Sex-sorted canine sperm cryopreservation: Limits and procedural considerations

Sex-sorted canine sperm cryopreservation: limits and procedural considerations

Barbara Merlo[a*], DVM, PhD, ECAR Diplomate, Associate Professor

Daniele Zambelli[a], DVM, PhD, ECAR Diplomate, Associate Professor

Marco Cunto[a], DVM, PhD, Researcher

Iacono Eleonora[a], DVM, PhD, Associate Professor

Ludovica Nasi[a], DVM

Elisa Giaretta[a], PhD student

Giovanna Galeati[a], DVM, PhD, Associate Professor

Diego Bucci[a], DVM, PhD, Research grant student

Marcella Spinaci[a], DVM, PhD, Associate Professor

[a]Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.

[*]Corresponding Author at: Barbara Merlo, DVM, PhD, ECAR Diplomate, Researcher, Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.

Tel.: +39-051-2097567

E-mail: barbara.merlo@unibo.it

Abstract

The aim of this study was to define a protocol to store dog sperm pre- and post-sorting in order to obtain an insemination dose sufficient to allow the conception by artificial insemination. Experiment 1 and 2 were performed to evaluate the more appropriate extender for preserving at room temperature dog sperm pre and post sorting. Four extenders were tested: a) Tris-fructose-citrate (TFC), b) Tris-glucose-citrate (TGC), c) modified Tyrode's albumin-lactate-pyruvate medium (mTALP), d) third fraction of the ejaculate (after centrifugation at 5000 x g for 10 min) (III FRAC). Experiment 3 and 4 were performed to evaluate the ability of dog semen to withstand sex-sorting and freezing/thawing. mTALP was the best extender for canine sperm storage at room temperature (20-25°C) pre- (total motility: TFC 8.3 ± 1.7, TGC 50.0 ± 11.5, mTALP 70.0 ± 0.1, III FRAC 25.0 ±1 0.4, $P<0.05$) and post-sorting (total motility: TFC 7.3 ± 1.5, TGC 10.3 ± 1.5, mTALP 33.3 ± 6.7, III FRAC 8.7 ± 5.8, $P<0.05$), even if at 24h sorted sperm quality was impaired in all extenders tested herein. Sperm quality decreased after sorting (total motility: control 92.5 ± 0.9, sorted 52.9 ± 6.0, $P < 0,05$) and, especially, after freezing/thawing (total motility: frozen-control 25.7 ± 4.1, frozen-sorted 2.4 ± 1.2, $P < 0,05$). In conclusion mTALP is an appropriate medium for canine sperm storage pre- and soon after sorting (hours), but a long storage period of sexed sperm at room temperature is not adequate. Cryopreservation greatly impaired sperm quality and further studies are needed to optimize the freezing protocol for sexed dog sperm.

Keywords: dog, spermatozoa, sex-sorting, extender, cryopreservation

1. Introduction

Flow cytometric sperm sorting based on X and Y sperm DNA difference has been established as the only effective method to pre-determinate the sex of mammalian offspring before fertilization.

While sexing technique has already reached a commercial level in the bovine species, sorting efficacy has been demonstrated suggesting future applications in a variety of mammals (pig, horse, sheep, goat, cat, endangered species) [1].

The production of animals of a predetermined sex by sperm sexing is an interesting target also in dog reproduction as, according to the different fields of breeding and use of animals (purebred pet dog, working dogs such as guide dogs, rescue dogs, farm dogs), male or female are preferably sought by dog breeders and owners [2-5].

The possible association of reproductive technology, such as cryopreservation and artificial insemination (AI), with sex sorting of sperm could optimize the profitability of the breeding and should have a great potential application also for the preservation of endangered canids maintaining the best male-female ratio for the animal repopulation [6].

On the best of our knowledge only a couple of studies have been performed until now on dog sperm sexing [3,5].

