In vitro and in vivo assessment of skim milk with and without egg yolk on canine spermatozoa incubated at 4ºC

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Abstract

The aims of this study were to assess and compare the effect of skim milk with (MEY) and without (SMI) egg yolk on canine spermatozoa incubated at 4ºC *in vitro* and to evaluate the efficacy of MEY in *vivo*. Also, the effect of semen cooled storage before freezing was also evaluated *in vitro*. The ejaculates of 10 dogs were collected, pooled, centrifuged, and divided into 4 aliquots and diluted in one of the following 4 diluents: Prostatic fluid (PRO), commercial diluent (COM), SMI, or MEY. Extended samples were stored at 4°C and evaluated daily for 6 days. Percentage of total ($P < 0.01$) and progressive ($P < 0.01$) motility, intact acrosomes ($P < 0.05$), and positive endosmosis ($P < 0.01$) decreased over time in the diluents, with COM and PRO having as the best and the worst performances, respectively. Furthermore, MEY motility differed from PRO ($P < 0.01$) and SMI ($P < 0.01$) but not COM. Acrosome integrity was higher in MEY when compared to SMI ($P < 0.05$). Sixteen pairs of dogs were randomly assigned to either fresh undiluted semen ($n = 8$) or to 24-h cooled MEY diluted semen ($n = 8$). Seven (87.5%; $P > 0.1$) bitches from each group became pregnant and whelped normally. MEY extended semen samples were cooled for 2, 24, or 48 h at 4°C, and a second dilution was performed prior to freezing and thawing. Post-thaw total and forward sperm motility decreased with increasing cooled storage ($P < 0.05$), although no significant differences in total or forward motility, normal acrosomes, positive endosmosis, live spermatozoa, or positive endosmosis were found between 2 and 24 h storage. These *in vitro* and *in vivo* results show that MEY can be considered a simple, inexpensive, and efficient diluent for canine semen chilling. Furthermore, MEY could be successfully frozen after 1 day of cooled storage.

Keywords: artificial insemination, cooled semen, dog, frozen semen, pregnancy rate.

Introduction

Preserved semen shipment replaces the international transport of breeding animals. This is a financially reasonable and safer (i.e., reduces health risks and transport stress) technique compared to live animal movement. Furthermore, semen preservation facilitates the wide distribution of varied and superior genetics. However, international canine semen interchange is partially limited in most developing countries by the proportionally high cost of proprietary diluents. In these countries, dogs are still travelling long distances for breeding and heterogeneity is not guaranteed. In this aspect, low cost, user-friendly, efficient, and safe diluents are needed before semen preservation can be widely applied worldwide.

Contrary to frozen-thawed semen, chilled semen can be deposited intravaginally and still maintain good fertility. Handling and shipping of chilled semen is also easier and cheaper than frozen semen. Furthermore, chilling can be carried out in ordinary veterinary clinics, whereas freezing requires expensive equipment and trained personnel that are not always available. Furthermore, the success rate of artificial insemination (AI) is usually higher for chilled semen than for frozen semen when equally good methods for timing of the estrous cycle, and for AI, are used (Linde-Forsberg, 1995). In this context, cooling appears to be the logical initial approach to canine semen preservation in certain geographical areas. It would also be of great advantage if semen could be pre-cooled in ordinary clinics and sent to semen banks for freezing and permanent storage.

Although cooling involves a number of factors that may damage the sperm plasma membrane, canine semen can be diluted in extenders, cooled, and maintained at 4°C for several days. Extenders protect spermatozoa, allowing for motility and fertility conservation over time by stabilizing plasmalemma, providing energy substrates, and preventing deleterious effects of changes in pH and osmolarity over time (Linde-Forsberg, 1995).

