

Effect of egg yolk-free extender on canine sperm quality after cooling

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Abstract

Effectiveness of a commercial extender was compared to a conventional extender in cooling dog semen. Ejaculates ($n = 21$) from 7 dogs were evaluated (volume and pH, sperm motility, vigor, concentration, morphology, chromatin condensation, and membrane function). Samples were divided into 2 aliquots, centrifuged and the resulting pellet was diluted in the commercial extender without egg yolk or in the conventional extender (containing tris, citric acid, fructose, and egg yolk). Sperm progressive motility (PM), vigor, morphology, chromatin condensation, viability, and acrosomal integrity were assessed at 24, 48, and 96 hours after refrigeration at 4°C. There were no differences in the percentage of morphologically normal sperm and sperm with condensed chromatin. However, percentage of live sperm with intact acrosomes was greater ($p < 0.01$) in samples diluted in the commercial extender. Furthermore, period of storage had a declining effect ($p < 0.0001$) only on PM for samples diluted in both extenders; PM was greater ($p < 0.01$) before cooling than at 48 and 96 hours of cooled storage and was greater ($p < 0.05$) at 24 hours storage than at 96 hours storage. In conclusion, the commercial extender was effective and better preserved acrosome integrity than the conventional extender.

Keywords: Cryopreservation, extender, semen, dog

Introduction

The importance of artificial insemination in dogs has increased in the past decade.^{1,2} More specifically, the use of chilled semen for artificial insemination has gained interest, since processing and shipping of chilled samples are easier and cheaper than preparing frozen straws.³⁻⁵ Extenders commonly used for semen cooling are based on tris, citric acid and fructose or glucose, with the addition of egg yolk as a cryoprotective agent. These conventional extenders allow sperm quality to be maintained for up to 10 days.^{2,6} Despite the frequent use of these extenders, the addition of egg yolk involves risk of contamination by bacteria. Moreover, being a complex biological compound, egg yolk has a variable composition that impairs standardization of extender composition. Furthermore, it contains substances that may affect sperm motility and acrosome morphology, and could interfere with assays (e.g. motility evaluation by computer-assisted sperm analysis [CASA] systems⁷⁻⁹). Therefore, the addition of low-density lipoprotein (LDL) instead of egg yolk to semen extenders has been studied in chilled dog semen, with good results.^{10,11} However, obtaining LDL is a

difficult process that could not be adapted to an industrial scale, impairing its routine use.⁸ As an alternative, extenders with cryoprotective agents free of animal protein, such as soybean lecithin, have been evaluated. Some authors have reported similar or better results when cooling dog semen with extenders containing soybean lecithin compared to conventional extenders,¹²⁻¹⁴ whereas others have observed the contrary.¹⁵

The membrane phase transition that occurs when lowering temperatures leads to irreversible structural changes to sperm plasma membrane. Cholesterol has an essential function in maintaining membrane stability and increased cholesterol/phospholipid ratio decreased the temperature at which the transition phase occurs. Therefore, a fluid membrane can be maintained at lower temperatures.^{9,16} Cyclodextrin-cholesterol (CLC) complex addition to semen extenders enables transferring cholesterol into plasma membrane, promoting membrane stability. In dogs, addition of low concentrations of CLC complex to the commercial extender before cryopreservation improved semen quality after thawing.^{17,18} Similarly,

the addition of a CLC complex to an extender that contained egg yolk, improved postthaw motility, viability and acrosomal integrity in frozen-thawed dog semen samples.¹⁹ To authors' knowledge, there are no reports regarding use of CLC complex for semen cooling in dogs. Therefore, the aim of the present study was to assess the effectiveness of a commercial extender free of egg yolk that has a CLC complex for cooling dog semen.

Materials and methods

Population under study

This study was approved by the Ethics Committee of the School of Veterinary Sciences of Buenos Aires University. Clinically and reproductively healthy male dogs (n = 7) of various breeds (4 Beagle, 1 Terranova, 1 French Bull dog, and 1 Dachshund) with a mean age of 4 ± 1.1 years, were included in the study. Dogs were housed in pens in the Faculty of Veterinary Sciences of the University of Buenos Aires (Argentina) or reared as pets at homes. They were fed commercial dog food and had water *ad libitum*. All dogs had proven fertility and a body condition score of 5 (scale of 1-9).

Semen collection

Ejaculates (n = 21) were obtained via digital manipulation method, and only the sperm-rich fraction of the ejaculate was collected.²⁰ Ejaculates were obtained from the same animal every 2 months.