One of the major limitation for the use of sperm sexing in the dog, as well as in other species, is the long sorting time necessary in order to obtain the number of sexed spermatozoa necessary for artificial insemination (AI) (approximately 150-200 million motile spermatozoa) [7]. Rodenas et al.,[5] reported that is possible to collect 14-16 x 10^6 dog sexed spermatozoa per hour in the best samples; therefore conventional AI is not feasible in this species. Moreover it has been reported that spermatozoa can be damaged during the sorting process [1]. The possibility to overcome this limitation by reducing both the number of spermatozoa and the distance from the site of fertilization has been demonstrated by Meyers et al. [3] producing offspring of the desired sex in one bitch by intrauterine low-dose insemination using sexed spermatozoa.

87 Furthermore, the number of spermatozoa requested in dog for a intrauterine low-dose insemination
88 (at least 50 million) [7] is very high considering the sorting time and, therefore, sperm cells have to
89 be stored properly at room temperature waiting to be sexed.
90 Another problem to solve is the storage of sexed semen, as the bitch can be far from the sorting
91 facilities or more than one insemination could be required. Until now no data about storage of
92 canine sexed semen are available in the literature.
93 Cryopreservation permits long-term sperm storage, but it has to be taken in mind that sexed
94 spermatozoa are cells already stressed by the sorting procedure and usually present a greater
95 susceptibility to cryopreservation procedures compared to unsexed semen.
96 The aim of this study was to define: 1) an appropriate extender in order to liquid store dog sperm
97 pre- and post-sorting; 2) evaluate if sexed canine spermatozoa are able to withstand
98 freezing/thawing procedure. For this purpose motility, viability and acrosome integrity were
99 assessed in unsorted and sorted sperm either maintained at room temperature or cryopreserved.
100
101 **2. Materials and methods**
102
103 ***2.1 Experimental design***
104
105 This study was divided into 4 experiments.
106 As the sorting process is slow, the aim of the experiment 1 was to evaluate which of four extenders
107 is the more appropriate for storing at room temperature dog spermatozoa waiting to be sexed. Total
108 motile spermatozoa, movement, viability and acrosome integrity were evaluated at 0, 2, 4, 6, 8, and
109 24h.

In the experiment 2, the best extender from experiment 1 (mTALP, see below) was used for diluting pre-sorted spermatozoa while the four extenders evaluated in the experiment 1 were tested for sperm storage at room temperature (20-25°C) after sorting. The above mentioned parameters were evaluated at 0, 2, 4, 24h after sorting.

The aim of the experiment 3 was to evaluate if dog spermatozoa are able to withstand sexing procedure followed by freezing. For that purpose ejaculates from purebred dogs (Australian Shepherd) were analyzed: immediately after collection (CTR), after the sorting (SORT) and after freezing and thawing of sperm for all groups (CTR-FR, SORT-FR)**.**

In the experiment 4 the same protocol of the experiment 3 was performed on semen from mixed breed dogs.

2.2 Extenders

All reagents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated.

Extenders tested in experiment 1 were Tris-fructose-citrate (TFC) (3% wt/vol Tris, 1.7% wt/vol citric acid, 1.25% wt/vol fructose, 0.06% wt/vol Na benzyl-penicillin, 0.1% wt/vol streptomycin sulphate in distilled water), Tris-glucose-citrate (TGC) (2.4% wt/vol Tris, 1.4% wt/vol citric acid, 0.8% wt/vol glucose, 0.06% wt/vol sodium benzyl-penicillin, 0.1% wt/vol streptomycin sulphate in distilled water), modified Tyrode's solution (mTALP) (3.3 mM $MgCl_2$, 99 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.35 Mm NaH_2PO_4, 1 Mm sodium pyruvate, 21.6 mM DL lactic acid, 0.125 mM Kanamycin in distilled water), third fraction (III FRAC) of the ejaculated after centrifugation at 5000 x g for 10 min.