Milk is a commonly used component of semen extenders in most species, having good performance both *in vitro* and *in vivo* (Maxwell and Salamon, 1993; Batellier *et al*., 2001). Skim milk proteins buffer semen pH and may also chelate any heavy metal ions (Jones and Martin, 1973; Maxwell and Salamon, 1993; Batellier *et al*., 2001). The addition of egg yolk to skim milk extender improves the viability of spermatozoa during chilled storage (Salamon and Maxwell, 2000) as its phospholipid fraction provides protection to sperm and acrosomal membranes against cold shock (Jones and Martin, 1973; White, 1993). It has also been

speculated that milk and egg yolk have a synergistic, protective effect on sperm because of their analogous action of the sequestration of seminal plasma proteins. In this context, skim milk with or without egg yolk could be an inexpensive, practical diluent for cooling canine semen. There is only one report of skim milk tested as a diluent for cooling canine semen with acceptable *in vitro* results (Rota *et al*., 1995). Skim milk and egg yolk also showed post-thaw semen parameters comparable to those obtained by a Tris based buffer in this species (Rota *et al*., 2001). Although the potentially improving effect of egg yolk supplementation to skim milk has not been described in this species yet, neither has the effect of cooled storage of milk based diluents before freezing. Furthermore, pregnancy rate and prolificacy have not been assessed for this diluent (skim milk-egg yolk) in dogs.

The aim of this study was twofold: first, to assess and compare the effect of skim milk with and without egg yolk on canine spermatozoa incubated at 4ºC *in vitro* and to evaluate the efficacy of skim milk-egg yolk on canine spermatozoa fertility incubated at 4ºC *in vivo*; second, to evaluate the effect of cooled storage using this diluent before freezing *in vitro*.

Materials and Methods

Experiment 1

Animals

Ten 1- to 5-year-old cross and pure bred (5 German shepherds), privately owned dogs weighing 11.5 to 32 kg were used for these studies. The animals were fed with different dry commercial feed and trained to ejaculate by digital manipulation (Linde-Forsberg, 1995) before the start of the experiments. All research protocols were approved by the Institutional IACUC.

Semen collection

The second and third fractions of each of the dogs' ejaculates were collected by digital manipulation in separate, calibrated plastic tubes (Linde-Forsberg, 1995). Immediately after collection, the volume, sperm motility, sperm concentration, morphology, and acrosome integrity of each semen sample were defined (see Semen evaluation below). Total sperm number was assessed in an aliquot of semen diluted 1:100 using an improved Neubauer hemocytometer counting chamber and the total sperm output was calculated by multiplying the concentration by the volume of the second fraction. Morphology was evaluated by smearing a drop of semen on a glass slide, allowing the slide to dry, staining with Giemsa stain, and examining more than 100 spermatozoa in the sample under 200X magnification. Only ejaculates with a sperm concentration $>100 \times 10^6$ spermatozoa/ml, progressive sperm motility $>70\%$, normal acrosome integrity $>85\%$, and normal sperm morphology $>85\%$ were included in the study.

Semen processing

The second fractions of the ejaculates were pooled, centrifuged at 450G for 10 min, and the supernatant was removed. The resultant sample was divided into 4 aliquots. Each fourth was diluted 1:3 to 1:6 (volume:volume) with each of the different extenders or with a corresponding volume of autologous prostatic fluid (third fraction of the ejaculate). The sperm concentration in the extended semen samples varied from 75 to 100 x 10^6/ml. Extended samples were stored in screw-cap closed sterile vials, placed into a glass tube filled with water, then moved to a refrigerator at 4°C and left for six days. For consistency of the results, this experiment was repeated 5 times at weekly intervals.

Semen extenders

The following extenders were used: 1) Prostatic fluid (PRO): autologous prostatic fluid was used as a negative control; 2) Commercial semen diluent® (COM): distilled water, glucose, fructose, sodium citrate, Tris, non-disclosed proprietary components, gentamicin, and 20% egg yolk, used as a positive control; 3) Skim milk (SMI; Table 1); and 4) Skim milk-egg yolk (MEY): 80% SMI and 20% egg yolk to test the effect of its supplementation in combination with milk. All the extenders were prepared fresh before each trial.

Table 1. Extenders (skim milk, SMI; skim milk-egg yolk, MEY) compared for dog semen stored at 4°C.

