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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