh-extended semen samples was 2238.1 ± 28. The recovered sperm

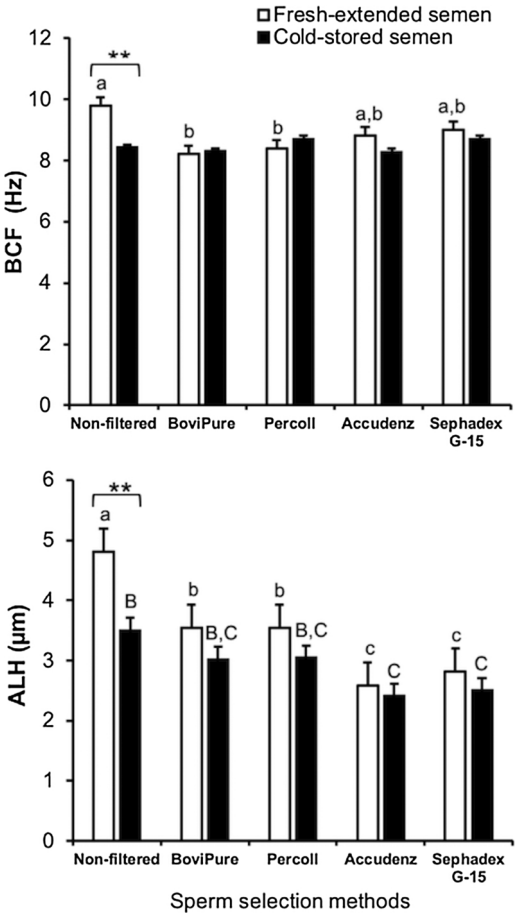

Fig. 4. Flagellar beat frequency (BCF) and amplitude of lateral head displacement (ALH) in non-filtered samples and after sperm selection using different procedures for fresh-extended and cold-stored semen.
[a,b,c]Different superscripts for values of the fresh − extended semen indicate differences with use of different selection procedures ($^{a,b}P < 0.05$, $^{b,c}P < 0.01$ and $^{a,c}P < 0.001$).
[B,C] Different superscripts associated with values for the cold − stored semen indicate differences between selection procedures ($^{B,C}P < 0.01$).
**Asterisks indicate differences in values between fresh-extended and cold-stored semen within each selection procedure ($^{**}P < 0.001$).

Table 1
Plasma and acrosomal membrane integrity in fresh-extended and cold-stored semen (mean ± SEM).

Fluorescence parameters	Non-filtered semen		Sperm selection procedures			
			BoviPure®	Percoll®	Accudenz®	Sephadex G-15®
%PIAI	1	71.6 ± 3.4	74.7 ± 2.4	74.5 ± 3.3	71.2 ± 3.7	78.6 ± 3.6
	2	66.9 ± 2.7	77.8 ± 2.5	71.5 ± 3.1	73,8 ± 3.1	75.3 ± 2.9
%PIAD	1	2.3 ± 0.8	1.5 ± 0.8	1.0 ± 0.6	1.6 ± 0.6	1.3 ± 0.5
	2	1.9 ± 0.5^{a,b}	1.6 ± 0.5^{a,b}	2.1 ± 0.7^{a}	1.7 ± 0.4^{a,b}	1.5 ± 0.4^{b}
%PDAI	1	20.4 ± 2.8	21.6 ± 2.0	22.3 ± 2.9	24.9 ± 3.2	16.5 ± 3.0
	2	24,2 ± 2.2	17.2 ± 2.0	23.2 ± 2.5	21.7 ± 2.5	18.7 ± 2.3
%PDAD	1	5.8 ± 0.9^{A}	2.7 ± 0.6^{B}	2.3 ± 0.7^{B}	2.3 ± 0.5^{B}	3.6 ± 0.9^{A,B}
	2	7.1 ± 1.4^{a}	3.5 ± 0.9^{a,b}	3.8 ± 0.7^{a,b}	2.9 ± 0.8^{b}	4.0 ± 0.9^{a,b}

[1] Fresh-extended semen samples; [2] Cold-stored semen samples; %PIAI, spermatozoa percentage with intact plasma and acrosome membranes; %PIAD, spermatozoa percentage with intact plasma membrane and damaged acrosome; %PDAI, spermatozoa percentage with damaged plasma membrane and intact acrosome; and % PDAD, spermatozoa percentage with damaged plasma and acrosome membranes; [a,b,A,B] Values within a row in both fresh − extended and cold − stored semen samples with different superscripts, differ significantly (^{a,b} P < 0.05; ^{A,B} P < 0.01).

concentration was greater ($P < 0.001$) with BoviPure® method (838.0 ± 72) than with Percoll®, Accudenz® and Sephadex® (701.0 ± 82, 643.5 ± 42 and 523.5 ± 43, respectively).

