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Effect of equilibration time on the motility and functional integrity of canine spermatozoa frozen in three different extenders

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ABSTRACT

The present work aimed to assess the effect of equilibration time on post-thaw motility parameters of canine sperm frozen in three extenders: 6% low-density lipoproteins (LDL), 6% liposomes (LIPO), and 40% egg yolk plasma (EYP). A second experiment is aimed at evaluating the functional integrity of canine spermatozoa frozen in the three extenders at the best equilibration time found in the experiment one. In the first experiment, 20 ejaculates harvested from 7 dogs, were frozen in three extenders (LDL, LIPO, and EYP) after four equilibration times (30 min, 1 h, 3 h, and 6 h). The semen was evaluated after thawing using an image analyser (HT-IVOS 14.0). The 6 h equilibration time gave better results of motility and progressive motility in the three studied extenders. (LDL: 58.9% vs. 42.7%; LIPO: 54.4% vs. 31.9%; EYP: 55.4% vs 40.5% for motility 6 vs. 1 h). In the second experiment, 10 ejaculates taken from 6 dogs were frozen under the same conditions as the previous experiment, after 6 h equilibration time. The integrity parameters of the spermatozoal membrane (hypo-osmotic swelling test, and SYBR14/propidium Iodide staining), acrosome (FITC-*Pisium sativum* Aglutinin staining), and DNA (acridine orange staining) were evaluated at three different stages: post-dilution (T0), post-equilibration, and post-thawing. Post-thaw results were as follows: membrane integrity (HOSt: 62;6% vs 58% vs 64.4%; SYBR14/IP: 63.6% vs 57.9% vs 64.8%); acrosome integrity (FITC-PSA: 79.4% vs 74% vs 76.2%) and DNA integrity (Acridine-orange: 98.9% vs 98.5% vs 98.7%) respectively for LDL vs. LIPO vs. EYP. No significant difference existed between the extenders tested; thus 6%LIPO and 40%EYP could be good candidates for replacement of 6%LDL in the protection of canine sperm during the freeze-thaw process without altering motility and integrity parameters.

1. Introduction

Equilibration is an essential step in the cryopreservation of sperm. In addition to ice preventing action due to the cryoprotectant (glycerol), it enables the spermatozoal membrane to be more stabilized and adapted to low temperatures in preparation for freezing. These changes are produced by the cooling temperature (4 °C) and the membrane stabilizing agents (egg yolk or substitutes) contained in the diluents (Okano et al., 2004).

When freezing canine semen, glycerol is either added in a single step followed by chilling at 4 °C for 1 to 2 h according to the majority of published protocols (Anderson, 1975; Ström et al., 1997; Rota et al., 1999) or in two phases over a total period of one and half to 2 h according to others (Nizanski et al., 1997; Rota et al., 1997; Pena and Linde-Forsberg, 2000).

Many works were aimed at extending the time of chilling before glycerol addition and freezing of canine semen (Verstegen et al., 2002; Okano et al., 2004; Hermansson and Linde-Forsberg, 2006), but only few authors have studied the effect of glycerol-equilibration time.

It has been reported that the best equilibration time is 2 h in goat (Sundararaman and Edwin, 2008), between 2 and 3 h in boar (Yi et al., 2002) and 4 h for both bull (Leite et al. (2010) and dog (Igna et al., 2008) semen freezing. All these protocols used egg yolk-based extenders.

Due to several sanitary and practical reasons, the whole egg yolk (EY) has been replaced in semen preservation by its cryoprotective fraction which is the low density lipoproteins (LDL) extracted according to the method described by Moussa et al. (2002).

LDL-based extenders give good results for freezing canine (Bencharif et al., 2008; Varela Junior et al., 2009), bovine (Amirat et al., 2004) and equine (Moreno et al., 2013) sperm. However, the protocol of LDL

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extraction has some technological and sanitary limitations since it is laborious and time consuming, and could never be reproduced on an industrial scale nor sterilized in accordance with bio-health standards and LDL still contain products of animal origin.

In contrast to LDL, egg yolk plasma (EYP) is faster, easier and cheaper to extract from the whole egg yolk and can be sterilized and industrially scaled. Liposomes (LIPO) are exogenous lipids with numerous advantages over LDL. They have the possibility to be incorporated into clear, semi-synthetic, chemically defined, easily sterilized and ready to use extenders. Recent studies show the possibility of substituting LDL with EYP in stallion sperm freezing (Pillet et al., 2011); and with LIPO in stallion (Pillet et al., 2012), bull (Le Röpke et al., 2011; Le Guillou et al., 2013) and dog (Diaz et al., 2014) semen freezing.

It would be very interesting to investigate more the substitution of LDL with EYP and LIPO in dog semen freezing and to assess the Effect of equilibration time and its interaction with these three membrane stabilizing agents. To our knowledge, no related works exist in literature and we hypothesize that the optimal equilibration time can be different to what has been previously reported about EY-based extenders.

The current work has two objectives. The first is to assess the effect of varying the equilibration time on the motility parameters of canine sperm frozen in LDL, EYP and LIPO based extenders. The second is to evaluate the functional characteristics of canine sperm frozen in the same three extenders after determining the optimal equilibration time.