Extenders

The conventional extender contained: tris (3.025 grams); citric acid (1.7 grams); fructose (1.25 grams); penicillin 0.06 grams; egg yolk 20% (% volume/volume); and 100 ml of QSF distilled water.²¹ Egg yolk was from fresh eggs (laid on the same day of semen collection) from the Agrotechnical School of the Veterinary Facility. The commercial extender (Bioptis SA, Mont-le-Soie, Belgium) consisted of a HBSS/HEPES based medium with glucose, lactose, casein and a cyclodextrin-cholesterol complex (1.5 mg/ml of 2-hydroxypropyl-beta-cyclodextrin cholesterol complex, HP β CD-C), and free of animal protein (no egg yolk or milk).

Experimental design and semen cooling

Only those samples that had $\geq 200 \times 10^6$ total sperm/ejaculate, motility $\geq 70\%$, vigor ≥ 4 , and normal morphology $\geq 70\%$ were cooled. After evaluation of semen quality (volume, pH, sperm motility, vigor, concentration, morphology, chromatin condensation, and membrane function) samples were split in 2 aliquots and centrifuged (700 g for 6 minutes). The supernatant was discarded, after obtaining a drop that was observed with an optical microscope.

The resulting pellet of each aliquot was diluted with conventional extender or commercial extender in a ratio of 1:3.²¹ Afterwards, tubes containing diluted sperm were placed in a glass filled with water at room temperature before cooling at 4°C.

At 24, 48, and 96 hours after refrigeration, an aliquot of each tube was assessed for sperm motility and kinetic parameters,

vigor, morphology, chromatin condensation, viability, and acrosomal integrity.

Semen quality assessment

Semen volume was assessed with graduated tubes and pH with pH tapes (DF[®]). Progressive motility and sperm kinetic parameters (VCL, VSL, ALH, and LIN) were assessed by CASA (Androvision[®], Minitüb, Germany). Vigor was evaluated by subjective observation using a phase contrast microscope (100 x) and a warm stage (37°C). Sperm concentration was calculated using a Neubauer chamber. Briefly, semen was diluted 1:100 in a buffered saline formol solution. After 5 minutes, samples were gently mixed and the 2 chambers of the hemocytometer were filled. After 5 minutes, a sperm count was performed under an optical microscope.⁷ Sperm morphology was determined using Diff-Quick stain, evaluating a minimum of 200 sperm per sample with light microscopy (1,000 x). Defects were classified as primary and secondary, and further by the location of the defect (acrosomal, head, midpiece, and tail defects).²² Membrane function was evaluated using the hypoosmotic swelling test²³; a minimum of 200 sperm per sample was assessed using phase contrast microscopy (400 x). Briefly, 5 μ l of semen were added to 50 μ l of a hypoosmotic solution (150 mOsmolar) and incubated at 37°C for 30 minutes before observation.²³ Chromatin condensation was assessed by toluidine blue (TB) stain.²⁴ Briefly, semen was extended, air-dried and fixed with ethanol 96° for 2 minutes. Staining was carried out using a working solution of TB diluted 1:3 with pH 4 buffer, prepared on the day of use, from a stock solution of 1% TB (preserved at 4°C protected from the light). After 15 minutes, smears were washed with distilled water. A minimum of 200 sperm were evaluated using light microscopy. Viability and acrosome integrity were evaluated by the fluorescent dye Propidium iodide (PI) (viability) combined with the peanut agglutinin lectin labeled with fluorescein isothiocyanate (FITC-PNA) stain (acrosome integrity). Briefly, samples were incubated with FITC-PNA for 10 minutes at 37°C (final concentration: 20 μ g/ml). Afterwards, PI was added, and samples were incubated for another 10 minutes at the same temperature (final concentration: 10 μ g/ml).²⁵ Thereafter, 6 μ l of stained sperm was placed on a glass slide and covered with a coverslip. Samples were evaluated using bright field and epifluorescence microscopy (Leica Microsystems, Wetzlar, Germany). At least 100 sperm were assessed. The following sperm populations were defined: live sperm with intact acrosomes (no fluorescence in either head or acrosome cap); live sperm with reacted acrosomes (no fluorescence in the head and intense green fluorescence in the acrosome cap); dead sperm with intact acrosomes (red fluorescent head with no fluorescence in the acrosome cap); and dead sperm with reacted acrosomes (red fluorescent head with spotted or half-moon green fluorescence in the acrosome).²⁵ The percentage of each population was considered.

Data analysis

The normality of the data and variance homogeneity were assessed by the Shapiro-Wilk test and Levene's test, respectively. Sperm quality parameters between extenders and along time were analyzed by a factorial ANOVA (Generalized Linear Model, Infostat, Argentina). In all cases, the dog was set as a random effect. Values are expressed as mean \pm SD. Differences were considered significant when p values were < 0.05 .

