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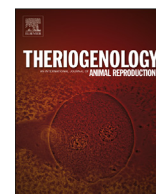

Effect of cushioned or single layer semen centrifugation before sex sorting on frozen stallion semen quality

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ABSTRACT

The aim of this study was to compare the effect of presorting centrifugation (cushioned [CC] or single-layer colloid [SLC]), with simple dilution (SD), on the quality of sex-sorted stallion semen before and after sorting and after freezing and thawing. Four ejaculates from each of two fertile stallions were collected 1 week apart and evaluated for percent total sperm motility (TM), percent viable acrosome-intact sperm (VAI), and DNA quality (percentage of DNA fragmentation index). Freezing caused, independently from CC and SLC treatments, a significant decrease of TM ($P < 0.05$) and VAI ($P < 0.05$) in both unsorted and sorted semen. On the other hand, sorting did not impair TM and VAI and, interestingly, improved DNA quality in all treatments only before freezing (28 vs 13, 28 vs 10, 22 vs 7 in SD, CC, and SLC for unsorted vs sorted groups, respectively; $P < 0.05$); this positive effect was lost in the same samples after freezing and thawing, suggesting that the freezing process reduces the DNA quality of sex-sorted sperm. Our results suggest that CC and SLC are not able to select those spermatozoa that possess a better ability to withstand sperm processing associated with sperm sorting and freezing.

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1. Introduction

Depending on the species, there are different reasons for performing gender selection before insemination primarily because of economic advantages of a particular sex. In horses, sex selection may also have an economic advantage such as the production of fillies in the Polo industry, or it may be rather subjective and due to a preference for the sex of a particular pedigree [1].

Commercial application of this process has been limited mainly by two factors: close proximity of the mare to the semen processing laboratory [2–4] and, more important, the poor quality of frozen-thawed sex-sorted stallion spermatozoa [1,2].

The development of procedures to cool and freeze sex-sorted sperm will be critical if this technology is to be embraced by the equine industry worldwide [1].

Pregnancy rates obtained with frozen-thawed sex-sorted stallion semen ranged between 0% and 16% when inseminating between 5 and 20×10^6 by hysteroscopic or rectally guided insemination [5,6]. Recently Gibb et al. [7] were able to achieve a pregnancy rate of 27% after insemination with frozen sex-sorted sperm, but there was a high incidence of early embryonic death.

Moreover, when used by intra cytoplasmic sperm injection for *in vitro* embryo production, the fertilizing ability of sex-sorted frozen-thawed spermatozoa is lower

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(range, 20%–30%), compared with nonsorted frozen-thawed sperm (range, 71%–83%) [8,9], even if the capability of establishing normal pregnancies is the same as nonsorted frozen-thawed semen [8].

Centrifugation of equine semen is commonly used to maximize sperm quality for semen preservation both cooled and frozen [10]. Cushioned and colloid centrifugation, in particular single-layer colloid centrifugation (SLC), are techniques extensively used for reducing sperm damage and selecting high-quality sperm, respectively [10–15].

The aim of this study was to investigate the effect of different presorting treatments such as CC and SLC, on the quality of sex-sorted stallion sperm before and after freezing and thawing.

2. Materials and methods

The experiment was approved by the Ethical and Scientific Committee of Alma Mater Studiorum, University of Bologna.

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan. Italy).

2.1. Semen collection and presorting treatments

Four ejaculates from two fertile stallions, one Trotter and one Connemara pony 15 and 18 years old, respectively, were collected 1 week apart by a Missouri model artificial vagina, equipped with a disposable liner and an online filter, after 1 week of a once-a-day collection for depleting extragonadal reserves. Sperm concentration was evaluated in the gel-free semen volume using a fluorescence-based instrument (NucleoCounter SP-100; ChemoMetec A/S, Allerød, Denmark) [13]. Each ejaculate was diluted in Kenney's extender supplemented with a modified high-potassium Tyrode's medium (KMT) extender [16] to a concentration of 100×10^6 sperm/mL and divided into three different treatments: simple dilution (SD), cushioned centrifugation (CC), and SLC.

