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Quality characteristics and fertilizing ability of ram sperm subpopulations separated by partition in an aqueous two-phase system

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ABSTRACT

Centrifugal countercurrent distribution (CCCD) in an aqueous two-phase system (TPS) is a resolute technique revealing sperm heterogeneity and for the estimation of the fertilizing potential of a given semen sample. However, separated sperm subpopulations have never been tested for their fertilizing ability yet. Here, we have compared sperm quality parameters and the fertilizing ability of sperm subpopulations separated by the CCCD process from ram semen samples maintained at 20 °C or cooled down to 5 °C. Total and progressive sperm motility was evaluated by computer-assisted analysis using a CASA system and membrane integrity was evaluated by flow cytometry by staining with CFDA/PI. The capacitation state, staining with chlortetracycline, and apoptosis-related markers, such as phosphatidylserine (PS) translocation detected with Annexin V, and DNA damage detected by the TUNEL assay, were determined by fluorescence microscopy. Additionally, the fertilizing ability of the fractionated subpopulations was comparative assessed by zona binding assay (ZBA). CCCD analysis revealed that the number of spermatozoa displaying membrane and DNA alterations was higher in samples chilled at 5 °C than at 20 °C, which can be reflected in the displacement to the left of the CCCD profiles. The spermatozoa located in the central and right chambers (more hydrophobic) presented higher values ($P < 0.01$) of membrane integrity, lower PS translocation ($P < 0.05$) and DNA damage ($P < 0.001$) than those in the left part of the profile, where apoptotic markers were significantly increased and the proportion of viable non-capacitated sperm was reduced. We have developed a new protocol to recover spermatozoa from the CCCD fractions and we proved that these differences were related with the fertilizing ability determined by ZBA, because we found that the number of spermatozoa attached per oocyte was significantly higher for spermatozoa recovered from the central and right chambers, in both types of samples. This is the first time, to our knowledge that sperm recovered from a two-phase partition procedure are used for fertilization assays. These results open up new possibilities for using specific subpopulations of sperm for artificial insemination or in vitro fertilization, not only regarding better sperm quality but also certain characteristics such as subpopulations enriched in spermatozoa bearing X or Y chromosome that we have already isolated or any other feature.

1. Introduction

The availability of spermatozoa with a high fertilizing potential is critical for the development of reproductive technologies in domestic animals. In ovine, the seminal doses usually used in artificial insemination (AI) are refrigerated, and the obtained fertility rate it is not very high. This could be due to the high sensitivity of ram spermatozoa to cold-shock stress that results in reduced membrane integrity and motility [1–3].

Moreover, cold-shock induces a series of sperm changes similar to those observed during in vitro capacitation. Capacitation is a complex set of sperm modifications undergone in the female reproductive tract whereby sperm acquire the ability to suffer the acrosome reaction and, finally, to fertilize the oocyte [4]. Whether these modifications occur far away of the oocyte, spermatozoa will not be able to achieve its function. Therefore, this premature capacitation related to cold-shock, also called cryocapacitation [5,6], would result in decreased fertilization rate [7–9].

We have already shown that refrigeration also accounted for an increase in apoptotic-related markers in ram spermatozoa [10].

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Certain characteristics of apoptotic somatic cells such as DNA fragmentation, PS translocation, mitochondrial impairment, or the presence of active caspases have been shown in ejaculated human [11,12], bull [13] and ram [14,15] spermatozoa. The presence of apoptotic sperm in seminal doses could also be one of the reasons for poor fertility, as it has been reported in human [16,17] and bull [13]. This is extremely important for the improvement of ram semen cryopreservation protocols, as the high cryoinjury produced in ram semen may be related, at least in part, to an apoptosis-related phenomenon.

Given that fertilization is a process that requires several sperm capacities, the combination of different analysis techniques of sperm quality and/or functionality, would allow us to increase the capacity to predict fertility rate. As many of the steps that occur during the fertilization process depend on changes in the sperm surface, partition in aqueous TPSs can be very useful in the analysis of sperm surface characteristics related to fertility. This technique is based on different cell surface affinity for immiscible aqueous solutions of polymers, such as dextran and polyethylene glycol (PEG) [18,19]. The upper phase is rich in PEG and is relatively more hydrophobic than the lower phase, which is rich in dextran. When cells are added to the system, they partition between the interface and the PEG-rich upper phase. The extent of partition is dependent on the cell surface properties. Thus, in the same TPS, cells having different surface characteristics will partition to a different extent.

The selectivity and separation resolution can be improved several fold by using multistep partition procedures. Counter-current distribution (CCD) is a chromatographic process with one stationary (lower) phase and one mobile (upper) phase. The cell sample is partitioned in one system and the two phases are then, in a systematic way, brought into contact with fresh opposite phases. However, the loss of viability due to dilution and washing during the separation process is always a major technical problem for sperm cells [20]. Thus, the long period of time necessary for phase separation at unit gravity [18] may increase cell death during the separation process. Shorter separation procedures can be carried out by using different equipments in which centrifugation speeds up the phase separation process (CCCD, for centrifugal countercurrent distribution) [21]. As a consequence of the separation procedure, fractions located further to the left or right of the CCCD profiles will contain cells with higher affinity for the lower dextran- or upper PEG-rich phase, respectively.

We have shown that CCCD in an aqueous TPS is a resolute technique revealing sperm heterogeneity [22–24], the functional variability in response of spermatozoa, which is known to be an inherent characteristic of sperm cells highly involved in successful fertilization [25,26]. This heterogeneity appears to be associated with different membranes integrity states of the fractionated cells in the CCCD process [24,27], and also with different maturation states [25]. Moreover, CCCD has been revealed as a useful tool for the estimation of the fertilizing potential of a given semen sample, taking into account the heterogeneity of the profile together with the viability values in the central chambers [28]. These results suggest the hypothesis that sperm subpopulations separated by CCCD should have different surface properties that would be a reflex of differences not only in sperm quality parameters but also in their fertilizing potential.

