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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Mari, G., Bucci, D., Love, C.C., Mislei, B., Rizzato, G., Giaretta, E., et al. (2015). Effect of cushioned or single layer semen centrifugation before sex sorting on frozen stallion semen quality. THERIOGENOLOGY, 83(6), 953-958 [10.1016/j.theriogenology.2014.11.031].

Availability:
This version is available at: https://hdl.handle.net/11585/515794 since: 2015-09-30

Published:

DOI: http://doi.org/10.1016/j.theriogenology.2014.11.031

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(Article begins on next page)

10 November 2026

This is the final peer-reviewed accepted manuscript of:

Mari G, Bucci D, Love CC, Mislei B, Rizzato G, Giaretta E, Merlo B, Spinaci M.

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

Theriogenology 2015;83(6):953-8.

The final published version is available online at:

https://doi.org/10.1016/j.theriogenology.2014.11.031

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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 compare the effect of pre-sorting centrifugation (cushioned (CC), or single-layer colloid (SLC)), to 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 one week apart and evaluated for percent total sperm motility (TM), percent viable-acrosome intact sperm (VAI) and DNA quality (% DFI). 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.

Keywords

Sexed semen, horse, cushioned centrifugation, single-layer colloid centrifugation

1. Introduction

Depending on the species, there are different reasons for performing gender selection prior to insemination primarily due to 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 (2,3,4) 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 $20x10^6$ by hysteroscopic or rectally-guided insemination (5,6). Recently Gibb et al. were able to achieve pregnancy rate of 27% after insemination with frozen sex-sorted sperm, but there was a high incidence of early embryonic death (7).

Moreover, when used by ICSI for *in vitro* embryo production, the fertilizing ability of sex-sorted frozen-thawed spermatozoa is lower (range-20- 30%), compared to non-sorted frozen-thawed sperm (range-71-83%) (8,9), even if the capability of establishing normal pregnancies is the same as non-sorted 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, are techniques extensively used for reducing sperm damage and for selecting high quality sperm respectively (10-15).

The aim of this study was to investigate the effect of different pre-sorting treatments such as cushioned and single-layer colloid centrifugation, on the quality of sex-sorted stallion sperm before and after freezing and thawing.

2. Materials and methods

The experiment was approved by the Ethic-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 pre-sorting treatments

Four ejaculates from two fertile stallions, one Trotter and one Connemara pony 15 and 18 years old respectively, were collected one week apart by a Missouri model artificial vagina, equipped with a disposable liner and an online filter, after one 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 KMT extender (16) to a concentration of 100 x 10^6 sperm/mL and divided into three different treatments: simple dilution (SD), cushioned centrifugation (CC) and single-layer colloid centrifugation (SLC).

For CC, 39mL of extended semen were first loaded in a 50mL glass conical tube, then 1mL 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; 20mL extended semen were gently layered onto

20mL of the gradient solution in a 50mL glass conical tube. CC and SLC semen samples were then centrifuged simultaneously at 300 x g for 20' (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 5mL sperm pellet was resuspended in KMT extender to 100 x 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 x 10^6 sperm/ML.

2.2. *Flow cytometric sperm-sorting and freezing procedure*

Aliquots of 1mL of semen of each treatment (100 x 10^6 sperm/mL) were incubated with 10 µl of Hoechst 33342 (5 mg/mL) 0.09-mM final concentration for 1h and 30 min at 35°C in the dark. Just prior to 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 since 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 x 10^6 spermatozoa per tube, the samples were centrifuged at 800 X *g* for 20 min and resuspended in Heitland extender (18) with 3% egg yolk and 3% glycerol, packed in 0,25mL straws and frozen in a floating styrofoam box system 6 cm under liquid nitrogen vapour for 20'. Samples were thawed in water bath at 35°-37°C for 30'' and immediately analyzed.

2.3. *CASA*

Motility evaluation was performed using a computer assisted sperm analyzer (Hamilton Thorn IVOS Vers 12.2L); semen was extended to 30 x 10^6 sperm/ML and thousand cells were analyzed using a fixed height Leja Chamber SC 20-01-04-B, Netherland. CASA settings from standard equine set-up were: frames x sec 60 Hz, N° of frames 45, Minimum contrast 70, Minimum Cell Size 4 pixel, Cell Size 6 pixel, Cell Intensity 106, Pat Velocity (VAP) 50 µm/sec, Straightness (%) 70%, VAP cut off 20µm/sec.

