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

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1 EFFECT OF CUSHIONED OR SINGLE LAYER SEMEN CENTRIFUGATION BEFORE SEX SORTING ON  
2 FROZEN STALLION SEMEN QUALITY

3

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20

21 **ABSTRACT**

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

34

35 **Keywords**

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

37

## 38 **1. Introduction**

39

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

44 Commercial application of this process has been limited mainly by two factors: close proximity of  
45 the mare to the semen processing laboratory (2,3,4) and, more important, the poor quality of  
46 frozen-thawed sex-sorted stallion spermatozoa (1,2).

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

49 Pregnancy rates obtained with frozen-thawed sex-sorted stallion semen ranged between 0% and  
50 16% when inseminating between 5 and  $20 \times 10^6$  by hysteroscopic or rectally-guided insemination  
51 (5,6). Recently Gibb et al. were able to achieve pregnancy rate of 27% after insemination with frozen  
52 sex-sorted sperm, but there was a high incidence of early embryonic death (7).

53 Moreover, when used by ICSI for *in vitro* embryo production, the fertilizing ability of sex-sorted  
54 frozen-thawed spermatozoa is lower (range-20- 30%), compared to non-sorted frozen-thawed  
55 sperm (range-71-83%) (8,9), even if the capability of establishing normal pregnancies is the same as  
56 non-sorted frozen-thawed semen (8).

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

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

64

## 65 **2. Materials and methods**

66 The experiment was approved by the Ethic-scientific Committee of Alma Mater Studiorum,  
67 University of Bologna.

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

69

### 70 **2.1. Semen collection and pre-sorting treatments**

71 Four ejaculates from two fertile stallions, one Trotter and one Connemara pony 15 and 18 years old  
72 respectively, were collected one week apart by a Missouri model artificial vagina, equipped with a  
73 disposable liner and an online filter, after one week of a once a day collection for depleting  
74 extragonadal reserves. Sperm concentration was evaluated in the gel-free semen volume using a  
75 fluorescence-based instrument (NucleoCounter SP-100; Chemometec, A/S, Allerød, Denmark) (13).  
76 Each ejaculate was diluted in KMT extender (16) to a concentration of  $100 \times 10^6$  sperm/mL and  
77 divided into three different treatments: simple dilution (SD), cushioned centrifugation (CC) and  
78 single-layer colloid centrifugation (SLC).

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

82 For SLC, a 70% gradient solution was prepared using a silica particle solution (RediGrad, GE  
83 Healthcare Life, Sciences) diluted with a calcium-free buffer, and pH and osmolarity were adjusted  
84 to about 7.0 and about 300 mOsm/L respectively; 20mL extended semen were gently layered onto

85 20mL of the gradient solution in a 50mL glass conical tube. CC and SLC semen samples were then  
86 centrifuged simultaneously at 300 x g for 20' (Thermo IEC CL 10 Centrifuge). After centrifugation, in  
87 the CC sample the supernatant was aspirated, most of the cushion solution was removed by  
88 aspiration and the remaining 5mL sperm pellet was resuspended in KMT extender to  $100 \times 10^6$   
89 sperm/ML. In the SLC sample, supernatant and most of the gradient were discharged, the sperm  
90 pellet aspirated through a 100 mL pipette and resuspended in KMT extender to  $100 \times 10^6$  sperm/ML.

91  
92 **2.2.** *Flow cytometric sperm-sorting and freezing procedure*

93 Aliquots of 1mL of semen of each treatment ( $100 \times 10^6$  sperm/mL) were incubated with 10  $\mu$ l of  
94 Hoechst 33342 (5 mg/mL) 0.09-mM final concentration for 1h and 30 min at 35°C in the dark. Just  
95 prior to sorting, 1  $\mu$ l of food dye (FD&C#40, Warner Jenkinson, St. Louis, MO, USA) stock solution  
96 (25 mg/mL) was added to each sample to identify membrane damaged spermatozoa by quenching  
97 the Hoechst 33342 fluorescence. The samples were then filtered through a 60  $\mu$ m nylon mesh filter  
98 to remove debris or clumped spermatozoa. A MoFlo SX<sup>®</sup> flow cytometer (DakoCytomation Inc., Fort  
99 Collins, CO, USA) equipped with an argon laser (wavelength 351 nm at 150 mW) was used. All live  
100 cells were sorted since separation of X from Y-bearing spermatozoa was not an aim of the work.  
101 Sorted spermatozoa were collected in polypropylene tubes containing 500  $\mu$ l of 2.5% TEST-egg yolk  
102 buffer (17). After collection of  $8 \times 10^6$  spermatozoa per tube, the samples were centrifuged at 800  
103 X g for 20 min and resuspended in Heitland extender (18) with 3% egg yolk and 3% glycerol, packed  
104 in 0,25mL straws and frozen in a floating styrofoam box system 6 cm under liquid nitrogen vapour  
105 for 20'. Samples were thawed in water bath at 35°-37°C for 30'' and immediately analyzed.