Therefore, the aim of this study was to compare motility, membrane integrity, capacitation state and apoptosis-related markers, such as PS translocation and DNA damage, of sperm subpopulations separated by the CCCD process from ram semen samples maintained at 20 °C or cooled down to 5 °C. The temperature of 20 °C is the one usually used for maintaining seminal doses in routine ovine AI and it is also that at which the CCCD process is carried out, and 5 °C is the critical point of the cryopreservation process. Additionally, the fertilizing ability of the fractionated subpopulations was comparative assessed by ZBA.

2. Materials and methods

2.1. Sperm collection

Semen was collected from nine 3–5 year-old *Rasa Aragonesa* rams using artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding (ANGRA) were kept at the Veterinary School under uniform nutritional conditions. Based on the positive results from a previous study, sires underwent an abstinence period of two days, and second ejaculates were pooled and used for each assay, to avoid individual differences [29].

2.2. Sample preparation

Two aliquots of 1 ml of ram semen were diluted (6×10^8 cells/ml) in a dextran/swim-up medium (SM) [30] supplemented with sodium pyruvate up to 10 mM. One of them was supplemented with 5% glycerol and gradually cooled down to 5 °C using a programmable water bath at a cooling rate of −0.2 °C/min (total refrigeration process was 2 h 20 min), and the other one was maintained at 20 °C during the same time.

2.3. Evaluation of semen samples

Sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany).

Total and progressive sperm motility was evaluated by computer-assisted analysis using a CASA system (ISAS, Proiser SL, Valencia, Spain). Two drops of each sample were studied using a negative contrast-phase optical microscope (at 100× magnification) maintained at 37 °C. Five fields of each drop were recorded and processed. Our CASA system was based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field using a camera Basler (Ahrensburg, Germany). These consecutive photographs were taken in a time-lapse of 1 s, which implied a velocity of image capturing of one photograph every 40 ms. The average number of spermatozoa analyzed per field was two hundred.

Cell viability (membrane integrity) was assessed by double staining with 6-carboxyfluorescein diacetate (CFDA, 1 mM) and propidium iodide (PI, 0.75 mM) [31]. Two μl of each stain were added to 200 μl of diluted semen (6×10^6 cells/ml). Samples were incubated at room temperature in darkness for 15 min and evaluated by flow cytometry. All the measurements were performed on a Beckman Coulter FC 500 (IZASA, Barcelona) with CXP software. The argon laser and filters of 525 and 675 nm were used to avoid overlapping. Monitored parameters were FS log, SS log, FL1 (CFDA) and FL4 (PI). The side and forward light scatter parameters were gated so that only those cells possessing the light scatter characteristics of spermatozoa were analyzed for fluorescence. A minimum of 20,000 events was counted in all the experiments. Sperm cells that displayed green fluorescence were considered viable (CFDA+/PI−) while those with green and red (CFDA+/PI+) or only red fluorescence (CFDA−/PI+) were considered non-viable.

Sperm capacitation state was evaluated using the chlortetracycline (CTC) assay that we previously validated for the evaluation of capacitation and acrosome reaction like changes in ram spermatozoa [32] following the procedure already reported [33]. For the evaluation of CTC patterns, the samples were observed using a Nikon Eclipse E-400 microscope under epifluoresce illumination with a V-2A filter at 1000× magnification. At least 200 cells were counted in duplicate for each sample. Three sperm types were estimated [7]: not capacitated (even distribution of fluorescence

on the head, with or without a bright equatorial band), capacitated (with fluorescence in the anterior portion of the head) and acrosome-reacted cells (showing no fluorescence on the head). The use of ethidium homodimer in this staining allows us to differentiate between live and dead sperm in the three mentioned types.

The PS translocation from the inner to the outer portion of the plasma membrane is indicative of early stages of membrane disturbance [34]. In the present study, Annexin V (AnnV: Apoptosis Detection Kit, Annexin V-Cy3; Sigma, Madrid, Spain) was used to detect externalization of PS on the sperm plasma membrane. Dual staining with CFDA was used to differentiate three subpopulations of spermatozoa: intact (CFDA+/AnnV−), live with PS translocation (CFDA+/AnnV+), and dead (CFDA−/AnnV+) cells. Aliquots of 50 μl were diluted with 150 μl of 1× binding buffer (commercial kit) and stained with 5 μl CFDA (1 mM in DMSO) and 1 μl Annexin V. After 10 min incubation, 5 μl of formaldehyde (1% in PBS) was added. Samples were examined at 1000× magnification by epifluorescence microscopy; viable spermatozoa (CFDA+) were visualized in green with a standard fluorescein (Nikon B-2A) filter, and AnnV+ spermatozoa in red with a rhodamine (Nikon G-2A) filter.

The presence of apoptosis-like DNA strands break in ram sperm was evaluated by the TUNEL (terminal transferase-mediated dUDP nick end-labeling) assay using the In Situ Cell Death Detection Kit with fluorescein isothiocyanate (FITC)-labeled dUTP (Roche, Mannheim, Germany). Previously, sperm samples were fixed with 4% paraformaldehyde in PBS at room temperature for 1 h and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The rest of the procedure was carried out according to the manufacturer's instructions. Samples were observed under a fluorescent microscope with a standard fluorescein (Nikon B-2A) filter, at 1000× magnification.

