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Effect of Single layer centrifugation using Bovipure on frozen-thawed bovine sperm during a 6 hours survival test

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Abstract

Aim of the study: to evaluate the functional parameters of bovine spermatozoa before and after separation by single layer centrifugation using Bovipure gradient.

Material and Methods: Frozen semen from 8 bulls was used in this study. Thawed pellets were subjected to the separation process through gradient media according to the manufacturer's instructions. Qualitative assessment of semen was performed at initial time (T0) and during a 6h survival test (T6) before and after gradient centrifugation preparation. Beside conventional examination, the functional parameters of sperm such as acrosome integrity (using Spermac Stain), membrane potential (JC 1 fluorescent dye) and DNA fragmentation (HalomaxBos Taurus) were evaluated.

Results: An extremely high variability in percentages of sperm obtained after separation of thawed pellets was noted, the average sperm recovery (± SD) from thawed pellets after separation being 29.6 ± 13.9% of original sperm count. Sperm preparation by SLC selected functionally and morphologically high quality sperm compared to the control samples, the overall advantages of SLC-selected samples over the control group being particularly evident during the 6 h survival test.

Conclusions: This procedure selected spermatozoa that were motile, possessed good membrane integrity, high respiratory activity and additionally, maintain these characteristics during storage significantly better than control samples, prolonging its longevity.

Keywords: bull semen, single layer centrifugation; Bovipure

1. Introduction

In order to increase the outcomes of assisted reproduction technology, male gametes of high quality are essential and thus, different sperm selection methods were developed in order to separate specific sperm populations based on certain characteristic such as motility or sperm membrane integrity. Motile sperm are separated from non-motile and the seminal plasma, cryoprotective agents andother background materials such as debris are removed [1]. In the same time, the capacitation process of sperm is initiated [2]. Some of the most important sperm separation methods are: dilution and washing [3], density-gradient centrifugation using colloids such as Percoll [4], PureSperm or Bovipure® [5], filtration using Sephadex, glass wool [6] or self-migration techniques [7]. Parrish et al. (1995) [8], applied the density gradient protocol for the in vitro fertilization with bull semen while Somfai et al. (2002) [9] reviewed the results obtained with the swim up and gradient centrifugation methods by various authors and compared them. Furthermore, the majority of authors cited preferred the use of colloid gradient centrifugation, which retrieved a higher number of sperm cells with normal acrosome while Brandeis and Manuel (1993) reported that the swim up methods selected preferably motile sperm. Parrish et al. (1995) and Somfai et al. (2002) focused on these methods with the aim to prepare bull sperm for in vitro fertilization.

The routine sperm quality control after commercial freezing/thawing process is limited to the subjective assessment of sperm motility, despite its low correlation to fertility [11-12]. The purpose of the present study was to evaluate the functional parameters of bovine spermatozoa before and after separation by single layer centrifugation using Bovipure gradient. The Bovipure(Nidacon International AB, Gothenburg, Sweden) density gradient colloid is composed of colloidal silica particles (15-30 nm in diameter) coated with non-dialysablepolyvinylpyrolidone (PVP). Colloid density-gradient centrifugation clearly separates spermatozoa from foreign material such as extender particles, cells and bacteria. The morphological selection of spermatozoa in the prepared population varies, with most tail, and mid-piece defects being primarily excluded [13].

2. Material and Methods

Biological material

Commercial frozen semen from 8 bulls used for artificial insemination (A.I) from a bull stud in Romania was used in this study. From each bull, 4 straws from the same batch were mixed in order to perform the experiments.