Freezing extender for the first step was TGC supplemented with 20% (v/v) egg yolk, 3% (v/v) glycerol (EYTGC1), while for the second step TGC was supplemented with 20% (v/v) egg yolk,

7% (v/v) glycerol and 1 % (v/v) Equex STM Paste (Nova Chemical Sales Inc., Scituate, Ma, USA) (EYTGC2).

2.3 Semen collection and evaluation

A total of 14 adult mixed breed and purebred male dogs between 1 and 7 years of age were enrolled in these experiments, between January 2008 and March 2013. The three fractions of the ejaculates were collected in calibrated plastic vials by digital manipulation. The experiment was approved by the Ethic-scientific Committee of Alma Mater Studiorum, University of Bologna.

The sperm-rich fraction was evaluated for volume, concentration, total sperm motility, movement, sperm morphology and membrane integrity to estimate sperm viability.

The volume was measured by a calibrated micro-pipette and sperm concentration was determined with a Bürker chamber, after dilution of the sperm suspension 1:40 with buffered formol saline (BFS) to immobilize spermatozoa.

The percentage of total motile spermatozoa and movement score, based on the type of the forward movement of sperm (scale of 0–5; 0 = no forward movement, 5 = steady, rapid forward progression) [8] were subjectively estimated at a phase contrast microscope (x400) (Axiolab, Zeiss, Italy) equipped with a warming plate (37°C) (Thermo Plate, Tokai Hit, Japan). Percentages of morphologically normal spermatozoa were determined at the same microscope (x1000) after dilution of semen 1:1 with BFS, and at least 200 spermatozoa per sample were examined.

In order to evaluate plasma membrane integrity, 25 µL of semen were incubated with 2 µL of a 300 µM propidium iodide (PI) stock solution, 2 µL of a 10 µM SYBR-14 (green) stock solution, both obtained from the live/dead sperm viability kit (Life Technologies, Molecular Probes®, Monza, Italy) for 5 min at 37°C in the darkness. Aliquots of the stained suspensions were placed on clean microscope slides, overlaid carefully with coverslips and at least 200 spermatozoa per sample were

159 scored with Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop,
160 The Netherlands). Spermatozoa stained with SYBR-14 and not stained with PI were considered as
161 viable. Spermatozoa SYBR positive and PI positive and those SYBR negative / PI positive were
162 considered as cells with non-intact membranes or dead as an estimate of sperm viability.
163 Acrosome integrity was measured with a FITC-coniugated lectin from Pisum sativum (FITC-PSA)
164 which labelled acrosomal matrix glycoproteins. Briefly, spermatozoa were washed twice in PBS,
165 resuspended with ethanol 95% and fixed at 4°C for 30 min. Samples were dried in heated slides and
166 incubated with FITC-PSA solution (5 µg PSA-FITC/1 mL H_20) for 15 min in darkness. After
167 staining samples were washed in PBS and mounted with Vectashield mounting medium with PI
168 (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with the above
169 mentioned microscope and at least 200 spermatozoa per sample were scored.
170 The presence of a green acrosomal fluorescence was considered indicative for an intact acrosome,
171 while a partial or total absence of fluorescence is indicative of acrosome disruption or acrosome
172 reaction.
173

174 ***2.4 Sperm sorting***

175

176 Sperm samples were diluted with mTALP to 100 x 10^6 spermatozoa/mL. Aliquots of 1 mL of
177 diluted semen were then transferred into Falcon tubes and stained with 10 µl of 5 mg/mL Hoechst
178 33342 stock solution for 1 h at 35° C in the dark. Just prior to sorting, 1 µL of food dye (FD&C#40,
179 Warner Jenkinson, St. Louis, MO, USA) stock solution (25 mg/mL) was added to each sample. The
180 samples were then filtered through a 60 µm nylon mesh filter to remove debris or clumped
181 spermatozoa.
182 A MoFlo SX® flow cytometer/sperm sorter (DakoCytomation Inc., Fort Collins, CO, USA)
183 equipped with an argon laser (wavelength 351 at 150 mW) was used. Dulbecco's phosphate