2.4. Centrifugal counter-current distribution

We designed the counter-current distribution machine on the basis of that invented by Akerlund [21]. The apparatus contains 60 chambers arranged in a circle, which allows transfers of the upper (mobile) phases relative to the lower (stationary) phases. CCCD is performed during centrifugation by keeping the denser (bottom) phases in the outer half while the lighter (upper) phases are in the inner half of each chamber. As no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 upper over 60 bottom batch phases. Each transfer in this centrifugal-enhanced CCCD includes the following: first, the phases are shaken at unit gravity to mix them thoroughly and they are then separated by centrifugation (1000 × *g*). After the phases have separated and while they are still rotating at full speed (1000 × *g*), the upper (inner) phases are transferred to the next chambers. After deceleration, a new cycle can be performed. A detailed scheme of the device was already reported by Akerlund [21].

The TPS used consisted of 5.5% (w/w) dextran T500 (Mr 500,000) from Pharmacosmos A/S (Holbaek, Denmark), 2% (w/w) PEG; (Mr 6000) from Sigma Chemical Co (Madrid, Spain), 10.5% (w/w) Ficoll 400 (Mr 400,000) from GE Healthcare Bio-Sciences (Uppsala, Sweden) and polymer-free medium named Phosphate HEPES Sucrose medium or PHS: 0.25 M sucrose, 0.1 mM EGTA, 4 mM sodium phosphate, 5 mM glucose, 10 mM HEPES and 2 mM KOH; final pH 7.5.

In each experiment the volume of the system loaded in chambers was the estimated amount required to maintain the desired volume of the bottom phase (0.7 ml). Two cell samples (each approximately 2×10^8 cells) were loaded simultaneously in chambers 0 and 30, and 29 transfers were carried out. In these conditions, both samples were directly comparable. The whole process took 1 h at 20 °C.

After the run, the content of each chamber was diluted with an equal volume of PHS to yield a single-phase suspension, which was collected and the cells counted under a light microscope. Partition results are expressed as the percentage of cells counted in each chamber with respect to the value obtained in the chamber containing the highest number of cells. For viability, CTC, Annexin-V and TUNEL assays, cells from three consecutive chambers throughout the run were pooled, centrifuged to allow sperm recovery (750 × *g*, 10 min), and stained as indicated above. Results are expressed as the percentage of cells in each sample that were viable, live non-capacitated, with inverted PS or damaged DNA. The viability area represents the total number of viable cells recovered after the CCCD process, which corresponds to the $\sum_0^{29}$% viable cells recovered in each chamber with respect to the number of cells in the chamber containing the maximum.

The TPS used in this study contained a low phosphate concentration (4 mM) and thus, sperm population partitioning was mainly due to hydrophobicity rather than charge. As a consequence of the separation procedure, sperm cell populations with a marked affinity for the lower dextran-rich phase (mainly due to a low hydrophobicity and, consequently, lower viability), partition in the left part of the profile. Sperm cells which partition almost equally in both phases distribute in the central sector, and sperm populations with a high affinity for the upper PEG-rich phase (mainly due to a high hydrophobic, which means high viability), partition in the right sector of the profile. The CCCD profiles presented in this paper are representative of four different experiments that showed a high degree of similarity. Profiles were divided for further analysis in three fractions corresponding to chambers 0–9, 10–19 and 20–29, and the presented results are mean values of the studied parameters in each fraction.

2.5. Sperm recovering from the two-phase system

In order to establish an appropriate protocol to recover cells freed from polymers after the CCCD procedure, we carried out a comparative analysis using two consecutive washes with different centrifugal forces (1000, 1700 or 2500 × *g*) and times for the second washing (20 or 5 min), and two volumes (20 or 4 ml) of medium for resuspending pellets. Aliquots of 20 ml of the TPS containing 7×10^7 spermatozoa were mixed with an equal volume of PHS (the usual way of disturbing the TPS after the CCCD run as indicated above) and centrifuged at the three indicated forces during 20 min. The pellets were re-suspended in either 20 or 4 ml of PHS and centrifuged again for 20 or 5 minutes respectively (see Fig. 1). In vitro sperm quality parameters were determined as indicated above, and the recovery rate was calculated (n° cells/n° initial cells) in each supernatant and pellet after the first and the second centrifugation (sn1, p1, sn2 and p2, respectively). According to the obtained results, we set the final protocol one centrifugation at 1700 × *g*, 20 ml PHS, 20 min, and the second one at 1700 × *g*, 4 ml PHS, 5 min as highlighted in Fig. 1. Pellets obtained after the second centrifugation with the optimized protocol were finally re-suspended in different media: PHS, FM (*Fertilization medium* consisting of Synthetic Oviductal Fluid (SOF) without glucose [35]) supplemented with 2% (V/V) of oestrous sheep serum, 10 μg/mL of heparin, and 1 μg/mL of hypotaurine or MRM (*Motility Recovery Medium*: patent pending), alone or supplemented with either fructose 1.5 mg/ml, or bovine serum albumin 5 (MRMa5) or 10 mg/ml (MRMa10). Viability, PS translocation and motility (total and progressive) were evaluated immediately after dilution and after 30 min of incubation at 39 °C. The samples diluted with FM were maintained in an incubator with 5% CO_2 so as to maintain stable pH value.

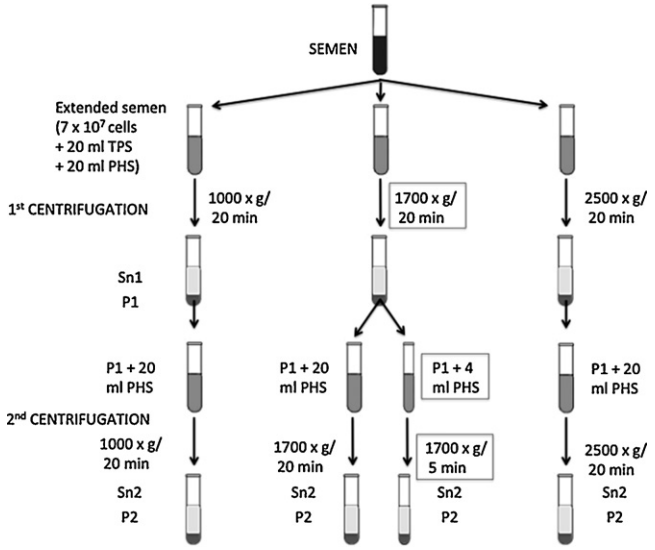

Fig. 1. Schematic representation of the experimental design for developing an optimal protocol of sperm recovering from the TPS. Sn, supernatant; P, pellet. The optimized established protocol is highlighted with squares.