2.4. *Viability and acrosome integrity*

Sperm acrosome intactness assay (fluoresceinated Pisum sativum-PSA/propidium iodide-PI) was used for evaluating viability and acrosome integrity by flow-cytometry (19). Viable, plasma membrane intact sperm (VAI) were those cells that did not acquire the PI, while non-viable sperm were those that fluoresce red due to PI uptake. Fifty microliters from each treatment were diluted with 133µl of Dulbecco phosphate buffer saline solution (D-PBS, Invitrogen Gibco,® Carlsbad, CA, USA) and stained with 2µl of PI (Invitrogen Molecular Probes, Eugene, OR, USA; 2.4 mM working solution) and 0.05 mg/mL PSA . Samples were incubated at room temperature in the dark for 10 min, and then 20µl of the stained sample were mixed with 400µl of D-PBS and subjected to analysis. A flow rate of approximately 300 events/sec 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 (SCSA)*

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 (max two weeks). The sperm samples were handled individually and were thawed in a 35 to 37°C water bath. Immediately following thawing (30 to 60 sec), a 2-7uL aliquot o f semen was diluted to 200 uL in a buffer solution (0. 186g disodium EDTA, 0.790 g Tris-HCl, 4.380 g NaCl in 500 mL deionized water,pH7.4). This was mixed with 400 uL of acid-detergent solution(2.19gNaC~ 1.0 mL of2N HCl solution, 0.25 mL Triton-X, qs. 250 mL deionized water). After 30 sec, 1.2 mL of the acridine orange (AO) solution was added (3.8869 g citric acid monohydrate, 8.9429 g NazH}lO... 4.3850 g NaO, 0.1700 g disodium EDTA, 4 ug/mL acridine orange stock solution (1mglmL),qs.500mLwater,pH6.0). The sample was covered with aluminum foil and placed in the flow cytometer, and allowed to pass through the tubing for 2 min prior to 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 100-200cells/sec. A total of 5000

events was evaluated for each sample. Sperm from a control stallion were used as a biologic control to standardize instrument settings between days of use. The flowcytometer was adjusted such that the mean green fluorescence was set at 500 channels (Fl-l @ 500) and mean red fluorescence at 150 channels (Fl-3@150).. Data were acquired in a list-mode and analysis was performed using WinList™ software (Verity Software House, Topsham, ME, USA). 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 were 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 Lillefors 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: D, CC and SLC treatments; unsorted and sorted samples; pre- and post-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 pre-sorting treatments (D, CC, SLC) that were sorted and frozen ($P>0.05$). Freezing induced a significant decrease in TM compared to unfrozen samples ($P<0.05$) while TM was similar in unsorted and sorted samples ($P>0.05$) (Tab. 1).

3.2. Viability and acrosome integrity

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

3.3. Sperm Chromatin Structure Assay (SCSA)

The results on DNA quality are summarized in Table 1.

Pre-freeze sorted samples had lower % DFI than pre-freeze unsorted samples ($p<0.05$); however, the % DFI post-thaw among pre-sorting treatments in unsorted, sorted and sorted frozen groups did not differ.

SLC showed a significantly lower % DFI ($P<0.05$) compared to D in unsorted frozen samples.

4. Discussion

The aim of this study was to investigate the effect of different pre-sorting treatments, cushioned centrifugation and single-layer colloid centrifugation compared to simple dilution, on sorted stallion sperm quality before and after freezing.

Cushioned centrifugation and colloid single-layer centrifugation were chosen as treatments, compared to simple dilution, because, as reported before, these procedures are becoming a commonplace in processing stallion semen (10). Other procedures have been reported for

188 concentrating sperm (20) or for selecting high quality sperm (15), but centrifugation, with or without
189 cushion, and colloid centrifugation, particularly using a single-layer, have been the most used in
190 processing stallion semen for cooling and freezing.
191 Samples obtained after these treatments differ from the untreated samples being a selected
192 population of sperm obtained with colloid centrifugation, and reduction of the amount of seminal
193 plasma after cushioned centrifugation.
194 Centrifugation of stallion semen can be harmful, especially when spermatozoa are packed tightly at
195 the bottom of the tubes after vigorous centrifugation, so different solutions layered at the bottom
196 of the tubes has been used to provide a "cushion" for spermatozoa during centrifugation (21).
197 Cushioned centrifugation can be used to provide a high sperm harvest while maintaining sperm
198 function, and the volume of "cushion" solution can be reduced to 1mL in conical bottom-tubes
199 without impairing sperm harvest or semen quality (10). Recently Len et al. (22) compared sperm
200 recovery rate, sperm motility (total and progressive), sperm plasma membrane integrity, and
201 acrosomal integrity after cushioned or noncushioned centrifugation of equine semen extended in a
202 commercial semen extender and subjected to higher centrifugal forces (900 and 1800 x g) than that
203 commonly recommended (400–600 g) for noncushioned centrifugation and lower centrifugation
204 time (10 min) than commonly used for cushioned centrifugation (20 minutes), and they found
205 optimal recovery rate and sperm quality after 900 x g non cushioned centrifugation. The aim of the
206 present study was not to optimize sperm recovery rate but to centrifuge semen trying to avoid
207 sperm damage, and this is the reason why cushioned centrifugation was performed with low
208 centrifugation force (300 x g). Processing sperm in this way did not modify the quality of fresh semen
209 in terms of motility, viability-acrosome integrity and DNA integrity, in agreement with Edmond et
210 al. (23).
211 It has been reported that SLC selects sperm with higher progressive motility and better morphology,

