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Original article

Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): Effect on sperm quality, function and gene expression

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ABSTRACT

This study assesses the effect of bovine sperm (obtained from three bulls) separation using density gradients (Percoll and BoviPure) and Swim-up on sperm function and gene expression. Sperm evaluations included the plasma membrane integrity (SYBR14/PI), acrosomal integrity (PNA-FITC/PI), oxidative stress (ROS; CH2FDDA), DNA fragmentation (TUNEL assay) and mitochondrial membrane potential (ΔYm; TMRM) using flow cytometry. Sperm motility was evaluated by computer-assisted sperm analysis (CASA) and gene expression using RT-qPCR. The results showed that separation by Percoll achieves a higher proportion of sperm with intact plasma and acrosomal membranes (89.8 and 87.5%, respectively) than the unseparated control (70.3 and 62.4%, respectively), as well as by Swim-up (74.9 and 63.3%, respectively) and BoviPure (83.3 and 80.4%, respectively). No differences were observed in the proportion of spermatozoa with high $\Delta\Psi$m between Percoll and BoviPure (84.3% and 83.5%, respectively), which were higher than Swim-up and the unseparated control (72.8% and 43.8%, respectively). The ROS levels were higher in the spermatozoa separated by Percoll and no differences were observed in the sperm DNA integrity between all groups. The motility analysis showed that the separation methods improve ($p < 0.05$) total and progressive motility compared to the control, with Percoll proving the most efficient in this regard. Finally, the gene expression analysis of leptin (*LEP*), aromatase cytochrome P450 (*CYP19*) and protamine I (*PRM1*), after validation of 6 reference genes, showed no differences between groups. In conclusion, bovine sperm separation using density gradient improves the parameters of motility and sperm function without affecting the gene expression.

1. Introduction

Assisted reproduction techniques (ARTs) in humans and animals depend on developing methods that can efficiently separate the motile sperm fraction from the other semen components. Selecting good quality, high density sperm that maintain their fertilization potential is a key factor for the success of ARTs, since dead and abnormal sperm exert toxic and lithic effects on normal sperm and thus have a negative impact on fertility [1]. Sperm selection methods mainly separate the motile from the immotile sperm, eliminate the seminal plasma, diluents and cryoprotectants [2,3], and significantly improved sperm quality, thereby increasing the progressive motility and normal morphology. The first method described for separating spermatozoa consisted only of washing the sperm cells by centrifugation. Later, Swim-up was described and more sophisticated sperm separation methods were designed that obtained a high number of motile spermatozoa to protect sperm function and reduce environmental effects such as reactive oxygen species (ROS). These methods include: Swim-up with Hyaluronic acid [4], Sephadex columns, glass wool filtration, migration/sedimentation and centrifugation in density gradients, among others (reviewed by Henkel and Schill [5]).

Percoll is a commercial medium used to separate cells and subcellular particles by means of density gradients, and it has been widely used in the separation of animal spermatozoa and other type of cells. Percoll is composed of colloidal silica particles coated with non-dialyzable polyvinylpyrrolidone (PVP). Centrifugation in Percoll density gradient separates the spermatozoa according to

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their state of maturity and integrity from the diluents, cells and bacteria [4,6]. In bovines, separation by density gradients has improved semen quality, mainly in cases of high viscosity, poor quality or in cryopreserved semen [1]. However, an endotoxic effect of the PVP present in Percoll has been described, which is why it was withdrawn from human ARTs in 1996 [4,7].

BoviPure is one of the products developed to replace Percoll for use in the purification and separation of bovine spermatozoa. BoviPure is an iso-osmotic salt solution that contains colloidal silica particles coated with silane [8]. There are several studies that have used BoviPure for sperm selection in *in vitro* fertilization (IVF) [9–11], but none has assessed the effect of this compound on spermatozoa, except by Samardzija et al. [8], who performed a partial analysis of sperm quality parameters separated by BoviPure and its effects on the embryonic developmental rate after IVF [8,12].

Although Swim-up is the oldest method, it is still the simplest and the most economical method widely used in IVF laboratories around the World, particularly in human ARTs. The method is based on the movement of sperm from the pellet to the uppermost part of the supernatant medium. The technique obtains a high percentage of motile sperm, after separation, most with normal morphology. Nevertheless, the number of separated sperm is very low. In addition, it has been described that the Swim-up method significantly reduces the percentage of sperm with normally condensed chromatin [13], and that the close contact between cells in the pellet, cell remains and leukocytes produces high levels of ROS, which are detrimental to the sperm [14].

