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Sex-sorted canine sperm cryopreservation: Limits and procedural considerations

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ABSTRACT

The aim of this study was to define a protocol to store dog sperm before and after sorting 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 before and after sorting. Four extenders were tested: (1) Tris-fructose-citrate (TFC), (2) Tris-glucose-citrate (TGC), (3) modified Tyrode's albumin lactate pyruvate medium (mTALP), and (4) third fraction of the ejaculate (after centrifugation at 5000× *g* for 10 minutes; III FRAC). Experiment 3 and 4 were performed to evaluate the ability of dog semen to withstand sex sorting and freezing/thawing. Modified Tyrode's albumin lactate pyruvate medium was the best extender for canine sperm storage at room temperature (20 °C–25 °C) before (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 after 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 24-hour 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 before 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.

1. Introduction

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

Although sexing technique has already reached a commercial level in the bovine species, sorting efficacy has been reported suggesting future applications in a variety of mammals (pig, horse, sheep, goat, cat, and 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, and farm dogs), male or female is 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].

To 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 limitations for the use of sperm sexing in the dog and in other species is the long sorting time necessary to obtain the number of sexed spermatozoa

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necessary for AI (approximately 150–200 million motile spermatozoa) [7]. Rodenas et al. [5] reported that it is possible to collect 14 to 16 $\times$ 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 reported by Meyers et al. [3] producing offspring of the desired sex in one bitch by intrauterine low-dose insemination using sexed spermatozoa.

Furthermore, the number of spermatozoa requested in dog for a intrauterine low-dose insemination (at least 50 million) [7] is very high considering the sorting time and, therefore, sperm cells have to be stored properly at room temperature waiting to be sexed.

Another problem to solve is the storage of sexed semen, as the bitch can be far from the sorting facilities or more than one insemination could be required. Until now, no data about storage of canine sexed semen are available in the literature.

Cryopreservation permits long-term sperm storage, but it has to be taken in mind that sexed spermatozoa are cells that are already stressed by the sorting procedure and usually present a greater susceptibility to cryopreservation procedures compared with unsexed semen.

The aim of this study was to (1) define an appropriate extender to liquid store dog sperm before and after sorting and (2) evaluate if sexed canine spermatozoa are able to withstand freezing/thawing procedure. For this purpose, motility, viability, and acrosome integrity were assessed in unsorted and sorted sperm either maintained at room temperature or cryopreserved.

2. Materials and methods

2.1. Experimental design

This study was divided into four experiments.

As the sorting process is slow, the aim of the experiment 1 was to evaluate which of four extenders is the more appropriate for storing at room temperature the dog spermatozoa waiting to be sexed. Total motile spermatozoa, movement, viability, and acrosome integrity were evaluated at 0, 2, 4, 6, 8, and 24 hours.

In the experiment 2, the best extender from experiment 1 (modified Tyrode's albumin lactate pyruvate medium [mTALP], see the following) was used for diluting presorted spermatozoa, whereas the four extenders evaluated in the experiment 1 were tested for sperm storage at room temperature (20 °C–25 °C) after sorting. The above mentioned parameters were evaluated at 0, 2, 4, and 24 hours 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 and 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 benzylpenicillin, 0.1% wt/vol streptomycin sulfate 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 benzylpenicillin, 0.1% wt/vol streptomycin sulfate in distilled water), 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), and third fraction (III FRAC) of the ejaculated after centrifugation at 5000$\times$ g for 10 minutes.

Freezing extender for the first step was TGC supplemented with 20% (v:v) egg yolk, 3% (v:v) glycerol (EYTGC1), whereas 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 aged between 1 and 7 years 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 micropipette, and sperm concentration was determined with a Bürker chamber, after dilution of the sperm suspension 1:40 with buffered formol saline 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 ($\times$400; Axiolab; Zeiss, Italy) equipped with a warming plate (37 °C; Thermo Plate; Tokai Hit, Japan). Percentages of morphologically normal spermatozoa were determined at the same microscope ($\times$1000) after dilution of semen 1:1 with buffered formol saline, and at least 200 spermatozoa per sample were examined.