212 increases the shelf life of stored semen and improves survival and fertility of frozen semen (15).
213 However, in our study we did not find any increase in the percentage of total motility and viable
214 acrosome intact sperm following SLC treatment in unsorted and sorted semen, either pre and post-
215 freezing,.

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

221 During SLC, SP is retained on the top of the colloid, removing some components that are beneficial
222 to fertilization such as cysteine–rich secretory proteins (CRISP) and non-protein constituents, such
223 as cholesterol, that may protect the spermatozoa during *in vitro* storage (15).

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

229 Our results suggest that processing stallion sperm by either CC or SLC does not improve the quality
230 (i.e. sperm motility, viability-acrosome integrity and DNA integrity) of fresh semen. In addition, pre-
231 sorting treatment did not improve the quality of post-thaw sperm compared to simple dilution.,
232 Similar to a previous study (25), these results suggest that CC and SLC cannot select a sperm
233 population that are more resistant to the sorting and freezing procedure.

234 The only significant positive effect of pre-sorting treatments was observed in unsorted frozen semen
235 in which the percentage of spermatozoa bearing damaged DNA was significantly reduced in SLC
236 treated sperm. This data agree with the results of Hoogewis et al (26), who reported a significant
237 positive effect of SLC on DFI after freezing and thawing; in that study, however, a different colloid
238 solution was used.
239 Our results indicate that the freezing procedure induces, independently from CC and SLC
240 treatments, the most serious damage in term of total motility and viability, causing a significant
241 decrease of these parameters in both unsorted and sorted semen. On the other hand, the sorting
242 procedure does not impair sperm quality relatively to these parameters in agreement with previous
243 observations (27,28).
244 The sorting procedure improved DNA quality, as assayed by SCSA, in all groups (D, CC and SLC) prior
245 to freezing. Bochenek et al. (29) reported no detrimental effects of sex sorting on sperm chromatin,
246 whereas the positive effect of sorting on the DNA integrity of the sperm cell population was
247 described in bull (30,31); all these observations agree with the fertility results reported with fresh
248 sex-sorted semen (1-4).

249 In both bull and stallion spermatozoa almost all the DNA is packaged by protamine and this could
250 be one of the reasons for the relative insensitivity to sorting damage of the chromatin in these
251 species compared with human spermatozoa (32,33). However, not only did we find an absence of
252 DNA damage but also an increase in the percentage of DNA intact sperm after sorting. An
253 explanation could be found in the effectiveness of the discarding system of membrane damaged
254 cells by gating out those spermatozoa that present Hoechst33342 fluorescence quenched by
255 FD&C#40. A correlation between sperm viability and DNA integrity was demonstrated in bulls by
256 Gosalvez et al. (31) who observed that a large proportion of DNA damaged spermatozoa are
257 accumulated in the wasted population due to 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 to non-sorted frozen semen reported by Colleoni et al (8), when sex-sorted frozen sperm were used by ICSI for *in vitro* embryo production.

Cryprotectants different from glycerol, as dimethyl formamide (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.

Acknowledgements

The authors wish to thank Fondazione Sfameni for financially supporting EG.

Work supported by a Bologna University grant (RFO).

Conflict of interest

The authors declare that no competing interests exist

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Table 1. Effect of pre-sorting treatments, sorting procedure and freezing on total sperm motility (TM), percentage of viable acrosome intact spermatozoa (VAI) and on the percentage of spermatozoa with damaged DNA (DFI).
D- simple dilution; CC- cushion centrifugation; SLC- single layer colloid centrifugation.

	TM			VAI			DFI		
	Pre-sorting Treatment			Pre-sorting Treatment			Pre-sorting Treatment		
Semen treatment	**SD**	**CC**	**SLC**	**SD**	**CC**	**SLC**	**SD**	**CC**	**SLC**
Pre-freeze									
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}
Post-thaw									
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$^{a\,A}$	28.1 ± 6.6$^{a\,AB}$	22.0 ± 6.4$^{a\,B}$
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.

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