The %SM after Sephadex and Percoll treatment was greater ($P < 0.001$) than in the non-filtered semen and after Accudenz

Table 2
Sperm morphological abnormalities (mean ± SEM) in fresh-extended and cold-stored semen.

Morphological abnormalities		Non-filtered semen	Sperm selection methods			
			BoviPure®	Percoll®	Accudenz®	Sephadex G-15®
Abnormal heads (%)	1	1.4 ± 0.3	0.4 ± 0.1	1.1 ± 0.4	0.4 ± 0.1	1.1 ± 0.3
	2	1.4 ± 0.3^{a}	1.2 ± 0.4^{a,b}	0.6 ± 0.2^{a,b}	0.6 ± 0.2^{a,b}	0.3 ± 0.1^{b}
Loose heads (%)	1	1.9 ± 0.4^{a}	1.0 ± 0.3^{a}	1.1 ± 0.4^{a}	0.5 ± 0.2^{b}	1.6 ± 0.4^{a}
	2	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	0.6 ± 0.3	0.4 ± 0.1
Abnormal midpieces (%)	1	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
	2	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.9 ± 0.4	0.3 ± 0.2
Abnormal tails (%)***	1	2.6 ± 0.8^{B}	1.4 ± 0.2^{B}	1.0 ± 0.3^{B}	4.9 ± 0.7^{A}	1.5 ± 1.4^{B}
	2	1.8 ± 0.4^{B}	2.7 ± 0.9^{B}	7.3 ± 1.8^{A}	6.0 ± 0.6^{A}	2.2 ± 0.6^{B}
Coiled tails (%)	1	6.6 ± 0.8	4.8 ± 0.7	3.9 ± 0.9	5.5 ± 1.1	6.0 ± 1.3
	2	18.9 ± 3.0	17.8 ± 3.4	10.5 ± 2.1	16.7 ± 2.3	21.7 ± 3.4
Cytoplasmic droplets (%)	1	1.2 ± 0.4	1.4 ± 0.5	0.4 ± 0.4	0.7 ± 0.5	0.6 ± 0.4
	2	1.7 ± 0.5	1.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.4	0.6 ± 0.3
Total abnormalities (%)	1	1 4.1 ± 1,2^{A}	9.1 ± 1.1^{A}	7.5 ± 1.1^{B}	12.0 ± 1.5^{A}	10.9 ± 1.4^{A}
	2	24.9 ± 2.8	24.2 ± 3.3	19.8 ± 2.8	25.3 ± 2.8	25.4 ± 3.7

[1] Fresh-extended semen samples; [2] Cold-stored semen samples; ***Interaction between factors (fresh-extended and cold-stored semen and type of selection procedures) at P < 0.001; [a,b,A,B] Values within a row with different superscripts show difference significantly between values of sperm selection methods ([a,b] P < 0.05; [A,B] P < 0.01).

treatment, respectively. The %PSM after Sephadex filtration was greater ($P < 0.05$) than after BoviPure treatment (Fig. 2). The VSL value of Sephadex filtered sperm and of non-filtered sperm were both greater ($P < 0.01$) than after Accudenz treatment. The %WOB after Sephadex filtration ($P < 0.001$) and after Percoll ($P < 0.05$) treatment were greater than in non-filtered semen. The %LIN after Sephadex filtration was greater than after BoviPure ($P < 0.05$) and Percoll ($P < 0.01$) treatment (Fig. 3). The ALH after Sephadex and Accudenz treatment was less than in non-filtered semen ($P < 0.001$) and after Percoll ($P < 0.01$) and BoviPure ($P < 0.01$) treatments (Fig. 4).

In non-refrigerated semen, the percentage of sperm with a damaged plasma membrane and acrosome was greater in non-filtered semen compared with cells after all DGC treatments, except after Sephadex filtration ($P < 0.05$) (Table 1).

There was an interaction between factors (non-refrigerated/cooled semen and type of sperm selection procedure) for the percentage of abnormal sperm tails ($P < 0.001$). The percentage of sperm with loose heads was less ($P < 0.05$) after Accudenz treatment compared with the others sperm selection procedures. The percentage of sperm with abnormal tails after Accudenz treatment, however, was greater ($P < 0.001$) than when the other selection procedures were used. The percentage of total abnormalities after Percoll treatment was less ($P < 0.01$) than in non-filtered semen and after the other sperm selection methods (Table 2).