Sperm preparation using Bovipure

Thawed pellets were subjected to the separation process through gradient media, according to the manufacturer's instructions in order to obtain functionally and morphologically high quality sperm. Briefly, the materials were brought at room temperature and the appropriate amount of solutions was prepared according to the instructions. The sperm pellet was extended with BoviWash^{TM} 1:1. Using a sterile pipette, the BoviPure^{TM} density gradient was placed into a conical centrifuge tube in order to form the bottom layer. Using a new sterile pipette, the extended sperm pellet was layered on top of the BoviPure^{TM} layer. The tubes were centrifuged at 300 x g for 25-30 minutes at room temperature without using the breaks. When the time elapsed, a sterile Pasteur pipette was used to aspirate, in a circular movement from the surface, everything except the pellet and 1-2 mm of the BoviPure Layer. A new sterile pipette was used to transfer and re-suspend the sperm pellet in 0.2 mL BoviWash^{TM} in a new sterile tube and analyze.

Sperm quality and functionality assessment

Qualitative assessment of semen was performed at the initial time (T0) and during a 6h survival test (T6) before and after gradient centrifugation preparation. Beside conventional examination, the functional parameters of the ejaculates such as acrosome integrity, membrane potential and DNA fragmentation were evaluated.

Sperm concentration was determined with a haemocytometerNeubauer. Progressive motility of semen was subjectively assessed by visual estimation under a microscope. Membrane integrity of spermatozoa was assessed by HOS test with modifications [14].In the HOS-2 test (Padrik, 1999), the proportion of FT spermatozoa with swollen tails was determined with 0.2% and 0.4% NaCl solutions (osmotic pressure 66 and 130 mOsm/kg). The semen straws were thawed in a water bath at 35°C for 20 s, and emptied into a test tube containing 1 ml of 0.2% and 0.4% NaCl solution. After incubating at room temperature (20−22°C) for 2 min, 0.2 ml of eosin was added to each test tube. A wet preparation of each concentration was examined under a phase contrast microscope. The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate.

In order to evaluate the sperm acrosome integrity, the dichromatic stain Spermac (FertiPro,Beernem, Belgium) was used, the sperm cells with reacted/abnormal acrosome being stained pink-red while the cells with normal acrosome presented a green post-acrosomal region.

Consequently, the mitochondrial function was determined by JC-1 mitochondrial Membrane Potential Detection Kit (Cell Technology, Inc., CA, U.S.A) [15]. Aliquots of 50 µl of semen were diluted in 150 µl of Tris containing 5 µl of JC-1 (0.15 mM in DMSO), incubated at 38^{o}C for 10 min and fixed with PBS containing 0.5% glutaraldehyde. A total of 200 spermatozoa were evaluated at x1000 magnification under oil immersion using a BP 450-490 nm excitation and LP 515 nm emission filters. Cells stained orange were classified as having high mitochondrial membrane potential, whereas cells stained green were classified as having low membrane potential.

For carrying out the Sperm Chromatin Dispersion test (SCD), samples were treated with the commercial kit Sperm Bos-Halomax (*Chroma Cell* SL, Madrid, *Spain*), following the producer protocol included in the kit. Briefly, 25 µl of sperm sample was added to a vial containing 50 µL of liquid low-melting agarose (provided by the kit) at 37◦C. Then, 25 µL of the solution (~3×10^{6}spz./mL) were placed on an agarose pre-treated slide also provided with the kit and cooled at 4◦C. The drop was covered with a coverslip (22×22 mm) and the slide was placed at 4◦ C for 5 min. Then, the coverslip was carefully removed and the slide was immediately put into lysing solution at ambient temperature. After 4 min the slide was washed for 5 min in distilled water and sequentially dehydrated in ethanol (70%, 90%,100%). After dehydration, slides were stained with Wright Stain (Sigma Aldrich) 1:1 in Phosphate Buffer pH 6.88 (Merck) and analyzed under bright field microscopy, x1000 using oil immersion as described by Garcia-Macias, 2006 [16].

In order to establish the DNA fragmentation index, the analysis of a minimum number of 500 s spermatozoa per sample is recommended. The criteria for classification are as follows: spermatozoa with fragmented DNA (those spermatozoa where the thickness of the halo is equal or greater compared with the length of the core minor diameter) and spermatozoa with non-fragmented DNA also called DNA intact (those spermatozoa without halos or those with a halo thickness of less than a third of the core minor diameter).