184 buffered saline (DPBS) served as sheath fluid and the instrument sheath pressure was 40 psi. Sorted
185 spermatozoa were deflected into 20 mL polypropylene tubes containing 500 µL of 2 % Tes-Tris-
186 egg yolk buffer [9]. After collection of a total of 20 x 10^6 sperm/sex (around 7 x 10^6 sperm/ tube),
187 the two populations were pooled (since sex predetermination was not an objective of the
188 experiment). The samples were divided in four aliquots in experiment 2 and after a centrifugation at
189 800 x g for 20 min the pellets were resuspended in the different extenders tested (TFC, TGC,
190 mTALP, III FRAC). In experiments 3 and 4, after centrifugation, the pellets were resuspended in
191 EYTGC1 .
192
193 ***2.5 Freezing and thawing***
194
195 After centrifugation at 300 g for 10 min (CTR group) and after the centrifugation performed in
196 order to concentrate sorted sperm (800 x g for 20 min) (SORT group), the supernatant was removed
197 and the sperm pellets (about of 40 x 10^6 spermatozoa/pellet) were resuspended in two steps in
198 freezing extender into a 1.5 mL conical centrifuge tube. Firstly, spermatozoa were diluted slowly at
199 room temperature (20-25°C) by dropwise addition of 250 µL of EYTGC1. After that, semen was
200 cooled to 4°C for 60 min in a controlled temperature refrigerator (about -0.3°C/min). Then the
201 second dilution with 250 µL of pre-cooled EYTGC2 was made at 4°C, reaching a final
202 concentration of 80 x 10^6 spermatozoa/mL. After an additional equilibration period of 15 min at
203 4°C, semen samples were loaded in plastic straws (0.5 mL) and the straws were heat sealed. Sperm
204 were frozen on liquid nitrogen vapors into a polystyrene box (36x33x30 cm) keeping straws at
205 distance of 7 cm from liquid nitrogen (4 cm) for 10 min, before plunging straws directly into liquid
206 nitrogen. Finally, the straws were transferred into a Dewar flask at -196°C.
207 After at least 4 d of storage, one straw of each sample was thawed by immersion in a 37 °C water
208 bath for 30 s and the content emptied into an 1.5 mL conical centrifuge tube for evaluations.

2.6 Statistical analysis

Values are reported as mean ± standard error of the mean (SEM). Data were checked for normality using a Shapiro-Wilk test and analyzed using a one-way ANOVA or a Kruskall-Wallis H test depending on distribution (IBM SPSS Statistics 21, IBM Corporation, Milan, Italy). When significant differences were found Tukey post-hoc test was performed. The level of significance was set at $P<0.05$.

3. Results

3.1 Experiment 1

Mean data from the evaluation of 4 ejaculates from 4 mixed breed dogs after collection were: volume 0.7 ± 0.3 mL, concentration 306 ± 108 × 10^6 spermatozoa/mL, total motility 86.7 ± 3.3%, movement 5.0 ± 0.0, and rate of morphologically normal spermatozoa greater than 90%.

The results of experiment 1 are reported in Figure 1.

After 24h storage at room temperature total sperm motility was higher in mTALP compared to the TFC and III FRAC (Fig.1A) ($P<0.05$). Sperm movement was significantly higher in mTALP compared to III FRAC at 6h (Fig.1B) ($P<0.05$). No differences were observed in the percentage of sperm with intact plasma membrane and sperm with intact acrosome in the different media (Fig.1C and D).

3.2 Experiment 2

234 Mean data from the evaluation of 4 ejaculates from 4 mixed breed dogs after collection were:
235 volume 1.6 ± 0.6 mL, concentration 154.3 ± 2.2 × 10 ^{6}spermatozoa/mL, total motility 91.7 ± 1.7%,
236 movement 5.0 ± 0.0, and rate of morphologically normal spermatozoa greater than 90%.