Ingredients	SMI	MEY
Ultra-high-temperature-treated skim milk (0% fat; Ilolay®, Argentina)	80%	80%
Benzyl penicillin	1 mg/ml	1 mg/ml
Dihydrostreptomycin sulphate	1 mg/ml	1 mg/ml
Egg yolk	---	20%

Semen evaluation

The following tests were performed immediately after mixing the fresh semen pellet with each extender (day 0) and then daily up to day 6 or up to <10% total motility. For each analysis, an aliquot of 150 µl of semen was collected from the 4°C preserved samples and slowly warmed at room temperature. Motility was assessed in a drop on a warmed glass slide and the percentage of total motile and progressively motile spermatozoa were subjectively assessed at 37°C in 5 µl at 400X magnification by two trained operators. Total and progressive motilities were the only determinations assessed in the prostatic diluent.

The number of live and dead spermatozoa was examined on nigrosin/eosin stained slides. Individual sperm cells were recorded as being either alive (unstained) or dead (stained). Plasmalemma integrity was evaluated by hypo-osmotic swelling (HOS) test. The percentage of curled/swollen spermatozoa (i.e., with an intact plasma membrane) was determined (England and Plummer, 1993). Acrosome normality was evaluated by Bengal Rose stain solution (Cardoso *et al*., 2007). Acrosomes were classified as normal or abnormal (detached, damaged, or lost). Percentage live/dead, HOS positivity, and acrosome normality were calculated on a total of 200 cells. Both the pH (pH-009 ATC, China) and osmolarity (Wescor, Inc mod. 5520, USA) were also measured.

Statistical analysis

All semen parameters (mean ± SEM) were compared among 3 diluents (COM *vs*. SMI *vs*. MEY) using a repeated analysis of variance followed by Tukey comparison test when significant differences were found (SPSS 17.0, SPSS, Chicago, IL, USA). In the case of total and progressive motilities, prostatic diluent (PRO) was also included in the analysis. Values were considered to be statistically significant when $P < 0.05$.

Experiment 2: Fertility and prolificacy of an egg yolk milk-based extender stored at 4°C for 24 h

Animals and semen collection and processing

Sixteen pairs (male and female) of healthy, 4 to 35 kg, 1.5- to 4.5-year-old, privately owned purebred dogs were used for this trial. Consent forms were signed by the clients. Semen collection, evaluation, and processing (group MEY) were carried out as explained in experiment 1.

Female estrus monitoring

Bitches were examined twice weekly for the presence of vulvar swelling and serosanguineous vaginal discharge as indicators of proestrus. After proestrus onset, vaginal smears were evaluated daily

until the first day of cytological diestrus. When vaginal cellular cornification was >80% (Olson *et al*., 1984), blood samples were collected daily by peripheral venipuncture for progesterone (P4) determination (Immulite®, Diagnostic Products Corporation, Los Angeles, CA, USA) until ovulation (P4 > 5 ng/ml) and then every other day until cytological diestrus. A single artificial insemination was performed on day 5 after the estimated luteinizing hormone (LH) peak (P_4 approximately 2.0 ng/ml) and P4 concentrations were between 7 and 20 ng/ml, depending on each case (Linde-Forsberg, 1995).

Artificial insemination

A minimum of 200×10^6 spermatozoa per AI was used in all cases. The females were randomly assigned to either fresh undiluted semen (UND; n = 8) or 24-h cooled MEY diluted semen (MEY; n = 8). The insemination dose was aspirated into a 5 ml sterile syringe. The vaginal deposition technique was carried out using a bovine plastic catheter (length 30 cm, width 4 mm) and the length was adjusted to the female's size. The tip of the pipette was introduced cranially to the farthest point possible (i.e., into the area of vaginal portion and paracervix (pseudocervix)). The hindquarters of the bitches were elevated and the extended semen was deposited into the vagina. The catheter was then withdrawn and the bitch was held with elevated hindquarters for 10 min with finger stroking and feathering the area of the vulva to prevent the reflux of semen (Linde-Forsberg, 1995).

Pregnancy diagnosis and litter size

In all cases, pregnancy was diagnosed by ultrasonography (Toshiba Core Vision Pro, Tokyo, Japan; Mattoon and Nyland, 1995) > 21 days after the estimated LH peak. Data concerning litter size at birth was provided by breeders.

Statistical analysis

Pregnancy rate was compared between groups (UND *vs*. MEY) by Fisher Exact Test. Values were considered to be statistically significant when $P < 0.05$.