2.6. Zona pellucida binding assays

Ewe ovaries were collected at the slaughterhouse and transported in sodium chloride 0.9% at room temperature to the laboratory. Ovaries were washed in saline solution and frozen at −20 °C until use [36]. The same day of assays, ovaries were thawed at room temperature and washed in saline solution. Oocytes were collected by slicing and puncture techniques [37]. Oocytes with no cumulus cells and intact zona pellucida were selected, distributed randomly and placed in wells of a four-well Petri dish, with 400 μl of fertilization medium (see Section 2.5), and kept at 39 °C and 5% CO_2 in an humidified atmosphere until their use. Spermatozoa were diluted in fertilization medium and added to the oocytes, with a final concentration of 1×10^6 cells/ml in each well. Wells were covered with mineral oil, and kept under a humidified atmosphere, with 5% CO_2 at 39 °C for 1 h. After incubation, oocytes were placed in a Petri dish with HEPES–TCM medium (Sigma–Aldrich, Co, USA), and washed by gentle pipetting to remove unattached spermatozoa [38]. Then, oocytes were fixed in glutaraldehyde 1.5% for 15 min and stained with Hoechst 33342 (1 μg/ml) for another 15 min at 37 °C. Groups of five to six oocytes were placed in a slide under a coverslip and examined with a fluorescence microscope at 400×. The number of zona pellucida attached spermatozoa per oocyte was counted and recorded.

2.7. Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM) of the number of samples. All data were analyzed using ANOVA test, and DMS as a post hoc test (SPSS Software, v.14.0). $P < 0.05$ was considered to be statistically significant. Area under the curve was calculated with Graphpad Prism 5 Software.

3. Results

3.1. Centrifugal countercurrent distribution analysis increased subtle differences between sperm samples maintained at different temperatures

We did not find significant differences neither in sperm membrane integrity (impermeability to propidium iodide) nor PS translocation between raw semen and samples incubated at 20 °C (Table 1). A sharped decrease in the proportion of viable non-capacitated sperm and a strong increment in the percentage of cells with damaged DNA could be observed in samples incubated at 20 °C, although differences were not statistically significant. Cooling down to 5 °C resulted in a slight reduction in membrane integrity but a strong and significant decrease in proportion of viable non-capacitated sperm. Apoptotic markers (PS inversion and DNA damage) showed a marked increment in latter samples compared with raw semen, although not significant (Table 1).

CCCD analysis was carried out to determine subtle differences between sperm surface properties of samples maintained at 20 °C or cooled down to 5 °C. Representative profiles (Fig. 2) evidenced a high heterogeneity in both samples, with the majority of the cell in the central part of the profile. The proportion of recovered spermatozoa with integral membrane and viable non-capacitated was reduced, and with inverted PS and damaged DNA was increased in both samples compared with values before CCCD (Table 1). Furthermore, not only sperm recovered viability, calculated as the area under the curve (AUC), was significantly lower in samples refrigerated at 5 °C than that maintained at 20 °C (296.52 ± 45.2 vs. 491.37 ± 49.5, $P < 0.05$, $n = 4$) but also an increase ($P < 0.01$) in damaged-DNA was found (Table 1).

To go further in the knowledge of differences between sperm surface characteristics, we divided the CCCD profile into three fractions, chambers 0–9, 10–19 and 20–29, and significant differences were observed (Table 2). In both samples (20 and 5 °C), spermatozoa located in the central (10–19) and right (20–29) chambers showed significantly higher values of membrane integrity ($P < 0.01$), lower PS translocation ($P < 0.05$) and DNA damage ($P < 0.001$) than in the left chambers (0–9). In samples maintained at 20 °C, the main sperm subpopulation was located in the central chambers while in samples cooled to 5 °C, the distribution profiles were slightly moved to the left, with a higher number of cells located in the first fraction (chambers 0–9) which indicates decreased hydrophobicity. In both samples, the number of spermatozoa in the right fraction was lower ($P < 0.05$) than in the other two fractions. Total recovered viability (AUC) in the central chambers was the unique significant difference between samples, which was higher ($P < 0.01$) in samples maintained at 20 °C (196.5 ± 23.5) than cooled down to 5 °C (99.9 ± 23.9) (Table 2).

3.2. A new protocol for high quality sperm recovery from two-phase system by centrifugal experiments

To determine whether the observed sperm quality differences were related to their fertilizing ability, an appropriate protocol for the recovery of certain sperm subpopulations from the CCCD chambers, freed from polymers, was required. For this purpose, we carried out comparative analyses mixing aliquots of the TPS with identical semen samples, and using different washing conditions as indicated in Fig. 1.

After the first washing, although no significant differences were found, the highest membrane integrity and the lowest PS translocation were found when the minimal centrifugal force ($1000 \times g$) was used (68.50 ± 7.4 and 36.25 ± 4.8% respectively, Table 3). After the second centrifugation, that was required to completely remove the polymers, values of membrane integrity and PS translocation were similar in samples centrifuged at either $1700 \times g$ or $2500 \times g$ (around 48%). We selected $1700 \times g$ for further experiments because it accounted for the highest total motility value in pellet 2 (40.29 ± 5.8), a suitable sperm recovering, and the results were less variable (lower SEM).