237 Four h after sorting, total motility and movement were significantly higher in semen resuspended in
238 mTALP (Fig.2 A e B) ($P<0.05$). The percentage of sperm with intact membranes in sorted group
239 resuspended in mTALP compared to TFC and TGC resulted higher at 0 and 4h (Fig. 2 C) ($P<0.05$).
240 The difference between mTALP and TGC was significant also after 24h ($P<0.05$). Regarding semen
241 resuspended in III FRAC, sorted spermatozoa presented a percentage of viable cells similar to
242 mTALP at 0h; this percentage, however, rapidly declined resulting the lowest already at 4h
243 ($P<0.05$). The resuspension of sorted semen in mTALP resulted in a percentage of acrosome intact
244 sperm higher than the other extenders at 2 and 4h (Fig. 2 D) ($P<0.05$).

245

246 ***3.3 Experiment 3***

247

248 Mean data from the evaluation of 8 ejaculates from 8 purebred dogs after collection were: volume
249 2.3 ± 0.5 mL, concentration 159 ± 22 × 10 ^{6}spermatozoa/mL, total motility 92.5 ± 0.9%, movement
250 5.0 ± 0.0, and rate of morphologically normal spermatozoa greater than 90%.
251 The sorting procedure induced a significant reduction of total motility and movement compared to
252 control semen evaluated immediately after collection (CTR) ($P<0.05$). Freezing caused a significant
253 decrease ($P<0.05$) of total motile sperm and movement in both CTR-FR and SORT-FR samples
254 (Tab. 1).

255 The percentage of sperm with intact membranes and that of acrosome intact cells did not differ
256 between CTR and SORT but freezing induced a significant reduction of these parameters in both
257 CTR-FR and SORT-FR samples ($P<0.05$).

3.4 Experiment 4

Mean data from evaluation of 8 ejaculates from 2 mixed breed dogs after collection were: volume 3.6 ± 0.2 mL, concentration 282 ± 21 × 10^6spermatozoa/mL, total motility 94.4 ± 0.6%, movement 5.0 ± 0.0, and morphologically normal spermatozoa greater than 90%.

A decrease of total motile sperm and sperm with intact membranes after sorting compared to control semen evaluated immediately after collection was observed ($P<0.05$). Freezing induced a reduction of total motile sperm, sperm with intact membranes and acrosome integrity in both unsorted and sorted sperm ($P<0.05$) (Tab. 1).

4. Discussion

This study demonstrates that the use of mTALP can be considered a suitable medium for the storage of dog semen at room temperature (20-25°C) before sorting. Even if all the parameters evaluated underwent a decrease after 24h storage, usually spermatozoa are sexed within the first hours after collection when sperm in mTALP maintained parameters similar to those of semen analysed immediately after collection. For this reason mTALP was used in the subsequent experiments for diluting dog semen pre-sorting.

Concerning the sex sorting procedure, even if it caused a decrease of sperm motility parameters, it did not seem to severely affect dog sperm membranes as viability and acrosome integrity were similar to that of control semen except for viability in experiment 4 that anyway resulted higher than 70%. Rodenas et al. [5] reported that, while the quality of spermatozoa immediately after sorting before centrifugation step, performed in order to concentrate the highly diluted sorted cells,