To evaluate plasma membrane integrity, 25 μL of semen was incubated with 2 μL of a 300-μM propidium iodide (PI) stock solution and 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 minutes 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 scored with the Nikon Eclipse E600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, the Netherlands). Spermatozoa stained with

SYBR-14 and not stained with PI were considered as viable. Spermatozoa SYBR positive and PI positive and those SYBR negative/PI positive were considered as cells with non-intact membranes or dead as an estimate of sperm viability.

Acrosome integrity was measured with a fluorescein isothiocyanate (FITC)-conjugated lectin from *Pisum sativum* (FITC-PSA) which labeled acrosomal matrix glycoproteins. Briefly, spermatozoa were washed twice in PBS, resuspended with ethanol 95%, and fixed at 4 °C for 30 minutes. Samples were dried in heated slides and incubated with FITC-PSA solution (5-μg PSA-FITC/1-mL H_20) for 15 minutes in darkness. After staining, samples were washed in PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with the above mentioned microscope, and at least 200 spermatozoa per sample were scored.

The presence of a green acrosomal fluorescence was considered indicative for an intact acrosome, whereas a partial or total absence of fluorescence is indicative of acrosome disruption or acrosome reaction.

2.4. Sperm sorting

Sperm samples were diluted with mTALP to 100×10^6 spermatozoa/mL. Aliquots of 1 mL of diluted semen were then transferred into Falcon tubes and stained with 10 μL of 5 mg/mL Hoechst 33342 stock solution for 1 hour 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. The samples were then filtered through a 60-μm nylon mesh filter to remove debris or clumped spermatozoa.

A MoFlo SX flow cytometer/sperm sorter (DakoCytomation Inc., Fort Collins, CO, USA) equipped with an argon laser (wavelength 351 at 150 mW) was used. Dulbecco's PBS served as sheath fluid, and the instrument sheath pressure was 40 psi. Sorted spermatozoa were deflected into 20 mL polypropylene tubes containing 500 μL of 2% TES-Tris egg yolk buffer [9]. After collection of a total of 20×10^6 sperm/sex (around 7×10^6 sperm/tube), the two populations were pooled (because sex predetermination was not an objective of the experiment). The samples were divided in four aliquots in experiment 2, and after a centrifugation at $800 \times g$ for 20 minutes, the pellets were resuspended in the different extenders tested (TFC, TGC, mTALP, and III FRAC). In experiments 3 and 4, after centrifugation, the pellets were resuspended in EYTGC1.

2.5. Freezing and thawing

After centrifugation at $300\times$ *g* for 10 minutes (CTR group) and after the centrifugation performed to concentrate sorted sperm ($800\times$ *g* for 20 minutes; SORT group), the supernatant was removed and the sperm pellets (about of 40×10^6 spermatozoa/pellet) were resuspended in two steps in freezing extender into a 1.5-mL conical centrifuge tube. First, the spermatozoa were diluted slowly at room temperature (20 °C–25 °C) by dropwise addition of 250 μL of EYTGC1. After that, semen was cooled to 4 °C for 60 minutes in a controlled temperature refrigerator (about −0.3 °C/min). Then the second dilution with 250 μL of precooled EYTGC2 was made at 4 °C, reaching a final concentration of 80×10^6 spermatozoa/mL. After an additional equilibration period of 15 minutes at 4 °C, the semen samples were loaded in plastic straws (0.5 mL) and the straws were heat sealed. Sperm were frozen on liquid nitrogen vapors into a polystyrene box (36 × 33 × 30 cm) keeping straws at a distance of 7 cm from liquid nitrogen (4 cm) for 10 minutes, before plunging straws directly into liquid nitrogen. Finally, the straws were transferred into a Dewar flask at −196 °C.

After at least 4 days of storage, one straw of each sample was thawed by immersion in a 37 °C water bath for 30 seconds and the content emptied into a 1.5-mL conical centrifuge tube for evaluations.

2.6. Statistical analysis

Values are reported as mean ± standard error of the mean. Data were checked for normality using a Shapiro–Wilk test and analyzed using a one-way ANOVA or a Kruskal-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 four ejaculates from four mixed-breed dogs after collection were volume, 0.7 ± 0.3 mL; concentration, $306 \pm 108 \times 10^6$ 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 24-hour storage at room temperature, total sperm motility was higher in mTALP compared with that in the TFC and III FRAC (Fig. 1A; $P < 0.05$). Sperm movement was significantly higher in mTALP compared with that in III FRAC at 6 hours (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, D).