Experiment 3: Quality of frozen-thawed semen stored in an egg yolk milk extender at 4°C for up to 48 h before freezing

Animals and semen collection

Same as in experiment 1.

Semen processing and evaluation

Pooled semen samples were divided into four aliquots. Each aliquot was diluted with the first extender

(ultra-high-temperature-treated skim milk [0% fat; Ilolay®, Argentina], 20% egg yolk, 1 mg/ml benzyl penicillin, 1 mg/ml dihydrostreptomycin sulphate and 0% glycerol) and after 2 (traditional protocol; Control), 24, or 48 h of equilibration at 4°C, a second dilution was performed (ultra-high-temperature-treated skim milk [0% fat; Ilolay®, Argentina], 20% egg yolk, 1 mg/ml benzyl penicillin, 1 mg/ml dihydrostreptomycin sulphate, 10% glycerol and Equex (Nova Chemical Sales Inc., Scituate, MA, USA) in 0.3 ml distilled water at 1%) to reach a final concentration of 100 x 10^6 spermatozoa∕ml, 20% egg yolk, 5% glycerol, and Equex at 0.5%. Then, the semen samples corresponding to the four aliquots were packed into 0.5 ml plastic straws which were placed horizontally on a rack situated above the surface of liquid nitrogen at a distance of 4 cm for 15 min. Finally, the straws were plunged into and stored in the liquid nitrogen until evaluation.

Approximately 2 weeks after freezing, the straws were thawed in a water bath at 37°C for 1 min and then poured into a glass tube pre-warmed to 37°C. After warming (37°C) for 5 min, the semen was analyzed to determine the percentages of total and forward sperm motility, live/dead, HOS positive, and acrosomal integrity. The techniques used to evaluate the

thawed semen were the same as those described previously in experiment 1. The experiment was repeated five times.

Statistical analysis

The sperm data (mean ± SEM; i.e., total and forward motility, live spermatozoa, and sperm cells with intact pasmalemma and acrosomes; all in percentages) were compared among treatments (2 *vs*. 24 *vs*. 48 *vs*. 72) using a repeated analysis of variance. When significant differences were found, the means were compared using the Tukey test. Values were considered to be statistically significant when $P < 0.05$.

Results

Experiment 1

Total ($P < 0.01$) and progressive ($P < 0.01$) motility decreased over time in the 4 diluents (Fig. 1, Inset), with COM and PRO yielding the best and the worst performance, respectively. Furthermore, MEY differed from PRO ($P < 0.01$) and SMI ($P < 0.01$), but not from COM.
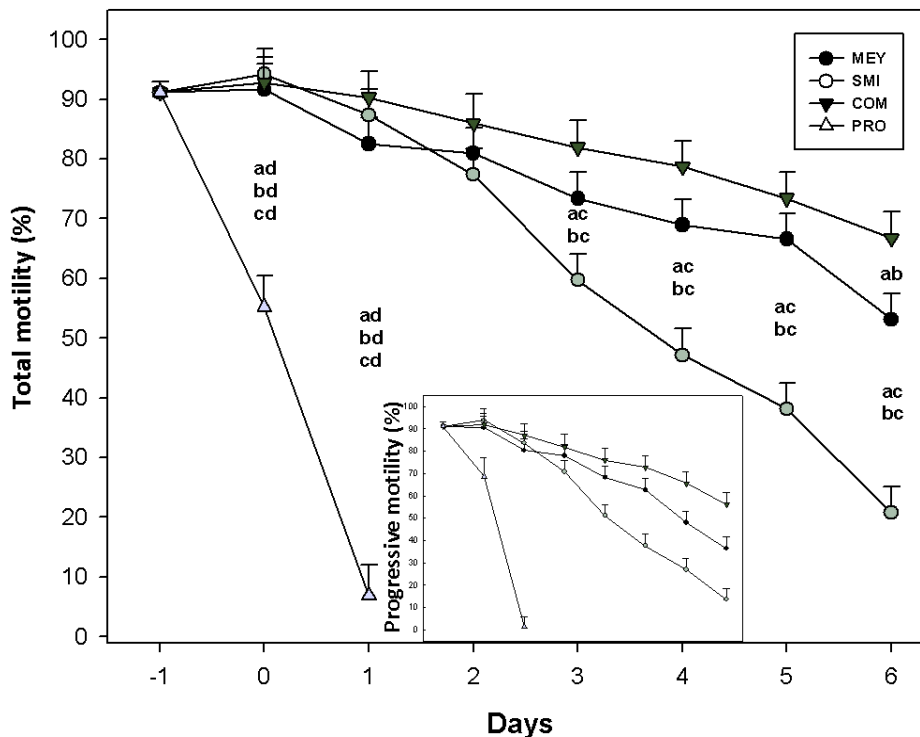