282 was similar to that of fresh semen samples, the high speed centrifugation of sexed sperm at 3000 x g
283 for 4 min resulted in a significant reduction of total motility, viability and sperm with intact
284 acrosome. In our study we performed a low speed centrifugation over a long period (800 x g for 20
285 min), basing on the protocol routinely used in our lab for horse and boar semen [10,11]. We did not
286 check viability and acrosome integrity before centrifugation step but the maintenance of a good
287 viability and acrosome integrity suggests that the centrifugation protocol after sorting used in this
288 study could be adequate for concentrating sexed spermatozoa in this species.
289 The bitch that has to be inseminated with sexed sperm can be far from the sorting facilities or
290 require multiple inseminations; for this reason it is very important to define a proper method to store
291 sexed sperm. As no data about storage of canine sexed sperm are available in literature, we decided
292 to evaluate the possibility of either liquid store sexed sperm at room temperature (20-25°C) or
293 freeze it.
294 In the experiment 2, among the four extender tested for liquid storage of dog sexed semen, mTALP
295 resulted in the maintenance of better sperm quality; this positive effect was particularly evident for
296 total motility. Anyway the low quality of sorted semen after 24h suggests the necessity of
297 optimizing an alternative storage method for long periods.
298 In experiment 3 the ability of sperm from 8 purebred dogs to withstand sexing procedure followed
299 by freezing was tested and freezing caused a significant reduction of all the parameters evaluated in
300 both unsorted and sorted sperm. SORT-FR semen was characterized by lower motility
301 characteristics but similar viability and acrosome integrity compared to CTR-FR. These results
302 suggest that the semen used in this experiment was mainly injured by freezing even if semen
303 susceptibility to freezing seems to be, in a certain manner increased, by sorting.

304 To exclude the hypothesis that the unsatisfactory quality of both unsorted and sorted frozen sperm
305 could be due to the fact that dogs of a single breed were enrolled in the experiment, the same
306 protocol was used for ejaculates from mixed breed dogs (experiment 4). The results evidenced that
307 sorting and subsequent freezing caused severe damage to spermatozoa. However the freezing

injuries to sperm observed could be due, at least in part, to the cooling and the second dilution with EYTGC2 made at 4°C performed in small volumes (500µl) that can increase the sperm susceptibility to cooling/freezing stress as performing the same procedure with higher volumes of unsorted semen (3 mL) significantly improved sperm quality (data not shown). This information must be considered in the future in order to optimize the procedure of freezing dog sorted semen; sex-sorted sperm in fact require modification of the basic protocol used for cryopreserving unsorted sperm due to the small volume of sex-sorted sperm at a low concentration compared to unsorted sperm [12].

4.1 Conclusions

In conclusion mTALP is an appropriate medium for canine sperm storage pre- and soon after sorting (hours), but a long storage period at room temperature is not tolerated by sex-sorted spermatozoa. Cryopreservation resulted in a great loss of sexed spermatozoa and further studies are needed to optimize the freezing protocol.

Acknowledgement

The authors wish to thank Società Italiana Produzione Sementi and Fondazione Sfameni for financially supporting Elisa Giaretta. Work funded by a Bologna University grant (RFO, Ricerca Fondamentale Orientata).

References

[1] Garner DL. Flow cytometric sexing of mammalian sperm. Theriogenology 2006;65:943-57.

[2] Garner D., Seidel GE Jr. Past, present and future perspectives on sexing sperm. Can J Anim Sci 2003;83:375–84.

[3] Meyers MA, Burns G, Arn D, Schenk JL. Birth of canine offspring following insemination of a bitch with flow-sorted spermatozoa. Rep Fertil Dev 2008;20: 213.

[4] Oi M, Yamada K, Hayakawa H, Suzuki H. Sexing of dog sperm by fluorescence in situ hybridization. J Reprod Dev 2013;59:92–6.

[5] Rodenas C, Lucas X, Tarantini T, Del Olmo D, Roca J, Vazquez JM, Martinez EA, Parrilla I. The effects of hoechst 33342 staining and the male sample donor on the sorting efficiency of canine spermatozoa. Reprod Domest Anim 2014;49:115-21.

[6] Johnston SD, Ward D, Lemon J, Gunn I, MacCallum CA, Keeley T, Blyde D. Studies of male reproduction in captive African wild dogs (Lycaon pictus). Anim Reprod Sci 2007;100:338–55.

[7] Payan-Carreira R, Miranda S, Niżański W. Artificial Insemination in dogs. In: Manafi M editor. Artificial insemination in farm animals, InTech; 2011, p. 51-78.