Results

Evaluation of raw semen samples

Mean sperm rich fraction volume was 1.4 ± 0.6 ml (range, 0.9 to 3 ml). Mean pH was 6.4; concentration was 472 ± 175 sperm/ml; and percentage of sperm with intact membrane was $90 \pm 8\%$. Mean sperm progressive motility, percentage of sperm with normal morphology and with condensed chromatin are provided (Table 1). Mean sperm kinetic parameters were: VSL 63.1 ± 4.4 $\mu\text{m}/\text{second}$, VCL 132.4 ± 25.8 $\mu\text{m}/\text{second}$, LIN $54 \pm 11\%$, and ALH 3.5 ± 0.4 μm . Sperm morphological abnormalities were bent tail, coiled tail, and detached head. All samples had acceptable sperm characters and samples were cooled and centrifuged; supernatant was free of sperm.

Evaluation of cooled stored samples

There were no significant differences in mean PM, percentage of sperm with normal morphology, and with condensed chromatin among samples (Table 1) neither in the sperm kinetic parameters among samples diluted in 2 extenders at any time point. Period of storage had a declining effect ($p < 0.0001$) only on PM for samples diluted in both extenders; PM was greater ($p < 0.01$) before cooling than at 48 or 96 hours of cooled storage and was greater ($p < 0.05$) at 24 hours storage than at 96 hours storage.

At 96 hours after cooling, the mean values for the conventional and the commercial extender, respectively, were as follows: VCL 123.9 ± 25.3 and 124.4 ± 34.5 $\mu\text{m}/\text{second}$; VSL 50.1 ± 9.6 and 56.4 ± 10.9 $\mu\text{m}/\text{second}$; LIN 42 ± 9 and $49 \pm 4\%$; and ALH 2.8 ± 0.6 and 2.9 ± 0.3 μm . Morphological abnormalities were like raw semen samples. Although there

were no differences in the percentage of total live sperm (both with intact and reacted acrosome) between extenders (Table 2), the percentage of live sperm with intact acrosomes was greater ($p < 0.01$) in samples diluted in the commercial extender.

Discussion

In the present study, the commercial extender was as effective as the conventional extender in preserving dog semen quality in chilled samples. The only parameter that was affected was PM that decreased to 51% in both extenders from 24 hours of storage to 96 hours. Similarly, PM of dog semen diluted in conventional extenders or in a commercial extender decreased to 56-67% after 4 days of cooled storage.^{15,26,27} After 48 hours of cooled storage, DNA (increase in DNA fragmentation) was observed.²⁸ Similar results were reported by TUNEL assay.²⁹ However, sperm chromatin integrity evaluated by SCSA method was not significantly affected after 8 or 10 days of cooled storage.^{13,30} In the present study, DNA fragmentation was not assessed, but chromatin condensation that is related to fertility was assessed.^{31,32} Chromatin condensation abnormalities have been related to decreased fertilization rates, and some authors have proposed that poorly condensed chromatin increases susceptibility to DNA fragmentation.³² In our research, chromatin condensation was not affected during the storage period in any of the extended samples, similar to that observed in horses and llamas.^{33,34} Differences between studies might be related to the techniques that have been employed that evaluated various processes (DNA integrity or chromatin compaction). To authors' knowledge, no other studies on chromatin condensation after cooling have been reported in the dog. Therefore, further research is needed to better understand DNA or chromatin damage after refrigeration.

Table 1. Mean sperm progressive motility (PM), mean percentage of sperm with normal morphology, and mean percentage of sperm with condensed chromatin for both extenders in raw samples and cooled samples at 24, 48, and 96 hours

Time (hours)	PM (%)		Sperm with normal morphology (%)		Sperm with condensed chromatin (%)	
	Conventional	Commercial	Conventional	Commercial	Conventional	Commercial
0	73 ± 8.1^a	73 ± 8.1^a	87 ± 5.7	87 ± 5.7	97 ± 1	97 ± 1.1
24	64 ± 4.9^{ac}	69 ± 6.6^{ac}	87 ± 5.9	88 ± 6.3	97 ± 1.4	97 ± 1.3
48	61 ± 7.1^{bc}	60 ± 7.2^{bc}	87 ± 6.5	89 ± 3.8	96 ± 1.3	97 ± 0.7
96	51 ± 7.1^b	51 ± 9.4^b	84 ± 7.1	83 ± 5.8	96 ± 1.1	96 ± 1.9

Means without a common superscript differed ($p < 0.05$)

Table 2. Mean percentage of total live sperm and with intact and reacted acrosome in samples at 24, 48, and 96 hours of cooled sample