3.2. Experiment 2

Mean data from the evaluation of four ejaculates from four mixed-breed dogs after collection were volume, 1.6 ± 0.6 mL; concentration, $154.3 \pm 2.2 \times 10^6$ spermatozoa/mL; total motility, 91.7 ± 1.7%; movement, 5.0 ± 0.0; and rate of morphologically normal spermatozoa greater than 90%.

Four hours after sorting, total motility and movement were significantly higher in semen resuspended in mTALP (Fig. 2A, B; $P < 0.05$). The percentage of sperm with intact membranes in sorted group resuspended in mTALP compared with TFC and TGC resulted higher at 0 and 4 hours (Fig. 2C; $P < 0.05$). The difference between mTALP and TGC was significant also after 24 hours ($P < 0.05$). Regarding semen resuspended in III FRAC, sorted

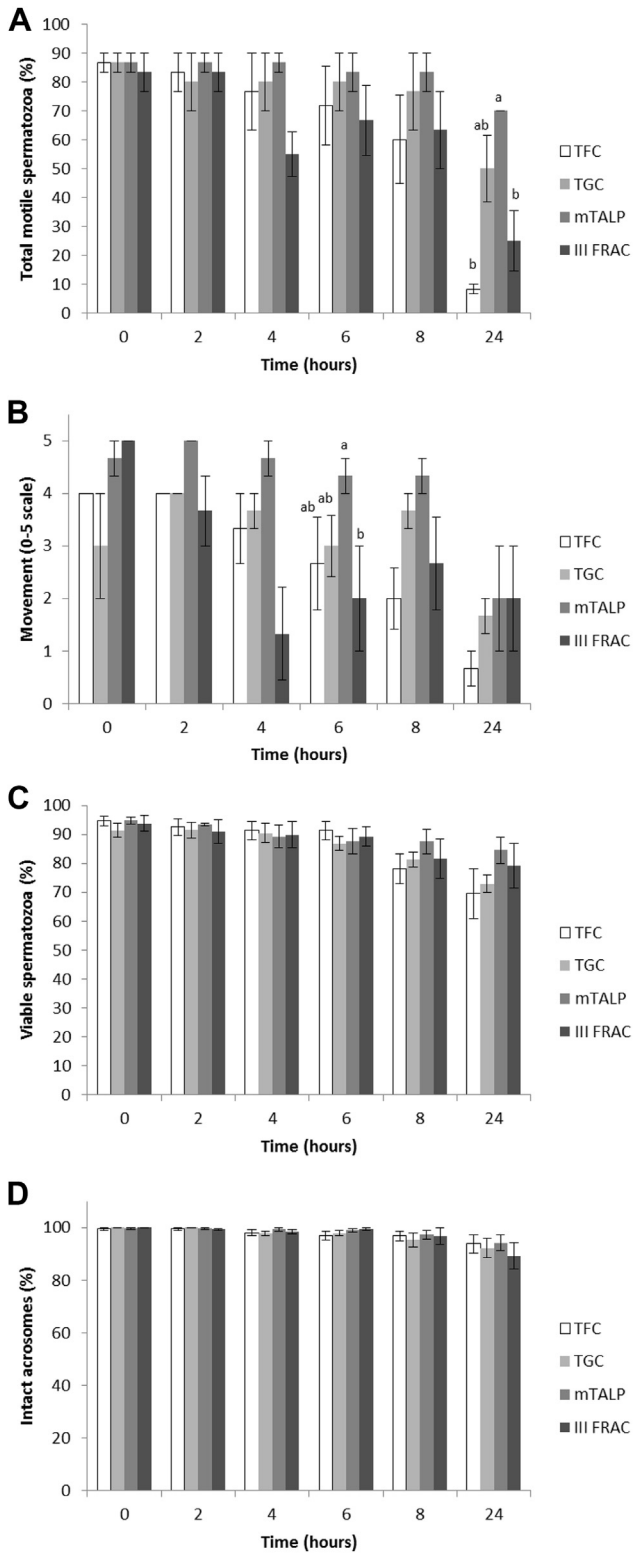

Fig. 1. (A) Total sperm motility, (B) movement, (C) membrane integrity as an estimate of sperm viability, and (D) acrosome integrity evaluated in dog semen stored at room temperature (20 °C–25 °C) in different extenders (n = 4). Different letters on the same type bars indicate a significant ($P < 0.05$) difference. Values are expressed as mean ± standard error of the mean. III FRAC, third fraction of the ejaculate; mTALP, modified Tyrode's solution; TFC, Tris-fructose-citrate; TGC, Tris-glucose-citrate.