3.2. Sperm selection in cold-stored semen samples

Similar to non-refrigerated semen samples, the concentration of sperm (x 10^6 sperm/ml) recovered in cooled samples was greater ($P < 0.001$) after BoviPure treatment than after Accudenz, Sephadex and Percoll treatments (67.0 ± 6.1 compared with 55.5 ± 6.6, 52.5 ± 5.8 and 43.0 ± 7.2, respectively).

The %SM after Accudenz treatment was less ($P < 0.001$) than in non-filtered semen and after all other selection methods treatments. The %PSM after Sephadex filtration was greater than in non-filtered ($P < 0.05$) semen and after BoviPure ($P < 0.05$), Percoll ($P < 0.05$) and Accudenz ($P < 0.001$) treatments (Fig. 2). The VCL ($P < 0.001$), VSL ($P < 0.01$) and VAP ($P < 0.001$) values after Accudenz treatment were less than in non-filtered semen and after all other sperm selection methods treatments (Fig. 3). The %WOB after Sephadex filtration ($P < 0.001$) and Percoll ($P < 0.01$) treatment were greater than in non-filtered semen. Furthermore, the %LIN and %STR after Sephadex filtration were greater ($P < 0.01$) than in non-filtered semen and after all DGC treatments (Fig. 3). The ALH after Sephadex and Accudenz treatments was less ($P < 0.01$) than in non-filtered semen (Fig. 4).

For the cold-stored semen, the percentage of sperm with an intact plasma membrane and damaged acrosome after Sephadex filtration was less ($P < 0.05$) than with the Percoll treatment. Furthermore, the percentage of sperm with a damaged plasma and acrosome membranes after Accudenz treatment was less (P < 0.05) than non-filtered semen (Table 1).

The percentage of abnormal heads after Sephadex filtration was less ($P < 0.05$) than non-filtered semen. The percentage of abnormal tails after Sephadex and BoviPure treatments and non-filtered semen were also less ($P < 0.01$) than after Percoll and Accudenz treatments (Table 2).

4. Discussion

In non-refrigerated semen, the use of all sperm selection treatments appeared to reduce the percentage abnormalities and percentage of sperm cells with a damaged plasma membrane and/or acrosome membrane, although the effects were small and not all pairwise comparisons with non-filtered sperm were significant, and the use of Accudenz had a negative effect on the % abnormal

sperm tails. Regarding motility, only the Percoll and Sephadex treatments resulted in a greater percentage of motile sperm. The BoviPure and Accudenz treatments had negative effects on percentage of motile sperm in non-refrigerated and in cold-stored semen, respectively. The percentage progressively motile sperm was only greater with use of Sephadex filtration and not with use of the DGC methods. Also, several of the CASA motility characteristics were greater with Sephadex filtration (e.g., progression ratio values) than after using DGC selection procedures (with a number of comparisons beings significant). This suggests that Sephadex filtration results in a more desirable ram sperm quality than with use of the DGC procedures. For many of the measured variables, Accudenz centrifugation had a negative effect on sperm quality.

For ram semen, it has been reported that centrifugation of semen is very harmful to sperm (García-López et al., 1996). Sperm selection procedures without centrifugation, therefore, could be beneficial for sperm quality of the treated semen. For this reason, alterative techniques, such as the dextran-swim up method, have been developed in rams (García-López et al., 1996; Pérez-Pé et al., 2001; Grasa et al., 2004). It, therefore, was hypothesized that filtration methods, such as Sephadex G-15® filtration, may be a useful technique for successfully selecting sperm in rams. Marti et al. (2006) found that there was no satisfactory selection of relatively greater quality sperm by filtration of ram semen when using a 5 μm pore Millipore filter. But Sephadex filtration appears to involve a different mechanism of trapping sperm with relatively lesser fertilizing capacity. Graham and Graham (1990) reported that Sephadex columns essentially trap non-motile sperm, while also more specific binding may be involved (e.g., capacitated sperm, see references in Ahmad et al., 2003) or of sperm with a damaged acrosomal membrane (Maurya and Tuli, 2003). The results obtained in the present study are consistent with the results of other studies where Sephadex filtration was used (Graham and Graham, 1990; Bollendorf et al., 1994; Ahmad et al., 2003; Januskauskas et al., 2005; Bussalleu et al., 2008; Lee et al., 2009) and also consistent with the finding in the present study where there were lesser percentages of (i.e. removal of part of the) immotile sperm with Sephadex filtration.