106  
107 **2.3.** *CASA*

108 Motility evaluation was performed using a computer assisted sperm analyzer (Hamilton Thorn IVOS  
109 Vers 12.2L); semen was extended to  $30 \times 10^6$  sperm/ML and thousand cells were analyzed using a  
110 fixed height Leja Chamber SC 20-01-04-B, Netherland. CASA settings from standard equine set-up  
111 were: frames x sec 60 Hz, N° of frames 45, Minimum contrast 70, Minimum Cell Size 4 pixel, Cell Size  
112 6 pixel, Cell Intensity 106, Pat Velocity (VAP) 50  $\mu$ m/sec, Straightness (%) 70%, VAP cut off 20 $\mu$ m/sec.

113

114        **2.4.**        *Viability and acrosome integrity*

115 Sperm acrosome intactness assay (fluoresceinated *Pisum sativum*-PSA/propidium iodide-PI) was  
116 used for evaluating viability and acrosome integrity by flow-cytometry (19). Viable, plasma  
117 membrane intact sperm (VAI) were those cells that did not acquire the PI, while non-viable sperm  
118 were those that fluoresce red due to PI uptake. Fifty microliters from each treatment were diluted  
119 with 133µl of Dulbecco phosphate buffer saline solution (D-PBS, Invitrogen Gibco,® Carlsbad, CA,  
120 USA) and stained with 2µl of PI (Invitrogen Molecular Probes, Eugene, OR, USA; 2.4 mM working  
121 solution) and 0.05 mg/mL PSA . Samples were incubated at room temperature in the dark for 10  
122 min, and then 20µl of the stained sample were mixed with 400µl of D-PBS and subjected to analysis.  
123 A flow rate of approximately 300 events/sec was used and a total of 5000 events were evaluated  
124 per sample. List-mode data were analyzed by WinList™ software (Verity Software House, Topsham,  
125 ME, USA).

126

127        **2.5.**        *Sperm Chromatin Structure Assay (SCSA)*

128 Sample preparation and processing, as well as flow cytometer adjustments, were performed as  
129 previously described (20). Briefly, an aliquot of each semen samples was immediately frozen and  
130 stored in a -20°C freezer until analysis (max two weeks). The sperm samples were handled  
131 individually and were thawed in a 35 to 37°C water bath. Immediately following thawing (30 to 60  
132 sec), a 2-7uL aliquot o f semen was diluted to 200 uL in a buffer solution (0. 186g disodium EDTA,  
133 0.790 g Tris-HCl, 4.380 g NaCl in 500 mL deionized water,pH7.4). This was mixed with 400 uL of acid-  
134 detergent solution(2.19gNaC~ 1.0 mL of2N HCl solution, 0.25 mL Triton-X, qs. 250 mL deionized  
135 water). After 30 sec, 1.2 mL of the acridine orange (AO) solution was added (3.8869 g citric acid  
136 monohydrate, 8.9429 g NazH}lO... 4.3850 g NaO, 0.1700 g disodium EDTA, 4 ug/mL acridine orange  
137 stock solution (1mg/mL),qs.500mLwater,pH6.0). The sample was covered with aluminum foil and  
138 placed in the flow cytometer, and allowed to pass through the tubing for 2 min prior to counting  
139 of the cells. The cell flow rate was placed on the high setting for the machine, which, based on sperm  
140 concentration in the solution, resulted in an actual flow rate of 100-200cells/sec. A total of 5000



141 events was evaluated for each sample. Sperm from a control stallion were used as a biologic control  
142 to standardize instrument settings between days of use. The flowcytometer was adjusted such that  
143 the mean green fluorescence was set at 500 channels (FI-I @ 500) and mean red fluorescence at  
144 150 channels (FI-3@150).. Data were acquired in a list-mode and analysis was performed using  
145 WinList™ software (Verity Software House, Topsham, ME, USA). The percent of sperm with  
146 abnormal DNA was defined by the parameter DNA fragmentation index (DFI).