Then, trying to optimize time and cell recovery, the volume of PHS added to the first pellet after centrifugation at $1700 \times g$ was reduced to 4 ml (instead of 20 ml) and samples were then

Table 1
Percentage of sperm with integral membrane, viable non-capacitated, PS translocation and DNA damage: initial values in raw semen and in samples maintained at 20 °C or 5 °C, analyzed before and after the CCCD process (mean values of all chambers). Results are expressed as mean values ± SEM of the number of experiments ($n = 4$). Different superscript letters between columns indicate significant differences ($P < 0.05$).

Sperm (%)	Raw semen	Pre-CCCD		Post-CCCD	
		20 °C	5 °C	20 °C	5 °C
Membrane integrity	75.50 ± 2.6[a]	72.00 ± 1.0[a]	66.00 ± 1.0[a,c]	38.54 ± 6.1[b]	35.05 ± 9.6[b,c]
Viable, non-capacitated	67.09 ± 3.0[a]	35.50 ± 18.5[a,c]	25.50 ± 15.5[b,c]	9.62 ± 4.2[b,c]	9.83 ± 5.0[b,c]
Inverted PS	34.00 ± 3.9[a]	39.13 ± 5.6[a]	53.38 ± 7.1[a,c]	75.96 ± 6.2[b,c]	75.50 ± 7.5[b,c]
Damaged DNA	4.89 ± 0.81[a]	10.54 ± 1.8[a]	21.66 ± 5.5[a]	20.18 ± 2.1[a]	27.18 ± 5.7[b]

Table 2
Total number of cells, percentage of sperm with integral membrane, viable non-capacitated, PS translocation, DNA damage and recovered viability calculated as the area under the curve (AUC), in samples maintained at 20 °C and 5 °C, after the CCCD process in the specified chambers (0–9, 10–19 and 20–29). Results are expressed as mean values ± SEM of 4 experiments. Different superscript letters indicate significant differences between fractions in the same sample ($P < 0.05$). Different superscript numbers indicate significant differences between samples in the same fraction ($P < 0.05$).

Spermatozoa	Chambers 0–9		Chambers 10–19		Chambers 20–29	
	20 °C	5 °C	20 °C	5 °C	20 °C	5 °C
Total number ($\times 10^7$)	7.87 ± 1.2[a]	9.13 ± 1.4[a]	10.21 ± 1.4[a]	7.87 ± 0.5[a]	3.57 ± 1.1[b]	3.41 ± 1.3[b]
Membrane integrity (%)	15.97 ± 7.7[a]	16.52 ± 8.4[a]	50.40 ± 8.4[b]	40.69 ± 12.5[b]	49.26 ± 2.6[b]	47.94 ± 10.2[b]
Viable, non capacitated (%)	5.12 ± 1.8[a]	5.00 ± 1.5[a]	11.37 ± 7.1[a]	9.37 ± 7.6[a]	12.37 ± 5.6[a]	13.12 ± 7.1[a]
Inverted PS (%)	97.25 ± 1.4[a]	94.00 ± 2.8[a]	63.25 ± 13.2[b]	72.13 ± 7.2[a,b]	67.38 ± 11.3[b]	62.21 ± 12.3[b]
Damaged DNA (%)	48.73 ± 4.8[a]	48.83 ± 4.3[a]	6.33 ± 0.4[b]	10.83 ± 4.5[b]	5.50 ± 1.2[b]	8.16 ± 4.6[b]
AUC	21.2 ± 5.4[a]	26.8 ± 7.8[a]	196.5 ± 23.5[b,1]	99.9 ± 23.9 [b,2]	51.4 ± 11.7[a]	32.0 ± 4.2[a]

Table 3
Percentage of total motile and progressively motile sperm, membrane integrity and PS translocation; cell concentration in the initial sample (before centrifugation) and in supernatants (Sn) and pellets (P) obtained after the first (1) and the second (2) centrifugation at 1000, 1700 and 2500 × *g* (20 min each). Percentage of cell recovery is related to the initial cell concentration. Results are expressed as mean values ± SEM of the number of experiments showed in brackets after each centrifugation. nd: not detected.

	Centrifugal force (× *g*) (*n*)	Total motile spermatozoa (%)	Progressively motile spermatozoa (%)	Sperm with membrane integrity (%)	Sperm with PS translocation (%)	Concentration ($\times 10^6$ cells/ml)	Cell recovery (%)
Initial	1000 (4)	82.50 ± 7.5	48.00 ± 5.4	70.00 ± 2.4	33.25 ± 2.9	2898.75 ± 385.2	–
	1700 (6)	83.71 ± 1.5	43.00 ± 4.1	71.29 ± 1.8	31.50 ± 2.1	2911.25 ± 306.6	–
	2500 (4)	84.00 ± 8.0	46.00 ± 4.7	67.50 ± 3.9	33.00 ± 3.0	3031.25 ± 586.5	–
Sn1	1000 (4)	22.00 ± 13.0	3.50 ± 3.5	43.00 ± 7.0	45.25 ± 7.4	0.50 ± 0.2	28.01 ± 15.5
	1700 (6)	44.66 ± 15.3	3.00 ± 0.0	46.75 ± 5.3	51.75 ± 6.7	0.40 ± 0.1	27.05 ± 8.8
	2500 (4)	nd	nd	45.00 ± 7.02	56.67 ± 12.5	0.54 ± 0.21	29.53 ± 15.9
P1	1000 (4)	62.75 ± 11.6	14.75 ± 4.2	68.50 ± 7.4	36.25 ± 4.8	93.67 ± 63.8	52.21 ± 20.6
	1700 (6)	71.20 ± 9.2	15.40 ± 3.6	59.38 ± 2.7	49.57 ± 5.6	67.65 ± 27.1	64.55 ± 10.5
	2500 (4)	60.50 ± 10.7	11.67 ± 5.2	59.33 ± 10.1	42.25 ± 5.9	147.15 ± 63.8	70.67 ± 15.3
Sn2	1000 (4)	10.00 ± 6.0	4.66 ± 3.7	32.75 ± 12.2	36.25 ± 7.6	1.27 ± 0.6	15.81 ± 7.8
	1700 (6)	34.67 ± 6.6	16.00 ± 8.5	49.29 ± 6.7	49.57 ± 5.6	2.74 ± 2.1	11.29 ± 1.9
	2500 (4)	29.66 ± 20.1	10.66 ± 10.1	64.67 ± 15.9	49.25 ± 5.9	0.44 ± 0.1	9.48 ± 5.6
P2	1000 (4)	30.75 ± 16.5	17.25 ± 11.1	41.25 ± 13.4	48.00 ± 3.4	60.70 ± 43.1	32.47 ± 3.9
	1700 (6)	40.29 ± 5.8	13.00 ± 3.7	48.71 ± 5.0	48.86 ± 2.7	72.16 ± 30.0	56.40 ± 10.0
	2500 (4)	33.25 ± 15.2	16.25 ± 8.3	50.00 ± 7.6	48.33 ± 7.4	115.32 ± 46.7	75.08 ± 21.0