Figure 1. Percentage (Mean + SEM) of total and progressive (Inset) motility of dog semen stored in three different extenders (commercial, COM; skim milk, SMI; skim milk-egg yolk, MEY; control, PRO) at 4°C for six days. Different letters within the same day indicate differences ($P < 0.05$) between groups.

Percentage of normal acrosomes (P < 0.05; Fig. 2) and positive endosmosis (P < 0.01; Table 2) diminished in the 3 treatments throughout the study period. While there were no significant differences in the percentage of endosmosis among the 3 extenders, acrosome integrity was higher in MEY when compared to SMI (P < 0.05). The pH remained within the normal physiological range in the 3 groups throughout the study period (P > 0.1). The remaining semen characteristics such as live spermatozoa (P > 0.1) and osmolarity (P > 0.1) slightly declined throughout the study, with no differences among extenders (Table 2).

Experiment 2

Seven (7/8; P > 0.1) bitches from each group became pregnant and whelped normally at term. Litter size ranged from 4 to 12 puppies depending on dam´s size, which was within the expected number for each breed.

Experiment 3

Post-thaw total and forward sperm motility decreased (P < 0.05) with increasing cooled storage time before freezing, although no significant differences in total and forward motility, normal acrosomes, positive endosmosis, live spermatozoa, and positive endosmosis were found between 2 and 24 h storage (Table 3).
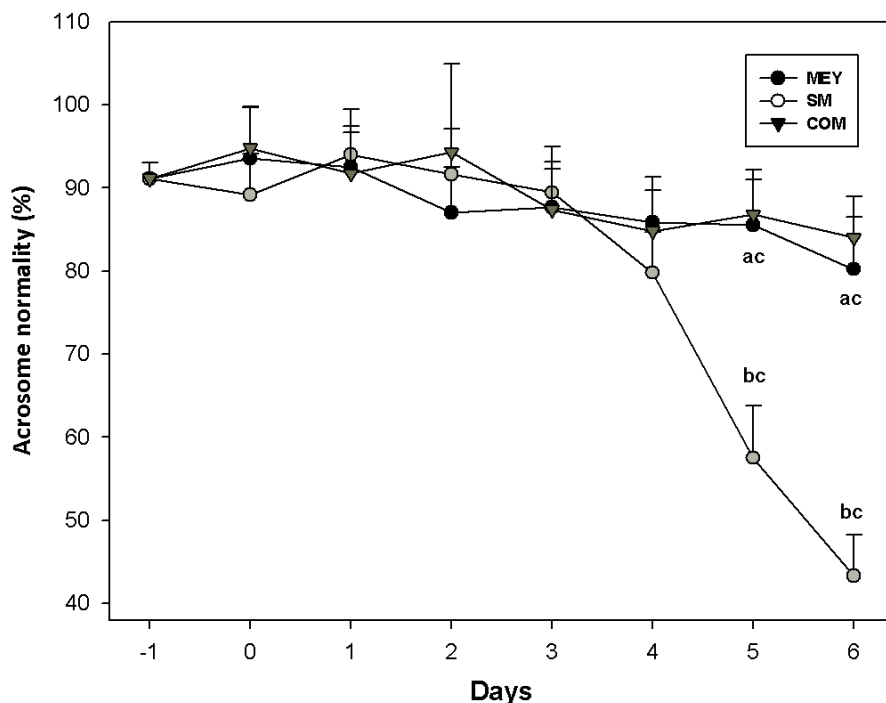

Figure 2. Percentage of normal acrosomes (mean + SEM) of dog semen stored in three different extenders (commercial, COM; skim milk, SMI; skim milk-egg yolk, MEY) at 4°C for six days. Different letters within the same day indicate differences (P < 0.05) between groups.