2. Material and methods

2.1. First experiment: study of motility parameters

2.1.1. Semen collection

Twenty five ejaculates were taken from 7 different dogs: 6 beagles from the Department of Reproductive Pathology and 1 privately owned Malinois, aged between 2 and 7 years. The owner of the dog included in the study provided written consent. All experimental procedures were carried out in compliance with the ethical committee of ONIRIS, National Veterinary School of Nantes.

Semen collection was limited to one person to avoid extraneous effects on sperm quality. The collection of semen was fractioned and only the sperm rich fraction was examined.

2.1.2. Examination of sperm quality

Mass motility was microscopically assessed and graded from 0 to 5 on the MILOVANOV scale (0: immobile spermatozoa and 5: presence of waves) and spermatozoal concentration measured using a photometer calibrated for dog semen (SpermaCue®, Minitube, Tiefenbach, Germany). Only ejaculates with a mass motility of 4 or more and a minimal concentration of around 200×10^6 spermatozoa per ml were frozen.

2.1.3. Extraction of LDL from chicken egg yolk

The method used to extract the LDL was developed by Moussa et al. (2002) and a purity of 97% was achieved. This technique is protected by a patent (No. 0100292) that was submitted jointly by the Veterinary School of Nantes and the INRA of Nantes.

2.1.4. Extraction of plasma from chicken egg yolk

The technique used to fractionate the chicken EY into granules and plasma was done as described by MacBee and Cotterill (1979) through repeated ultra centrifugations to obtain the EYP fraction with a satisfactory degree of purity.

2.1.5. Preparation of liposomes

Liposomes are artificial vesicles composed of one or several concentric lipid bilayers, which have the ability to encapsulate molecules. They are produced according to a patent-protected protocol (IMV-Technologies, l'Aigle, France). The phospholipid combination used in our study was commercial lecithins E80S, (Lipoïd GMBH, Frigenstrasse4, D-67065 Ludwigshafen, Germany), composed of 74.1% phosphatidylcholine (PC), 13.3% phosphatidylethanolamine (PE), 1% sphingosine (SPH) and 1.9% lysoPC. After dissolution of commercial phospholipids into chloroform, we obtained phospholipid films which were rehydrated in an extender base solution for 12 h before sonication and extrusion (mini-Extruder, AVANTI Poloar Lipids, Inc.) through polycarbonate Cyclopore Track etched membranes (pore size 0.1 μm, Whatman). After measuring the size of vesicles (ranging from 120 to 150 nm), liposomes were stored at 4 °C until use.

2.1.6. Preparation of the extenders

Three semen extenders for cryopreservation of spermatozoa were prepared using a common basic diluent with the addition of 6% LDL (control), 6% LIPO, or 40% EYP (Table 1).

2.1.7. Semen dilution

Twelve cryotubes were placed in a water bath (37 °C); 100 μl of the 3 studied extenders (6%LDL, 6%LIPO, and 40%EYP) were then added to the tubes (four tubes for each extender); 100 μl of semen was placed in each cryotube. Depending on the sample concentration, the volume of each extender was topped up to obtain a final concentration of 100×10^6 spz/ml in each cryotube.

2.1.8. Equilibration

The 12 cryotubes were then equilibrated in a refrigeration case at 4 °C.

At the end of each one of the 4 studied equilibration times (30 min, 1 h, 3 h and 6 h), the corresponding 0.25 ml straws (IMV Technologies, Aigles, France) were filled manually and plugged with polyvinyl alcohol powder then placed horizontally on a metal ramp and maintained in at 4 °C for 30 min.

2.1.9. Freezing

In the first phase, the canine semen was frozen in liquid nitrogen vapours (4 cm above the level of the liquid nitrogen) for 10 min. The straws were then immersed vertically in liquid nitrogen for storage in nitrogen vats.

2.1.10. Thawing

The straws were immersed directly in a water bath at 37 °C for 30 s. Each batch was analysed 10 min later. Before to be analysed, semen was diluted with EasyBuffer B® solution (IMV-Technologies, Aigle, France) to obtain an appropriate concentration (25×10^6 spz/ml) for the image analysis.

Table 1
Composition of 6% LDL, 40% EYP and 6% LIPO based extenders.

Component	Quantity		
	6%LDL (Canifreeze®)	40% egg yolk plasma	6% liposomes
Tris (g)	3.026	3.026	3.026
Citric acid (g)	1.700	1.700	1.700
Fructose (g)	1.250	1.250	1.250
Glycerol (ml)	32.00	32.00	32.00
Penicillin-streptomycin (IU/g)	10^{6-1}	/	/
Penicillin G sodium (g)	/	0.0695	/
Streptomycin sulphate (g)	/	0.100	0.010
Enrofloxacin 10% (ml)	/	/	0.80
LDL (g)	16.5	/	/
Egg yolk plasma (ml)	/	40	/
Phospholipid powder (g)	/	/	0.546
Bi-distilled water	*QS* 100 mL		

Legend: LDL: low density lipoproteins; LIPO: liposomes; EYPlasma: egg yolk plasma; *QS*: (*Quantum Satis*): quantity sufficient for.