For CC, 39 mL of extended semen was first loaded into a 50-mL glass conical tube; then, 1 mL of a cushion solution (Cushion Fluid, Minitube, Germany) was layered beneath the extended semen using a blunt-tipped 3.5-inch 18-ga spinal needle attached to a 20-mL sterile syringe.

For SLC, a 70% gradient solution was prepared using a silica particle solution (RediGrad, GE Healthcare Life Sciences) diluted with a calcium-free buffer, and pH and osmolarity were adjusted to about 7.0 and about 300 mOsm/L, respectively; 20-mL extended semen was gently layered onto 20 mL of the gradient solution in a 50-mL glass conical tube. Cushioned centrifugation and SLC semen samples were then centrifuged simultaneously at $300\times$ *g* for 20 minutes (Thermo IEC CL 10 Centrifuge). After centrifugation, in the CC sample, the supernatant was aspirated, most of the cushion solution was removed by aspiration and the remaining 5-mL sperm pellet was resuspended in KMT extender to 100×10^6 sperm/mL. In the SLC sample, supernatant and most of the gradient were discharged; the sperm pellet aspirated through a 100-mL pipette and resuspended in KMT extender to 100×10^6 sperm/mL.

2.2. Flow cytometric sperm-sorting and freezing procedure

Aliquots of 1 mL of semen of each treatment (100×10^6 sperm/mL) were incubated with 10 μL of Hoechst 33342 (5 mg/mL), 0.09-mM final concentration for 1 hour and 30 minutes at 35 °C in the dark. Just before sorting, 1 μL of food dye (FD&C#40, Warner Jenkinson, St. Louis, MO, USA) stock solution (25 mg/mL) was added to each sample to identify membrane-damaged spermatozoa by quenching the Hoechst 33342 fluorescence. The samples were then filtered through a 60-μm nylon mesh filter to remove debris or clumped spermatozoa. A MoFlo SX flow cytometer (DakoCytomation Inc., Fort Collins, CO, USA) equipped with an argon laser (wavelength 351 nm at 150 mW) was used. All live cells were sorted because separation of X from Y-bearing spermatozoa was not an aim of the work. Sorted spermatozoa were collected in polypropylene tubes containing 500 μL of 2.5% TEST-egg yolk buffer [17]. After collection of 8×10^6 spermatozoa per tube, the samples were centrifuged at $800\times$ *g* for 20 minutes and resuspended in Heitland extender [18] with 3% egg yolk and 3% glycerol, packed in 0.25-mL straws and frozen in a floating styrofoam box system 6 cm under liquid nitrogen vapor for 20 minutes. Samples were thawed in water bath at 35 °C to 37 °C for 30 minutes and immediately analyzed.

2.3. Computer-assisted sperm analyzer

Motility evaluation was performed using a computer-assisted sperm analyzer (Hamilton Thorne IVOS Version 12.2 L); semen was extended to 30×10^6 sperm/mL, and 1000 cells were analyzed using a fixed-height Leja Chamber SC-20-01-04-B, the Netherlands. Computer-assisted sperm analyzer settings from standard equine setup were frames per sec, 60 Hz; number of frames, 45; minimum contrast, 70; minimum cell size, 4 pixel; cell size, 6 pixel; cell intensity, 106; path velocity, 50 μm/s; straightness (%), 70%; and path velocity cut off, 20 μm/s.

2.4. Viability and acrosome integrity

Sperm acrosome intactness assay (fluoresceinated *Pisum sativum [FITC-PSA]*/propidium iodide [PI]) was used for evaluating viability and acrosome integrity by flow cytometry [19]. Viable acrosome-intact spermatozoa (VAI) were those cells that did not acquire the PI and FITC_PSA, whereas nonviable sperm were those that fluoresce red because of PI uptake. Fifty microliters from each treatment was diluted with 133 μL of Dulbecco's PBS solution (Invitrogen Gibco, Carlsbad, CA, USA) and stained with 2 μL of PI (Invitrogen Molecular Probes, Eugene, OR, USA; 2.4-mM working solution) and 0.05 mg/mL *P sativum*. Samples were incubated at room temperature in the dark for 10 minutes, and then, 20 μL of the stained sample was mixed with 400 μL of Dulbecco's PBS solution and subjected to analysis. A flow rate of approximately 300 events/s was used, and a total of 5000 events were evaluated per

sample. List-mode data were analyzed by WinList software (Verity Software House, Topsham, ME, USA).