Statistical analysis

Differences among treatments were evaluated using analysis of variance (ANOVA) following arcsin transformation (arcsin √P/100) of the percent values and Tukey's multiple-comparison test, using the INSTAT program for Windows (version 3.01).

3. Results

Sperm analysis of pellets from 8 AI bulls gave evidence of a good quality of insemination doses. The detected initial values generally ranged within conventional limits (Table 1). Thawed pellet spermatozoa were separated using density gradient media to obtain sperm of good function and morphology. The results of quality assessment of sperm fractions obtained after single layer centrifugation are shown in Table 1. All sperm parameters were significantly ($P < 0.001$) higher in SLC-selected samples in comparison to control samples at both time points. Mean value, standard deviation, maximum and minimum values were calculated for each of the parameters taken into study. An extremely high variance in percentages of sperm obtained after separation of thawed pellets was noted, the average sperm recovery (± SD) from thawed pellets after separation being 29.6 ± 13.9% of the original sperm count.

The percentage of progressive motile sperm did not fall below 18.8% after 6 hours of incubation in the control samples, a higher number of progressive (mean± SD) sperm cells being observed in the SLC selected samples ($p<0.001$).

Membrane integrity, which was demonstrated by a HOS-test with modifications, declined on average by 27.5% after 6 hours versus initial value in the unselected samples while in the selected samples, the decline between the two time periods, was on average 17.9% ($p<0.001$).

Morphological analysis of spermatozoa stained according to Hancock at the initial time point showed a mean total of 80% normal sperm (ranged between 66.8 and 91%). After the survival test, the percentage of cells with normal morphology ranged between 55.7 and 75.6 with a mean of 67.8. In the SLC selected samples, the normal morphology decreased from 90% at the initial time to 78.8 after 6 hours incubation. Furthermore, a decrease in the percentage of sperm cells with normal acrosome was observed, from 76% to 60.5 % in the unselected samples while in the selected samples, the percentage of sperm cells with normal acrosome decreased by 8.7% ($p<0.05$) after 6 hours of incubation compared to the initial time point. In addition, a higher percentage of sperm cells with high respiratory activity, as defined by the mitochondrial potential, was observed in the SLC-selected samples compared to the control one ($p<0.001$) at both initial time and after the survival test.

Regarding the DNA fragmentation index, the percentage of sperm cells with damaged DNA increased after the incubation, ranging from 8.5 to 12.6 with a mean value of 10.2 in the samples what were not processed by SLC. A significant difference was observed over time in both control and SLC-treated samples ($p<0.001$) (Table 1).

Parameter	Before SLC								After SLC							
	T0				T6				T0				T6			
	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
Progressive Motility (%)	59.5	14	67.5	47.5	30	12	44.3	18.8	84.7	8.3	89.8	63.6	65.8	10.5	74.3	47.8
Viability %	66.9	16	71.75	47.25	39.4	13	44.3	25.3	88	11	92	66	70.1	9	76.6	52.3
Nomal Morphology	80	12	91	66.8	55.7	14	75.6	60.4	90	15	95	67	78.8	8	82	54.34
Acrosome Integrity (%)	77	10	88	68	60.5	11	68	45.6	84	12	92	72	75.3	6	78.9	40.2
DFI%	5	1.4	6.9	3.44	10.2	2.4	12.6	8.5	3.2	0.88	5.21	2.33	5	1.2	6.7	3.5
Mitochondrial Potential (%)	56.5	11	60.8	24.8	30.6	9	38.3	22.3	68.5	8	73	46.3	48.4	12	53.1	37.6

Table1. Sperm analysis of thawed pellets before and after separation by single layer centrifugation using Bovipure colloid at T0 and T6 (6 hours incubation survival test)

T0 = initial time; T6= 6 hourse incubation (survival test); Mean= mean values; SD=standard deviation; Max=maximum value; Min= minimum value

4. Discussion

As it was expected, there was a progressive deterioration of all the studied parameters of sperm quality, over time. However, this deterioration was more evident in control samples, rather than in SLC-selected samples. Previous studies on stallion and buck semen have shown that SLC significantly improves the quality of sperm in different species [18-20]. In the present study, samples were incubated for 6 h, as long as they preserved their progressive motility.