Table 2. Parameters (mean + SEM) of dog semen stored in three different extenders (commercial, COM; skim milk, SMI; skim milk-egg yolk, MEY) at 4°C for six days.

	Seminal parameters											
	Live spermatozoa (%)			Endosmosis (%)			Osmolarity (mOsm)			pH		
Day	SMI	MEY	COM	SMI	MEY	COM	SMI	MEY	COM	SMI	MEY	COM
0	94.5 ± 7.4	91.6 ± 6.9	91.3 ± 4.9	93.3 ± 4.9	93.0 ± 4.9	95.1 ± 3.4	308.0 ± 9.1	309.1 ± 9.1	311.5 ± 8.0	6.7 + 0.0	6.6 + 0.0	6.7 + 0.0
1	82.8 ± 6.9	90.5 ± 6.9	81.6 ± 5.4	88.7 ± 4.9	87.7 ± 4.9	91.0 ± 2.4	312.0 ± 9.1	312.8 ± 9.1	316.5 ± 8.0	6.6 + 0.0	6.6 + 0.0	6.6 + 0.0
2	83.7 ± 6.9	89.1 ± 6.9	82.4 ± 6.3	76.5 ± 4.9	83.2 ± 4.9	85.2 ± 3.7	304.6 ± 9.6	319.6 ± 9.1	325.1 ± 11.4	6.6 + 0.0	6.6 + 0.0	6.6 + 0.0
3	76.9 ± 7.9	87.9 ± 7.3	77.2 ± 5.1	71.5 ± 5.4	69.1 ± 5.1	75.6 ± 2.4	299.8 ± 14.5	321.1 ± 11.3	326.0 ± 8.0	6.6 + 0.0	6.6 + 0.0	6.6 + 0.0
4	66.8 ± 6.9	82.5 ± 6.9	76.0 ± 5.1	59.1 ± 4.9	62.3 ± 4.9	64.1 ± 2.4	330.7 ± 9.6	328.7 ± 10.4	321.0 ± 8.0	6.6 + 0.0	6.6 + 0.0	6.6 + 0.0
5	61.4 ± 7.9	77.3 ± 7.3	75.2 ± 5.8	54.8 ± 5.4	54.5 ± 5.1	57.3 ± 3.5	338.8 ± 14.5	329.9 ± 12.6	307.0 ± 8.0	6.5 + 0.0	6.6 + 0.0	6.5 + 0.0
6	53.3 ± 7.9	55.3 ± 7.3	73.1 ± 5.1	36.5 ± 5.8	39.4 ± 4.6	40.4 ± 3.2	355.5 ± 14.5	363.5 ± 11.3	319.0 ± 8.0	6.6 + 0.0	6.5 + 0.0	6.6 + 0.0

Table 3. Percentage of frozen thawed parameters (mean + SEM) of milk-egg yolk diluted canine semen stored for 2, 24, or 48 h at 4°C before freezing.

	Seminal parameters				
Storage time (h)	Total motility	Progressive motility	Live spermatozoa	Endosmosis	Intact acrosome
2	67.4 ± 6.3[a]	63.2 ± 5.7[a]	65.0 ± 8.2	52.8 ± 6.3	93.8 ± 0.1
24	48.1 ± 6.1[a,b]	44.8 ± 5.7[a,b]	53.4 ± 8.2	46.0 ± 5.8	87.8 ± 0.1
48	40.0 ± 3.5[b]	34.2 ± 5.2[b]	53.2 ± 4.8	47.2 ± 4.9	88.0 ± 8.0

Different letters within the same row indicate differences (P < 0.05).