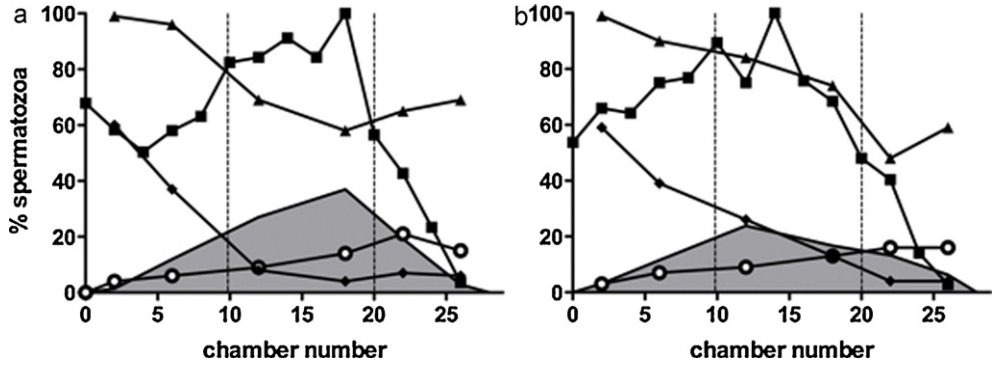

Fig. 2. Representative CCCD profiles of (a) fresh (maintained at 20 °C) and (b) refrigerated (up to 5 °C) samples. Percentage of maximum of cells (–■–); percentage of viable non-capacitated sperm (–○–), and percentage of spermatozoa with translocated PS (–▲–) and damaged DNA (–♦–). Dark area (■) represents total spermatozoa with membrane integrity.

Table 4
Percentage of total motile and progressively motile sperm, membrane integrity and PS translocation; cell concentration in the initial sample (before centrifugation) and in supernatants (Sn) and pellets (P) obtained after the first (1; 20 min) and the second (2; 5 min) centrifugation at 1700 × *g*. Percentage of cell recovery is related to the initial cell concentration. Results are expressed as mean values ± SEM of 6 experiments. nd: not detected.

	Total motile spermatozoa (%)	Progressively motile spermatozoa (%)	Sperm with membrane integrity (%)	Sperm with PS translocation (%)	Concentration ($\times 10^6$ cells/ml)	Cell recovery (%)
Initial	82.80 ± 2.5	44.50 ± 2.1	67.83 ± 2.6	32.83 ± 2.9	2690.00 ± 382.9	–
Sn 1	54.50 ± 20.5	nd	53.17 ± 4.5	51.83 ± 7.2	0.45 ± 0.1	34.13 ± 13.7
P1	48.33 ± 19.0	13.00 ± 5.8	58.33 ± 2.7	51.33 ± 4.9	29.17 ± 10.5	68.11 ± 23.8
Sn2	nd	nd	56.40 ± 5.0	48.50 ± 11.5	0.83 ± 0.1	14.40 ± 2.6
P2	66.70 ± 5.6	29.43 ± 5.4	54.38 ± 5.3	47.62 ± 5.0	103.50 ± 16.4	82.69 ± 26.0

Table 5
Percentage of total motile and progressively motile sperm, membrane integrity and PS translocation in pellet samples obtained after two washing (centrifugation at 1700 × *g*, 20 and 5 min, respectively) immediately after dilution (0 min) and after 30 min of incubation at 39 °C in PHS, MRM and FM. Results are expressed as mean values ± SEM of 4 experiments. Different superscripts within columns indicate significant differences ($P < 0.05$).

Incubation time	Medium	Total motile spermatozoa (%)	Progressively motile spermatozoa (%)	Sperm with membrane integrity (%)	Sperm with PS translocation (%)
0 min	PHS	26.75 ± 9.5	13.25 ± 3.3	59.50 ± 3.2	44.50 ± 3.1
	MRM	58.42 ± 8.8[a]	39.14 ± 9.1[a]	59.85 ± 2.7[a]	44.72 ± 3.1
	MRMf	52.80 ± 4.1	28.8 ± 2.8	45.20 ± 5.7	58.80 ± 4.8
	MRMa5	35.00 ± 4.5	22.33 ± 8.1	33.00 ± 3.0[b]	51.34 ± 8.3
	MRMa10	44.66 ± 5.1	26.00 ± 7.9	22.50 ± 1.5[b]	63.34 ± 3.3
	FM	14.83 ± 7.7[b]	7.83 ± 5.2[b]	42.83 ± 3.9	52.67 ± 5.4
30 min	PHS	38.50 ± 7.1	18.25 ± 5.1	62.50 ± 3.6[a]	44.50 ± 6.5
	MRM	49.57 ± 7.2[a]	31.28 ± 6.3	52.71 ± 4.4[a]	47.58 ± 3.1
	MRMf	42.60 ± 8.6[a]	22.20 ± 2.4	40.20 ± 5.7[b]	61.40 ± 1.9
	MRMa5	43.66 ± 14.7	29.33 ± 16.6	25.00 ± 5.0[b]	57.67 ± 7.7
	MRMa10	37.00 ± 9.1	22.66 ± 12.9	20.50 ± 5.5[b]	60.70 ± 11.2
	FM	8.16 ± 3.0[b]	3.16 ± 1.7	32.83 ± 2.4[b]	57.84 ± 5.0