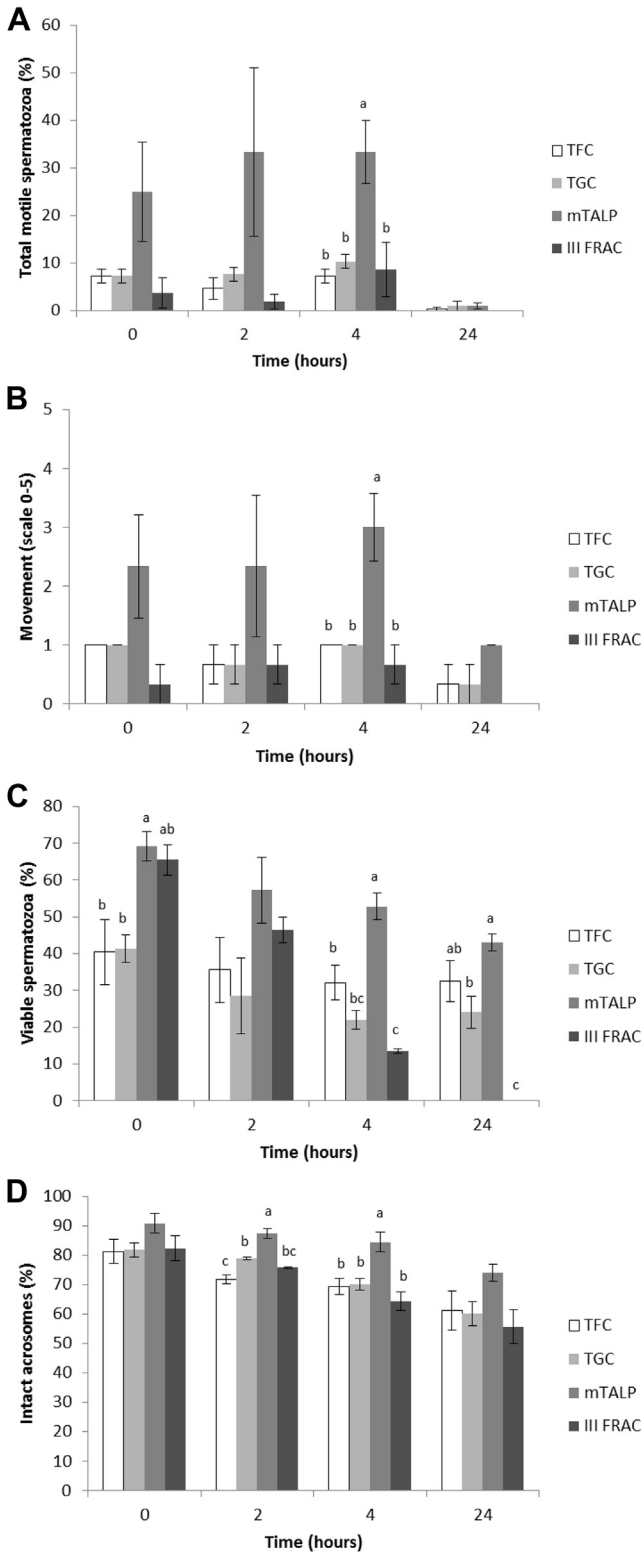

Fig. 2. (A) Total sperm motility, (B) movement, (C) membrane integrity as an estimate of sperm viability, and (D) acrosome integrity evaluated in sorted dog sperm resuspended and stored with different extender at room temperature (20 °C–25 °C) (n = 4). Different letters on the same type bars indicate a significant ($P < 0.05$) difference. Values are expressed as mean ± standard error of the mean. III FRAC, third fraction of the ejaculate; mTALP, modified Tyrode's solution; TFC, Tris-fructose-citrate; TGC, Tris-glucose-citrate.

spermatozoa presented a percentage of viable cells similar to mTALP at 0 hours; this percentage, however, rapidly declined resulting the lowest already at 4 hours ($P < 0.05$). The resuspension of sorted semen in mTALP resulted in a percentage of acrosome-intact sperm higher than that of the other extenders at 2 and 4 hours (Fig. 2D; $P < 0.05$).