A sharper progressive motility decrease after 6 h of incubation was obvious in control samples and more discrete in SLC-selected semen. After 6 h of incubation all SLC-selected samples maintained their progressive motility between 47.8 % and 65.8, compared to the control samples, where the mean value was 30%, ($p<0.001$).

A higher proportion of viable spermatozoa was observed in the SLC-selected samples when compared to control samples, at both time periods. This is also consistent with results from previous studies [18-21], suggesting that removal of unviable spermatozoa after preparation by density gradient method results in better longevity of the viable population. The increased longevity has been attributed to the fact that dead spermatozoa are a source of reactive oxygen species (ROS), that may cause damage to other spermatozoa [22] by attacking the polyunsaturated fatty acids (PUFA) of the sperm membrane [23,24]. Spermatozoa contain high concentrations of PUFA, therefore they are particularly sensitive to oxidative stress [23] causing their plasma membrane to lose its fluidity and integrity [24].

Moreover, the acrosome membrane can be damaged during semen handling and processing [9, 25] which may lead to sub-fertility in some bulls. In the present study, acrosome damage was not statistically different in control and SLC-selected samples, even though there was a numerical decrease after density gradient centrifugation with Bovipure. Since ROS leaking through unviable membranes and cause damage to other spermatozoa [22], we can assume that damaged acrosome membranes, both in dead and live cells, may be responsible for damaging other sperm cells in the ejaculate.

Previous studies [19, 20] have tested sperm chromatin integrity after density gradient centrifugation in stallions, showing a significant improvement in SLC-selected samples. In our study, we used the HalomaxBos-Taurus test to evaluate the degree of DNA damage. DNA damage was significantly different in control and SLC-selected samples.

Johannisson et al. [19] and Morrell et al. [20] suggested that SLC has many advantages, on one hand selects spermatozoa with intact chromatin since immature spermatozoa or those with damaged chromatin have a lower density and, removes seminal plasma along with dead spermatozoa and leukocytes in order to prevent further damaged caused by ROS. The latter is supported by studies done by Baumber et al. [22] in which the antioxidants catalase and glutathione reduced equine sperm DNA damage. In a study conducted by Morrel et al, 2014, [26] the use of single layer centrifugation with another specific colloid (Androcoll-B) of bull spermatozoa resulted in the selection of a sperm population that had higher mitochondrial membrane potential compared with the control samples. Additionally, other studies conducted [26] indicated that BoviPure® method has an enhanced capacity of selecting sperm for embryo production.

5. Conclusions

Semen preparation by the single layer gradient centrifugation with Bovipure significantly improved the quality of frozen-thawed bull semen. This procedure selected spermatozoa that were motile, possessed good membrane integrity, high respiratory activity and additionally, maintain these characteristics during storage significantly better than control samples,

prolonging their longevity. The overall advantages of SLC-selected samples over the control group were particularly evident after 6 hours of incubation. Thus, the preparation of bull semen by single layer centrifugation using Bovipure may be particularly useful in the AI industry in order to obtain semen of higher quality.

This method could enable the use of ejaculates of poor quality that would usually be discharged by selecting sperm cells with specific characteristics such as progressive motility, high DNA integrity and mitochondrial potential thus increasing the fertility potential. Prospective studies have shown that single layer centrifugation may reduce bacteria from fresh ejaculates before dilution; therefore this method could also help prevent antibiotic resistance.

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