MRMf: MRM + fructose 1.5 mg/ml; MRMa5 and MRMa10: MRM + bovine serum albumin 5 or 10 mg/ml respectively.

centrifuged for 5 min (instead of 20 min). The obtained results (Table 4) showed that the included modifications resulted in higher cell concentration in pellet 2 ($103.50 \pm 16.4 \times 10^6$ cells/ml; 82.69% of cell recovery related to the initial sample), together with higher motility (66.70 ± 5.6 and 29.43 ± 5.4% of total and progressively motile sperm, respectively) and membrane integrity (54.38 ± 5.3%) than the values obtained with 20 ml of PHS and 20 min each centrifugation (Table 3).

Next, trying to improve motility of sperm samples recovered from pellets, we assayed the effect of different media to resuspend the pellets obtained following the optimized protocol. Significant differences were observed even immediately after dilution (Table 5). MRM provided the highest values of total (58.42 ± 8.8%) and progressive (39.14 ± 9.4%) motility and membrane integrity (59.85 ± 2.7%), along with the lowest PS translocation value that was very similar to that in PHS (around 44%). Furthermore, differences were maintained after 30 min of incubation in these media, and MRM and PHS provided the best results regarding membrane state (high integrity and low PS translocation). Supplementation of MRM with fructose or bovine serum albumin did not enhance sperm quality results (Table 5).

3.3. Results of zona binding assays

In order to confirm sperm functionality, we performed an additional experiment to determine the zona pellucida binding ability as an indicator of the fertilizing capacity of the spermatozoa. We used MRM and PHS given that both media accounted for good sperm quality results (Table 5), and FM because it is the medium usually used for co-incubation with oocytes.

Samples diluted in FM, despite its low motility data (Table 5), resulted in a significantly higher number of spermatozoa bound per oocyte than samples diluted in the two other media (Table 6). The pH values of each medium were determined before (8.8, 7.4 and 7.5) and after incubation in CO_2 incubator (7.3, 7.0 and 7.5 for MRM, PHS and FM, respectively). Therefore, we used FM for the assessment of ZBA of sperm subpopulations recovered after the CCCD process.

Table 6
Mean value (± SEM) of sperm number bound per oocyte assessed by ZBA using sperm samples recovered from a TPS by optimized double centrifugation at 1700 × *g* (20 and 5 min, respectively) and diluted in PHS, MRM or FM. *N*: number of oocytes incubated. Different superscripts within columns indicate significant differences ($P < 0.001$).

Media	*N*	Spermatozoa/oocyte
PHS	70	4.73 ± 0.6[a]
MRM	72	5.54 ± 1.0[a]
FM	62	17.79 ± 2.5[b]

We obtained three fractions (chambers 0–9, 10–19 and 20–29) by using the optimized centrifugation protocol. The final pellets were diluted with FM to assess the ability of each sperm fraction to bind oocytes, carrying out a comparative analysis of samples maintained at 20 °C and 5 °C. The number of spermatozoa bound per oocyte was significantly higher ($P < 0.001$) in samples obtained from fractions 10–19 and 20–29 than that from fraction 0–9, at both temperatures (Table 7). Furthermore, the number of spermatozoa bound per oocyte in fraction 20–29 refrigerated up to 5 °C was higher than the corresponding fresh one ($P < 0.01$).

4. Discussion

The use of refrigerated seminal doses in ovine AI is more widespread than the use of frozen semen. The term refrigeration can refer to either dilution of the ejaculates and further maintaining at 15–20 °C, or a deeper cooling down to 5 °C which results in a much longer arrest of metabolic activity [39]. Rapid cooling down of spermatozoa, especially from 20 to 5 °C, results in cold shock injures, commonly associated with sperm membrane damage [40]. Therefore, in this study we used a slowly controlled decrease of

Table 7
Mean value (± SEM) of sperm number bound per oocyte assessed by ZBA using sperm recovered after the CCCD process from the obtained fractions (0–9, 10–19 and 20–29) by optimized double centrifugation at 1700 × *g* (20 and 5 min, respectively) and diluted in FM. *N*: number of oocytes incubated. Different superscript letters indicate significant differences between chambers in the same sample ($P < 0.001$); different superscript numbers indicate significant differences between samples in the same chambers ($P < 0.01$).

Sample	Chambers	*N*	Spermatozoa/oocyte
20 °C sample	0–9	61	$1.02 \pm 0.4^{a,1}$
	10–19	58	$2.93 \pm 0.6^{b,1}$
	20–29	56	$1.75 \pm 0.4^{b,1}$
5 °C sample	0–9	65	$0.26 \pm 0.0^{a,1}$
	10–19	63	$4.78 \pm 1.5^{b,1}$
	20–29	62	$4.81 \pm 0.8^{b,2}$

temperature and the inclusion of glycerol in the diluent to avoid, as far as possible, cryoinjury. Our results showed that despite the fact that the cooling process used in this study scarcely affected membrane integrity (evaluated as impermeability to propidum iodide), both treatments (incubation at 20 or 5 °C) resulted in a strong decrease in the percentage of viable non-capacitated sperm with an increment in apoptotic markers, PS translocation and DNA damage, specially in samples cooled up to 5 °C, although most changes are not statistically significant. This observation indicates that the assessment of other parameters rather than membrane integrity can be relevant given that other alterations that seriously affect sperm functionality can occur. The significant decrease in the percentage of viable non-capacitated sperm at 5 °C is consistent with the capacitation process associated with cold-shock [41], termed cryocapacitation.