3.3. Experiment 3

Mean data from the evaluation of eight ejaculates from eight purebred dogs after collection were volume, 2.3 ± 0.5 mL; concentration, $159 \pm 22 \times 10^6$ spermatozoa/mL; total motility, $92.5 \pm 0.9\%$; movement, 5.0 ± 0.0; and rate of morphologically normal spermatozoa greater than 90%.

The sorting procedure induced a significant reduction of total motility and movement when compared with those of control semen evaluated immediately after collection (CTR; $P < 0.05$). Freezing caused a significant decrease ($P < 0.05$) of total motile sperm and movement in both CTR-FR and SORT-FR samples (Table 1).

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

3.4. Experiment 4

Mean data from evaluation of eight ejaculates from two mixed-breed dogs after collection were volume, 3.6 ± 0.2 mL; concentration, $282 \pm 21 \times 10^6$ spermatozoa/mL; total motility, $94.4 \pm 0.6\%$; 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 with 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 ($P < 0.05$; Table 1).

4. Discussion

This study reports that the use of mTALP can be considered a suitable medium for the storage of dog semen at room temperature (20 °C–25 °C) before sorting. Even if all the parameters evaluated underwent a decrease after 24-hour storage, usually spermatozoa are sexed within the first hours after collection when sperm in mTALP maintained parameters similar to those of semen analyzed immediately after collection. For this reason, mTALP was used in the subsequent experiments for diluting dog semen before 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 those of control semen except for viability in experiment 4 that anyway resulted higher than 70%. Rodenas et al. [5] reported that, although the quality of spermatozoa immediately after sorting before centrifugation step, performed to concentrate the highly diluted sorted cells, was similar to that of fresh semen samples, the high-speed centrifugation of sexed sperm at $3000\times$ *g* for 4 minutes resulted in a significant reduction of total motility, viability, and sperm with intact acrosome. In our study, we performed a low-speed centrifugation over a long period ($800\times$ *g* for 20 minutes) on the basis of the protocol routinely used in our laboratory for horse and boar semen [10,11]. We did not check viability and acrosome integrity before centrifugation step, but the maintenance of a good viability and acrosome integrity suggests that the centrifugation protocol after sorting used in this study could be adequate for concentrating sexed spermatozoa in this species.

The bitch that has to be inseminated with sexed sperm can be far from the sorting facilities or requires multiple inseminations; for this reason, it is very important to define a proper method to store sexed sperm. As no data about storage of canine sexed sperm are available in the literature, we decided to evaluate the possibility of either liquid store the sexed sperm at room temperature (20 °C–25 °C) or freeze it.

In the experiment 2, among the four extender tested for liquid storage of dog sexed semen, mTALP resulted in the maintenance of better sperm quality; this positive effect was particularly evident for total motility. Anyway, the low quality of sorted semen after 24 hours suggests the necessity of optimizing an alternative storage method for long periods.

In experiment 3, the ability of sperm from eight purebred dogs to withstand sexing procedure followed by freezing was tested and freezing caused a significant

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

Group	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

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

reduction of all the parameters evaluated in both unsorted and sorted sperm. SORT-FR semen was characterized by lower motility characteristics but similar viability and acrosome integrity compared with CTR-FR. These results suggest that the semen used in this experiment was mainly injured by freezing even if semen susceptibility to freezing seems to be, in a certain manner increased, by sorting.

To exclude the hypothesis that the unsatisfactory quality of both unsorted and sorted frozen sperm could be due to the fact that dogs of a single breed were enrolled in the experiment, the same protocol was used for ejaculates from mixed-breed dogs (experiment 4). The results evidenced that 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 (500 μL) 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 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 because of the small volume of sex-sorted sperm at a low concentration compared with unsorted sperm [12].

4.1. Conclusions

In conclusion, mTALP is an appropriate medium for canine sperm storage before 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.

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