2.5. Sperm chromatin structure assay

Sample preparation and processing, as well as flow cytometer adjustments, were performed as previously described [20]. Briefly, an aliquot of each semen samples was immediately frozen and stored in a −20 °C freezer until analysis (maximum 2 weeks). The sperm samples were handled individually and were thawed in a 35 °C to 37 °C water bath. Immediately after thawing (30–60 seconds), a 2 to 7 μL aliquot of semen was diluted to 200 μL in a buffer solution (0.186-g disodium EDTA, 0.790-g Tris-HCl, 4.380-g NaCI in 500-mL deionized water, pH 7.4). This was mixed with 400 μL of acid detergent solution (2.19-g NaCl, 1.0 mL of 2N HCI solution, 0.25-mL Triton X, deionized water quantum sufficit to a final volume of 250-mL). After 30 seconds, 1.2 mL of the acridine orange solution was added (3.8869-g citric acid monohydrate, 8.9428 g Na_2HPO_4, 4.3850-g NaO, 0.1700-g disodium EDTA, 4 μg/mL acridine orange stock solution [1 mg/mL], quantum sufficit 500-mLwater, pH 6.0). The sample was covered with aluminum foil and placed in the flow cytometer and allowed to pass through the tubing for 2 minutes before counting of the cells. The cell flow rate was placed on the high setting for the machine, which, based on sperm concentration in the solution, resulted in an actual flow rate of 100 to 200 cells/s. A total of 5000 events were evaluated for each sample. Sperm from a control stallion were used as a biologic control to standardize instrument settings between days of use. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (Fl-l at 500) and mean red fluorescence at 150 channels (Fl-3 at 150). Data were acquired in a list mode, and analysis was performed using WinList software (Verity Software House). The percent of sperm with abnormal DNA was defined by the parameter DNA fragmentation index (DFI).

2.6. Experimental design

Spermatozoa were evaluated for motility, viable acrosome intactness, and chromatin integrity before (SD) and after the different treatments (CC, SLC); analysis was repeated after sorting aliquots of semen from the different treatments, and after freezing and thawing both sorted and unsorted semen.

2.7. Statistical analysis

The normal distribution of the data was checked using the Lilliefors test. In light of the normal distribution, the data were analyzed using an ANOVA model. When significant differences were found Tukey *post hoc* test was performed to assess the difference between: SD, CC, and SLC treatments; unsorted and sorted samples; before and after freezing. The level of significance was set at $P < 0.05$. All analyses were performed using R version 3.0.3. (Copyright 2014, The R Foundation for Statistical Computing).

3. Results

3.1. Motility evaluation

Percent total sperm motility (TM) was similar among presorting treatments (D, CC, SLC) that were sorted and frozen ($P > 0.05$). Freezing induced a significant decrease in TM compared with unfrozen samples ($P < 0.05$), whereas TM was similar in unsorted and sorted samples ($P > 0.05$; Table 1).

3.2. Viability and acrosome integrity

Percent of viable acrosome-intact spermatozoa (VAI) spermatozoa was similar among presorting treatments that were sorted and frozen ($P > 0.05$). Percent VAI was less in frozen-thawed samples than that in unfrozen samples ($P < 0.05$), whereas no effect of sorting was recorded both in unfrozen and frozen samples ($P > 0.05$; Table 1).

3.3. Sperm chromatin structure assay

The results on DNA quality are summarized in Table 1.

Prefreeze sorted samples had lower percentage of DFI than prefreeze unsorted samples ($P < 0.05$); however, the

Table 1
Effect of presorting treatments, sorting procedure, and freezing on total sperm motility (TM), percentage of viable acrosome-intact spermatozoa (VAI), and the percentage of spermatozoa with damaged DNA (DNA fragmentation index [DFI]).