Cooling of the semen (cold shock) or cooled storage (chilling injury) leads to reduced sperm quality in non-filtered semen (e.g., regarding %MS and %PSM). In addition, Accudenz centrifugation of cooled semen had a marked negative effect on sperm quality (e.g., %SM and %PSM).

The advantage of the Sephadex filtration method as compared with DGC methods used in the present study is that the use of Sephadex more effectively traps immotile sperm than with use of the DGC methods, or the DGC methods cause more damage to sperm cells (e.g., because of centrifugation). With the Sephadex method in the present study, a relatively lesser force (200 *g* during 10 min) centrifugation was used for recovering sperm in the pellet after filtration. For specific CASA sperm motility characteristics, ALH values were less after use of all sperm selection treatments in the present study, but most clearly after Accudenz centrifugation and Sephadex filtration. It is believed that greater values of ALH are not compatible with effective progressive movement of sperm cells (Santiago-Moreno et al., 2017). Effective progressive movement of the sperm is required to overcome the anatomical and physiological barriers in the female genital tract, such as folds of the cervix, and the cervical mucus, among others (Aitken et al., 1985). The value of ALH increases during sperm capacitation (Santiago-Moreno et al., 2017). Thus, a lesser ALH of the selected sperm populations would be consistent with trapping of prematurely capacitated sperm, as has been suggested as a mechanism of Sephadex filtration by Januskauskas et al. (2005). The greater values of %SM and %PSM together with lesser values of ALH in the present study for the Sephadex-filtrated semen suggest that Sephadex filtration selects non-capacitated ram sperm with greater progressive motility and enhanced capacity to cross the cervical barriers of ewes.

Use of the DGC methods in non-refrigerated semen resulted in lesser percentages of sperm with a damaged plasma membrane acrosome, compared with non-filtered semen. In cooled samples, this reduction was numerically greater, however, was only significant for the Accudenz-treated semen. There appeared to be greater detrimental effects on Accudenz-treated semen that resulted in a decrease in a majority of motility variables, and this was especially so for cooled semen. The use of Accudenz, therefore, is not a recommended method for ram sperm, at least according to the findings in the present study, unlike results obtained with use of Accudenz for human sperm centrifugation (Sbracia et al., 1996).

Sperm morphology is one of the most commonly assessed variables for characterizing sperm, because the percentage of normal sperm morphology is positively associated with fertility outcomes (Kot and Handel, 1987; Chenoweth, 2005). Most selection methods studied in the present study were effective for reducing the percentage of at least one type of sperm abnormality: Sephadex decreased the percentage of abnormal heads in cold-stored semen, Accudenz decreased the percentage of loose heads in fresh-extended semen, and Percoll decreased the percentage of total abnormalities in fresh-extended semen. Also for bull semen, the use of Sephadex filtration has been reported to reduce the percentages of abnormal sperm (Graham and Graham, 1990; Januskauskas et al., 2005). It, however, is important to consider that the frequency of such abnormalities was already less in the samples before filtration. In the present study, there were no significant differences between non-filtered semen and after BoviPure treatment, regarding the types of morphological abnormalities. This finding is consistent with a previous report in wild sheep where DGC was also used with silane-coated colloidal silica particles (Santiago-Moreno et al., 2014).

It is concluded that Sephadex filtration can be used to select ram sperm in fresh-extended and cold-stored semen, with an advantage compared with the tested DGC methods as to percentage progressively motile sperm and sperm motility characteristics. The rationale of selecting normal, motile sperm is that abnormal spermatozoa may increase ROS production, which could lead to a greater damage of normal sperm in semen (Henkel, 2011) during cold storage, freezing, and after insemination. In addition, after insemination, the presence of damaged or abnormal sperm may increase recruitment of phagocytes in the female genital tract. This occurred to a limited extent in pigs (Matthijs et al., 2000). In humans, Eisenbach (2003) suggested, and Oren-Benaroya et al. (2007) reported that sperm with enhanced maturity ('post-capacitated')were preferentially phagocytosed. In addition, sperm purification would allow adjusting the concentration of sperm doses to a more desirable number of normal and functional sperm cells. Future research must be conducted to evaluate whether fertility with fresh, cold-stored, or frozen-thawed ram semen can be improved by

using sperm selection prior to vaginal artificial insemination.

Conflict of interest

None of the authors have any conflict of interest to declare.

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