Therefore, the aim of the present study was to assess the effect of sperm separation in bovine species using density gradient (Percoll and BoviPure) and Swim-up methods on motility, plasma and acrosome membrane integrity and DNA damage, mitochondrial membrane potential, ROS, and gene expression.

2. Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.1. Sperm preparation and pretreatments

We used commercially available semen (Alta Genetics, Alberta Canada) from three bulls of different breeds (Jersey, Holstein Friesian and Fries-Hollands), all with proven *in vivo* fertility. Semen samples from the same batch of each different bull were thawed, pooled (three replicates for each assay) and separated by Percoll gradient (45:90) [15]; BoviPure gradient (according to the manufacturer's instructions) and Swim up according to Boguen et al. [16]. Separated sperm were counted on a hemocytometer to adjust the concentration to 1×10^6 sperm/ml. Then, sperm cells were washed once with Sp-TALP (sperm-Tyrode's albumin lactate pyruvate) medium and centrifuged for 5 min at 200×*g* and prepared for evaluation.

2.2. Analysis of sperm motility

We evaluated total and progressive sperm motility using the integrated sperm analysis system (ISAS®, Proiser, Valencia, Spain), by depositing an aliquot of 2 μL of sperm on a D4C16 slide (ISAS®, Proiser, Valencia, Spain). All the materials used to manipulate the sperm during the motility analysis were brought to a temperature of 37 °C beforehand. In each treatment, motility was evaluated in duplicate in 5 fields with approximately 200 sperm per field. This experiment was replicated 3 times.

2.3. Analysis of seminal parameters by flow cytometry

2.3.1. Evaluation of plasma membrane integrity

We determined spermatozoa plasma membrane using the LIVE/DEAD Sperm Viability kit (Molecular Probes, Eugene, OR, USA) measured by flow cytometry (FACS CANTO II Becton & Dickinson) using 1×10^6 spermatozoa in 400 μL of DPBS (dulbecco's phosphate buffered saline) containing 1 nM SYBR14 and 18 μM PI according to the manufacturer's instructions. A minimum of 1×10^4 spermatozoa was examined for each reading (n = 3).

2.3.2. Evaluation of the acrosome integrity

We assessed acrosomal membrane integrity using 1×10^6 spermatozoa in 400 μL of DPBS containing 0.3 μg/mL PNA-FITC (Peanut agglutinin [PNA] conjugated to fluorescein isothiocyanate [FITC]/PI-assay and 18 μM PI for 10 min, respectively at 38.5 °C in darkness. The sperm were washed once with DPBS and analyzed immediately.

2.3.3. Evaluation of DNA integrity

To evaluate DNA integrity, we performed the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using the *In Situ* Cell Death Detection Kit Fluorescein (Roche

Table 1
Information on the primers used for RT-qPCR.

Gene	Function	Accession N°	Product size (bp)	Primer sequence (5′–3′)	E (%)
ACTB	Cytosqueletal structural protein	AY141970	87	CCTCACGGAACGTGGTTACA TCCTTGATGTCACGCACAATTT	95.2
GAPDH	Oxidoreductase in glycolysis and gluconeogenesis	XM583628	119	TTCAACGGCACAGTCAAGG ACATACTCAGCACCAGCATCA	89.9
HPRT1	Purine nucleotide synthesis	AF176419	154	TGCTGAGGATTTGGAGAAGG CAACAGGTCGGCAAAGAACT	101
PPIA	Catalysis of the *cis*-trans isomerization of proline	NM_178320.2	203	CTGGCATCTTGTCCATGGCAAA CCACAGTCAGCAATGGTGATCTTC	107.9
SF3A1	Structural component of the splicing system	XM_878187.1	125	GCGGGAGGAAGAAGTAGGAG TCAGCAAGAGGGACACAAA	95.1
YWHAZ	Signal transduction	BM446307	120	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	89.3
CYP19	Monooxygenases which catalyze reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.	NM_174305.1	155	AACCATGGCCTGTGCCATTT TGGGTCTTTAGGGCTGCAGT	109.8
LEP	Endocrine functions, and is involved in the regulation of immune and inflammatory responses, hematopoiesis, angiogenesis and wound healing.	NM_173928.2	188	AAAACTGCGAGGCAGGAAAC TGCTTGATGGTCCAAAGGCT	102.6
PRM1	Compaction of sperm DNA	NM_174156.2	164	AAGATGTCGCAGACGAAGGA ATGTGGCAAGAGGGTCTTGA	92.9

E: PCR efficiency.