Discussion

This study attempted to identify a low cost, efficient, and practical canine semen extender so that chilled semen for AI could be widely available and more affordable for worldwide breeders of purebred dogs. Although pregnancy rates and prolificacy are the optimal ways to evaluate the efficacy of a semen extender, in experimental conditions, the combination of various tests usually correlates with potential fertility (Rota *et al*., 1995). Thus, spermatozoa must have good motility to reach the oviduct and an intact plasmalemma and acrosome for zona pellucida binding and oocyte fusion. Despite the fact that in this trial motility had to be subjectively estimated because computerized techniques (such as CASA) cannot be used for milk based extenders, this limitation was compensated for by having each sample evaluated by two trained observers and finally by the *in vivo* fertility tests.

The beneficial effects of egg yolk supplementation to this milk based extender were demonstrated in both motility and acrosome integrity evaluations. These results are in line with previous reports using other laboratory prepared chilling extenders in other species (Salamon and Maxwell, 2000) and also in dogs (Iguer-Ouada and Verstegen, 2001).

In this *in vitro* experiment, MEY showed a better performance than in a previous study in dogs (Rota *et al*., 1995), maintaining >70% of progressive motility up to day 2 and >80% normal acrosomes up to day 4. Conversely, plasmalemma integrity was lower than previously reported using this diluent (Rota *et al*., 1995). These differences could be due to the diluent components (i.e., milk and egg yolk), the animals used, or to the subjective evaluations carried out in both studies.

It is noteworthy that the changes in pH found in this experiment during the study period were within the physiological range reflecting good buffering capacity of both milk based extenders. These pH findings were in accordance with a previous report using a similar MEY extender (Rota *et al*., 1995). The acceptable *in vitro* semen parameters obtained guaranteed the following *in vivo* assessment of the MEY extender.

In line with the *in vitro* results, the 24 h cooled MEY diluted semen remained fertile, as pregnancy rate was normal (approximately 88%) and not different from that of fresh undiluted semen. Interestingly, prolificacy also seemed to be normal for each particular breed. The failure of one bitch to conceive cannot directly be attributed to semen processing as it occurred in both groups. Furthermore, a complete breeding examination was not carried out on any of the dogs, and other etiologies of infertility could have been present. With these results in mind, it would also be interesting to test the *in vivo* effect of 48 h storage of the MEY diluent.

It has already been demonstrated that ejaculated dog semen can be successfully frozen after 1-2 days of cooled storage in a Tris-glucose-egg-yolk extender (TEY; Hermansson and Linde-Forsberg, 2006; Santana *et al*., 2013). In the present study, it was confirmed that the same is true, at least for the first 24 h, for this milk-egg yolk chilling extender which could function as a prolonged equilibration previous to freezing in semen banks. For this reason, the exact same cooling extender (i.e., without glycerol) was used for the first dilution. Although the cooled storage time interval showed a gradual negative effect on thawed semen motility, during the first 24 h both total and progressive motility did not show any differences from the samples frozen by our conventional cryopreservation method. Furthermore, acrosomes, viability, and membrane functionality were not significantly affected by cooling time.

Using this milk based extender, post-thaw motility was slightly lower than that described for TEY diluent after 24 h cooled storage (Hermansson and Linde-Forsberg, 2006; Santana *et al*., 2013). These differences could be attributed to the fact that no thaw medium was used in this trial, and samples were directly assessed after thawing. Conversely, membrane and acrosome integrity were within the range previously described in the previously mentioned reports (Hermansson and Linde-Forsberg, 2006; Santana *et al*., 2013).

The results clearly demonstrate that cooling canine semen in this milk-egg yolk extender up to 24 h before freezing does not produce a decrease in semen quality when compared to semen frozen by a traditional procedure (i.e., with only 2 h cooling at 4°C). More studies are still necessary to find out if the fertilizing capacity of spermatozoa is affected by cold storage prior to freezing using this practical milk based extender.

According to these *in vitro* and *in vivo* results,

this milk-egg yolk extender could be considered not only simple to prepare and economically accessible, but also efficient, for canine semen chilling. A contribution to canine welfare, genetics, and reproduction will be made, particularly in countries where the costs of expensive commercial semen extenders limit the wide application of semen preservation. Furthermore, the semen diluted in this extender could be successfully frozen after 1 day of cooled storage. This way, worldwide canine semen interchange would benefit from the inclusion of new import countries. Importantly, for zoosanitary reasons, the source of these proteins (eggs and/or milk) must be controlled and should be tested prior to use.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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