Semen treatment	TM			VAI			DFI		
	Presorting treatment			Presorting treatment			Presorting treatment		
	SD	CC	SLC	SD	CC	SLC	SD	CC	SLC
Prefreeze									
Unsorted	55.8 ± 15.2^{a}	53.6 ± 15.8^{a}	64.1 ± 10.2^{a}	65.0 ± 11.0^{a}	66.6 ± 10.9^{a}	72.3 ± 8.6^{a}	27.9 ± 8.7^{a}	28.0 ± 9.0^{a}	21.5 ± 6.6^{a}
Sorted	57.1 ± 15.2^{a}	51.5 ± 15.8^{a}	53.9 ± 17.4^{a}	76.0 ± 5.4^{a}	74.0 ± 6.7^{a}	73.4 ± 5.2^{a}	12.7 ± 8.4^{b}	10.0 ± 5.4^{b}	7.4 ± 4.3^{b}
Postthaw									
Unsorted	16.7 ± 9.3^{b}	14.0 ± 8.5^{b}	15.2 ± 6.0^{b}	26.8 ± 9.9^{b}	20.0 ± 7.3^{b}	25.8 ± 7.6^{b}	32.9 ± 10.7^{aA}	$28.1 \pm 6.6^{a\ AB}$	22.0 ± 6.4^{aB}
Sorted	7.2 ± 6.6^{b}	6.0 ± 5.9^{b}	6.1 ± 4.6^{b}	27.1 ± 12.7^{b}	25.8 ± 10.6^{b}	26.1 ± 11.6^{b}	25.5 ± 10.6^{a}	24.3 ± 9.1^{a}	17.0 ± 5.1^{a}

[a,b]Within columns, superscripts are different ($P < 0.05$).
[A,B]Within row, superscripts are different ($P < 0.05$) in DFI values.
Abbreviations: CC, cushion centrifugation; SD, simple dilution; SLC, single-layer colloid centrifugation.

percentage of DFI postthaw among presorting treatments in unsorted, sorted, and sorted frozen groups did not differ.

Single-layer colloid centrifugation showed a significantly lower percentage of DFI ($P < 0.05$) compared with SD in unsorted frozen samples.

4. Discussion

The aim of this study was to investigate the effect of different presorting treatments, CC and SLC compared with simple dilution, on sorted stallion sperm quality before and after freezing.

Cushioned centrifugation and SLC were chosen as treatments, compared with simple dilution, because, as reported before, these procedures are becoming a common place in processing stallion semen [10]. Other procedures have been reported for concentrating sperm [20] or selecting high-quality sperm [15], but centrifugation, with or without cushion, and colloid centrifugation, particularly using a single-layer, have been the most used in processing stallion semen for cooling and freezing.

Samples obtained after these treatments differ from the untreated samples being a selected population of sperm obtained with colloid centrifugation, and reduction of the amount of seminal plasma after CC.

Centrifugation of stallion semen can be harmful, especially when spermatozoa are packed tightly at the bottom of the tubes after vigorous centrifugation, so different solutions layered at the bottom of the tubes have been used to provide a "cushion" for spermatozoa during centrifugation [21]. Cushioned centrifugation can be used to provide a high sperm harvest while maintaining sperm function, and the volume of "cushion" solution can be reduced to 1 mL in conical bottom tubes without impairing sperm harvest or semen quality [10]. Recently Len et al. [22] compared sperm recovery rate, sperm motility (total and progressive), sperm plasma membrane integrity, and acrosomal integrity after cushioned or noncushioned centrifugation of equine semen extended in a commercial semen extender and subjected to higher centrifugal forces (900 and 1800× g) than that commonly recommended (400–600 g) for noncushioned centrifugation and lower centrifugation time (10 minutes) than that commonly used for CC (20 minutes), and they found optimal recovery rate and sperm quality after 900× *g* noncushioned centrifugation. The aim of the present study was not to optimize sperm recovery rate but to centrifuge semen trying to avoid sperm damage, and this is the reason why CC was performed with low centrifugation force (300× *g*). Processing sperm in this way did not modify the quality of fresh semen in terms of motility, viability, acrosome integrity, and DNA integrity, in agreement with Edmond et al. [23].