The CCCD process, despite appropriate conditions and harmlessness of polymers, accentuated differences in all mentioned parameters between each sample and the initial values. The incubation at 5 °C accounted for higher numbers of spermatozoa displaying membrane and DNA alterations that was reflected in the displacement to the left of the CCCD profile. This could be influenced by the CCCD process, which could specially affect those cells more liable that would suffer alterations much easier.

When we analyzed sperm quality in the fractions obtained after dividing the CCCD profile in three parts, the obtained results showed important differences. Thus, those spermatozoa located in the central and right chambers (more hydrophobic) presented higher values of membrane integrity, and lower PS translocation and DNA damage than those with higher affinity for the lower dextran-rich phase (in the left part of the profile) in which apoptotic markers were significantly increased.

To know whether the observed sperm quality differences were related to fertilizing ability, we decided to test spermatozoa isolated from each fraction by using ZBA. For this purpose, several attempts were previously needed for optimizing a sperm recovery protocol from the aqueous TPS as we proved that polymers do not result innocuous for oocytes (data not shown) and highly reduce sperm motility and, consequently, gametes' binding. After different assays, we established two sequential centrifugations at 1700 × *g* as the appropriate washing protocol based not only on better sperm motility, but a lower variation of results. Centrifugal force can affect sperm quality characteristics in different extent, probably depending of the initial state of spermatozoa. With the aim to improve sperm motility of the obtained pellets, we assayed different dilution media. Despite significant lower results in sperm motility and membrane integrity in comparison with the other media, FM was chosen for pellet dilution after the second centrifugation given that the ZBA rate was significantly higher than that with MRM. This could be due to inability of MRM to maintain pH during co-incubation in CO_2 incubator (as evidenced by a sharp decrease in pH values measured before and after incubation) that may affect the ability of sperm to bind to the zona pellucida of oocytes. Another reason might be the high viscosity of the MRM (0.25 M sucrose) that would prevent o make difficult this binding. However, it is worth noting the good results in sperm quality, particularly motility, obtained with MRM, which suggests that this medium could be suitable for sample dilution in other assisted reproductive technologies such as AI that do no implies direct contact of medium with oocytes. It might be especially useful for diluting sperm samples with low motility rate.

The washing and diluting developed protocol is useful for obtaining suitable sperm samples, as we proved by testing the fertilizing ability of each sperm CCCD fraction using ZBA. We confirmed that the differences observed in sperm quality were related with the number of spermatozoa attached per oocyte that was significantly higher for spermatozoa recovered from the central and right chambers, in both types of samples. Regarding spermatozoa recovered from the right fraction, differences in ZBA between both samples are not related to viability, CTC staining, DNA damaged or PS translocation, because not significant differences were found in these parameters between samples at 20 or 5 °C in this fraction. According to previous results [5,6], it can be inferred that samples chilled at 5 °C contained a higher proportion of cryocapacitated sperm which, in turn, would result in higher ZBA score. Probably, the CTC staining is not able to reveal subtle changes associated with different capacitation degree that would explain variations in the number of sperm bound to oocyte.

The unique parameter significantly different between samples, in the same CCCD fraction, was viability in the central fraction that was higher in samples maintained at 20 °C than those cooled down to 5 °C. However, this difference was not reflected in different ZBA rate. In a previous study [28], we found that viability in the central fraction and the recovered viability area (AUC) correlate significantly with field fertility, probably as a result of the CCCD process, which could cause some damage to the cells, reflected in the viability loss of those cells more liable to suffer membrane alterations. Thus, recovered viability could reflect the resistance of the spermatozoa to stressful events, including those that would occur during insemination and the passage through the female genital tract. However, in the experiments carried out in the present study, spermatozoa did not suffer the stressful passage through the female genital tract, and the fertilizing ability was only measured as the number of spermatozoa attached to oocyte after a period of gamete incubation. Therefore, it seems logical that differences in sperm resistance are not translated into differences in ZBA results.

This is the first time, to our knowledge that sperm recovered from a two-phase partition procedure are used for fertilization assays. These results open up new possibilities for using specific subpopulations of sperm for AI or in vitro fertilization, not only regarding better sperm quality but also specific characteristics such as subpopulations enriched in spermatozoa bearing X or Y chromosome that we have already isolated [42] or any other feature.

5. Conclusions

Cooling ram sperm samples at 20 or 5 °C resulted in a significant decrease in the percentage of viable non-capacitated sperm with an increment in apoptotic markers, PS translocation and DNA damage, especially in samples chilled to 5 °C. CCCD analysis revealed that the number of spermatozoa displaying membrane and DNA alterations was higher in samples chilled at 5 °C than at 20 °C, which can be reflected in the displacement to the left of the CCCD profiles. The spermatozoa located in the central and right chambers (more hydrophobic) presented higher values of membrane integrity, lower PS translocation and DNA damage than those in the

left part of the profile, where apoptotic markers were significantly increased and the proportion of viable non-capacitated sperm was reduced. With the new protocol for sperm recovery from the aqueous TPS, we proved that these differences were related with sperm fertilizing ability. CCCD has been revealed as an efficient tool to separate sperm subpopulations with different fertilizing potential, so we can select high-fertility fractions for assisted reproductive techniques, which might result in higher fertility results.

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