Time (hours)	Live sperm with intact acrosome (%)		Live sperm with reacted acrosome (%)		Total live sperm (%)	
	Conventional	Commercial	Conventional	Commercial	Conventional	Commercial
24	$47 \pm 10^*$	63 ± 7	22 ± 14	18 ± 9	74 ± 19	79 ± 11
48	$46 \pm 14^*$	60 ± 7	21 ± 16	15 ± 8	73 ± 12	76 ± 10
96	$44 \pm 5^*$	59 ± 9	19 ± 5	12 ± 4	61 ± 9	71 ± 10

*Significant differences for live sperm with intact acrosome

As other parameters, sperm morphology is another important factor related to fertilization success.³⁵ In the present study, sperm morphology was not affected by cooled storage as reported.²⁶ On the contrary, various authors have reported a slight increase in the percentage of sperm with abnormal morphology at various locations (head, midpiece, acrosome, and tail defects) after cooled storage.^{2,36-38}

Other important factors for successful fertilization are sperm viability and acrosome integrity. Cooled storage promotes acrosomal reaction and thus, might affect fertility.³⁹⁻⁴¹ Some authors have reported an increase in the percentage of sperm with reacted acrosomes after cooled storage with commercial or conventional extenders, although this increase was observed after 10 days of storage in most reports.^{4,26,27,38,41} In the present study, no significant differences were observed in the percentage of live sperm with intact acrosomes from 24-96 hours of storage for both extenders. However, this percentage was greater in samples diluted in the commercial extender than in the conventional extender during all the storage period, whereas viability was not affected by the extender used. Therefore, the commercial extender would preserve acrosome integrity better than the conventional extender that could have a positive impact on fertility. This beneficial effect of the commercial extender could be related to the presence of a CLC complex that facilitates incorporation of cholesterol in sperm membranes, increasing stability.^{19,42} In fact, addition of 2 mg/ml of CLC complex to a conventional extender improved acrosome integrity after cryopreservation in dogs.¹⁹

In the present study, viability was not affected by the storage time, remaining ~ 60-70% after 96 hours of storage. Similarly, samples diluted with commercial or conventional extenders maintained their viability ~ 75% after 3 days of cooled storage.^{26,43}

The addition of components of animal origin (egg yolk and milk) to most extenders represent a potential risk of contamination and due to their complex biological composition, impair extender standardization.⁸ Thus, as an alternative to these components, addition to soybean lecithin to semen extenders has been evaluated. Better sperm PM was reported with soybean lecithin as a cryoprotective agent compared to egg yolk (mean PM of 67% with soybean lecithin versus 41% with egg yolk, after 4 days of cooled storage).¹² Similarly, other authors have reported better sperm PM, viability, and morphology after 8 or 10 days of storage when using soybean lecithin.^{13,44} On the contrary, PM and acrosome membrane integrity after 10 days of storage were better for extenders with egg yolk than those including various concentrations of soybean lecithin.¹⁵ Differences between studies might be related to various lecithin sources, concentration, and extender preparation. In fact, various soybean lecithin sources have varying composition and effects on sperm quality.^{13,45}

As the plasma membrane is a primary site of sperm damage when lowering temperature, the CLC complex has been used to replace egg yolk in semen.⁹ In stallions and donkeys, the addition of this complex to semen extenders improved sperm motility after 48 hours of cooled storage.^{46,47} In dogs, the addition of CLC complex to a cryopreservation extender improved PM in frozen-thaw samples.¹⁸ In the present study, semen diluted in a commercial extender that included this complex had a lower percentage of sperm with reacted

acrosome but PM and other studied parameters had no significant changes when compared to a conventional extender.

In conclusion, the commercial extender evaluated in this study maintained good sperm quality after 4 days of cooled storage. Throughout the study period, this extender preserved PM, kinematic parameters, and chromatin condensation in canine sperm, similar to the conventional extender, while offering the additional advantage of better preserving acrosome integrity. The CLC complex, known for its membrane-stabilizing properties, could protect the acrosome membrane, contribute to an overall improvement of sperm function and indirectly to fertility. Moreover, being free of egg yolk provides a more standardized and pathogen-free composition. Therefore, it would represent a more suitable option for cooling dog semen.

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Authors' contribution statement and agreement

MG designed and performed the study and wrote the manuscript; CA performed the study and collaborated in data analysis; EM performed the study; MM & MM critically revised and edited the manuscript; and NM performed the study, collaborated in data analysis and critically revised the manuscript. Authors have read and approved the final submission.

Conflict of interest

None to declare.

Data availability statement

Data can be obtained from the corresponding author based on reasonable request.

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