147

## 148 **2.6.** *Experimental design*

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

153

## 154 **2.7.** *Statistical analysis*

155 The normal distribution of the data was checked using the Lillefors test. In light of the normal  
156 distribution, the data were analyzed using an ANOVA model. When significant differences were  
157 found Tukey post-hoc test was performed to assess the difference between: D, CC and SLC  
158 treatments; unsorted and sorted samples; pre- and post-freezing. The level of significance was set  
159 at  $P < 0.05$ . All analyses were performed using R version 3.0.3. (Copyright © 2014, The R Foundation  
160 for Statistical Computing).

161

## 162 **3. Results**

### 163 *3.1. Motility evaluation*

164 Percent total sperm motility (TM) was similar among pre-sorting treatments (D, CC, SLC) that were  
165 sorted and frozen ( $P>0.05$ ). Freezing induced a significant decrease in TM compared to unfrozen  
166 samples ( $P<0.05$ ) while TM was similar in unsorted and sorted samples ( $P>0.05$ ) (Tab. 1).

167

### 168 *3.2. Viability and acrosome integrity*

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

173

### 174 *3.3. Sperm Chromatin Structure Assay (SCSA)*

175 The results on DNA quality are summarized in Table 1.

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

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

180

## 181 **4. Discussion**

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

185 Cushioned centrifugation and colloid single-layer centrifugation were chosen as treatments,  
186 compared to simple dilution, because, as reported before, these procedures are becoming a  
187 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.

258 The positive effect of the sorting procedure on DNA integrity was lost after freezing and thawing  
259 suggesting that the freezing process reduces the DNA quality of sex-sorted sperm. This could explain  
260 the low viability of embryos and the high rate of embryonic death observed with the use of  
261 cryopreserved sex-sorted semen (7), and also the lower level of fertilizing ability compared to non-  
262 sorted frozen semen reported by Colleoni et al (8), when sex-sorted frozen sperm were used by ICSI  
263 for *in vitro* embryo production.

264 Cryoprotectants different from glycerol, as dimethyl formamide (25) and antioxidants (34) have been  
265 shown to ameliorate the quality of semen and the effects of oxidative stress during the  
266 cryopreservation of sex- sorted equine sperm, so further studies are needed to optimize extender  
267 quality to reduce sperm damage during sorting and freezing procedures.

268

269

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## 273 **Conflict of interest**

274 The authors declare that no competing interests exist

275

276

277

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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.

Semen treatment	TM			VAI			DFI		
	Pre-sorting Treatment			Pre-sorting Treatment			Pre-sorting Treatment		
	SD	CC	SLC	SD	CC	SLC	SD	CC	SLC
<b>Pre-freeze</b>									
<b>Unsorted</b>	55.8 ± 15.2 <sup>a</sup>	53.6 ± 15.8 <sup>a</sup>	64.1 ± 10.2 <sup>a</sup>	65.0 ± 11.0 <sup>a</sup>	66.6 ± 10.9 <sup>a</sup>	72.3 ± 8.6 <sup>a</sup>	27.9 ± 8.7 <sup>a</sup>	28.0 ± 9.0 <sup>a</sup>	21.5 ± 6.6 <sup>a</sup>
<b>Sorted</b>	57.1 ± 15.2 <sup>a</sup>	51.5 ± 15.8 <sup>a</sup>	53.9 ± 17.4 <sup>a</sup>	76.0 ± 5.4 <sup>a</sup>	74.0 ± 6.7 <sup>a</sup>	73.4 ± 5.2 <sup>a</sup>	12.7 ± 8.4 <sup>b</sup>	10.0 ± 5.4 <sup>b</sup>	7.4 ± 4.3 <sup>b</sup>
<b>Post-thaw</b>									
<b>Unsorted</b>	16.7 ± 9.3 <sup>b</sup>	14.0 ± 8.5 <sup>b</sup>	15.2 ± 6.0 <sup>b</sup>	26.8 ± 9.9 <sup>b</sup>	20.0 ± 7.3 <sup>b</sup>	25.8 ± 7.6 <sup>b</sup>	32.9 ± 10.7 <sup>aA</sup>	28.1 ± 6.6 <sup>aAB</sup>	22.0 ± 6.4 <sup>aB</sup>
<b>Sorted</b>	7.2 ± 6.6 <sup>b</sup>	6.0 ± 5.9 <sup>b</sup>	6.1 ± 4.6 <sup>b</sup>	27.1 ± 12.7 <sup>b</sup>	25.8 ± 10.6 <sup>b</sup>	26.1 ± 11.6 <sup>b</sup>	25.5 ± 10.6 <sup>a</sup>	24.3 ± 9.1 <sup>a</sup>	17.0 ± 5.1 <sup>a</sup>

<sup>a,b</sup> within columns superscripts are different (P<0.05)

<sup>A,B</sup> within row superscripts are different (P<0.05) in DFI values.