[8] Howard JG, Bush M, Wildt DE. Semen collection, analysis and cryopreservation in nondomestic mammals. In: Morrow DA editor. Current therapy in theriogenology 2nd edn., Philadelphia: Saunders Company, 1986; p. 1047-53.

[9] Johnson LA. Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. Reprod Domest Anim 1991;26:309–314.

[10] Spinaci M, Chlapanidas T, Bucci D, Vallorani C, Perteghella S, Lucconi G, Communod R, Vigo D, Galeati G, Faustini M, Torre ML. Encapsulation of sex sorted boar semen: sperm membrane status and oocyte penetration parameters. Theriogenology 2013;79:575-81.

[11] Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Peña FJ, Mari G, Galeati G. Effect of sex sorting on stallion spermatozoa: heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci 2013;141:68-74.

[12] Bathgate R. Functional integrity of sex-sorted, frozen-thawed boar sperm and its potential for artificial insemination. Theriogenology 2008;70:1234–41.

359 **Table 1.** Seminal quality evaluations of sperm of purebred (n=8) and mixed breed dogs (n=8) after
360 collection (CTR), after flow cytometric sex-sorting (SORT), and after thawing of non-sorted and
361 sorted cryopreserved samples (CTR-FR, SORT-FR).

	Total motility (%)	**Movement (0-5)**	**Viability (%)**	**Acrosome intact sperm (%)**
		Purebred		
CTR	92.5 ± 0.9 [a]	5.0 ± 0.0 [a]	83.9 ± 1.8 [a]	94.5 ± 1.2 [a]
SORT	52.9 ± 6.0 [b]	2.1 ± 0.6 [b]	85.2 ± 6.8 [a]	90.6 ± 1.3 [a]
CTR-FR	25.7 ± 4.1 [c]	3.1 ± 0.4 [b]	31.3 ± 4.0 [b]	76.5 ± 2.8 [b]
SORT-FR	2.4 ± 1.2 [d]	0.6 ± 0.4 [c]	22.8 ± 4.5 [b]	78.0 ± 2.8 [b]
		Mixed breed		
CTR	94.4 ± 0.6 [a]	5.0 ± 0.0 [a]	91.4 ± 1.1 [a]	97.9 ± 0.5 [a]
SORT	54.4 ± 9.1 [b]	4.6 ± 0.2 [a]	71.3 ± 5.2 [b]	89.9 ± 3.9 [a]
CTR-FR	37.5 ± 4.1 [b]	5.0 ± 0.0 [a]	33.2 ± 2.8 [c]	33.9 ± 4.0 [b]
SORT-FR	9.4 ± 4.6 [c]	2.7 ± 0.5 [b]	12.2 ± 2.5 [d]	43.7 ± 3.4 [b]

362 [ab] Values with different superscripts within a column differ significantly ($P<0.05$)

363

Figure legends

Figure 1. (A) Total sperm motility, (B) movement, (C) membrane integrity as an estimate of sperm viability, and (D) acrosome integrity evaluated in dog semen stored at room temperature (20-25°C) in different extenders. (n=4)

TFC - Tris-fructose-citrate; TGC - Tris-glucose-citrate; mTALP - modified Tyrode's solution, III FRAC - third fraction of the ejaculate.

Different letters on the same type bars indicate a significant ($P<0.05$) difference. Values are expressed as mean ±SEM.

Figure 2. (A) total sperm motility, (B) movement, (C) membrane integrity as an estimate of sperm viability and (D) acrosome integrity evaluated in sorted dog sperm resuspended and stored with different extender at room temperature (20-25°C). (n=4)

TFC - Tris-fructose-citrate; TGC - Tris-glucose-citrate; mTALP - modified Tyrode's solution, III FRAC - third fraction of the ejaculated.

Different letters on the same type bars indicate a significant ($P<0.05$) difference. Values are expressed as mean ±SEM.