It has been reported that SLC selects sperm with higher progressive motility and better morphology, increases the shelf life of stored semen, and improves survival and fertility of frozen semen [15]. However, in our study we did not find any increase in the percentage of total motility and viable acrosome-intact sperm after SLC treatment in unsorted and sorted semen, either before and after freezing.

Cushioned centrifugation and SLC, followed by resuspension of sperm pellet, have as consequence the partial and total removal of seminal plasma (SP), respectively [15,21]. Seminal plasma has been shown to be detrimental to stallion spermatozoa during storage, and sperm motility, viability, membrane integrity, and fertility are better maintained when SP is diluted to 25% or less of its original volume, and this can be accomplished through centrifugation [21,24].

During SLC, SP is retained on the top of the colloid, removing some components that are beneficial to fertilization such as cysteine-rich secretory proteins and nonprotein constituents, such as cholesterol, that may protect the spermatozoa during *in vitro* storage [15].

Flow cytometric sex sorting of sperm is time consuming because sperm must pass individually through the laser beam. In this study, the interval between semen collection and sorting procedure of all different treatments was between 2 and 4 hours, during which the semen was held at room temperature, but differences in the amount of SP between samples (50%, ∼20%, and ∼0% in treatments SD, CC, and SLC, respectively) did not affect the sperm quality after sorting.

Our results suggest that processing stallion sperm by either CC or SLC does not improve the quality (i.e., sperm motility, viability, acrosome integrity, and DNA integrity) of fresh semen. In addition, presorting treatment did not improve the quality of postthaw sperm compared with simple dilution. Similar to a previous study [25], these results suggest that CC and SLC cannot select a sperm population that are more resistant to the sorting and freezing procedure.

The only significant positive effect of presorting treatments was observed in unsorted frozen semen in which the percentage of spermatozoa bearing damaged DNA was significantly reduced in SLC-treated sperm. These data agree with the results of Hoogewijs et al. [26], who reported a significant positive effect of SLC on DFI after freezing and thawing; in that study, however, a different colloid solution was used.

Our results indicate that the freezing procedure induces, independently from CC and SLC treatments, the most serious damage in terms of total motility and viability, causing a significant decrease of these parameters in both unsorted and sorted semen. On the other hand, the sorting procedure does not impair sperm quality relatively to these parameters in agreement with previous observations [27,28].

The sorting procedure improved DNA quality, as assayed by sperm chromatin structure assay, in all groups (SD, CC, and SLC) before freezing. Bochenek et al. [29] reported no detrimental effects of sex sorting on sperm chromatin, whereas the positive effect of sorting on the DNA integrity of the sperm cell population was described in bull [30,31]; all these observations agree with the fertility results reported with fresh sex-sorted semen [1–4].

In both bull and stallion spermatozoa, almost all the DNA is packaged by protamine, and this could be one of the reasons for the relative insensitivity to sorting damage of the chromatin in these species compared with human spermatozoa [32,33]. However, we found not only an

absence of DNA damage but also an increase in the percentage of DNA-intact sperm after sorting. An explanation could be found in the effectiveness of the discarding system of membrane-damaged cells by gating out those spermatozoa that present Hoechst 33342 fluorescence quenched by FD&C#40. A correlation between sperm viability and DNA integrity was reported in bulls by Gosálvez et al. [31] who observed that a large proportion of DNA-damaged spermatozoa are accumulated in the wasted population because of the simultaneous presence of membrane injury.

The positive effect of the sorting procedure on DNA integrity was lost after freezing and thawing suggesting that the freezing process reduces the DNA quality of sex-sorted sperm. This could explain the low viability of embryos and the high rate of embryonic death observed with the use of cryopreserved sex-sorted semen [7], and also the lower level of fertilizing ability compared with nonsorted frozen semen reported by Colleoni et al. [8], when sex-sorted frozen sperm were used by intra cytoplasmic sperm injection for *in vitro* embryo production.

Cryoprotectants differ from glycerol, as dimethylformamide [25] and antioxidants [34] have been shown to ameliorate the quality of semen and the effects of oxidative stress during the cryopreservation of sex-sorted equine sperm, so further studies are needed to optimize extender quality to reduce sperm damage during sorting and freezing procedures.

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Competing interests

The authors declare that no competing interests exist.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.11.031.

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