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The benefits of liposomes for chilling canine sperm for 4 days at 4 °C

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ABSTRACT

This study comprises 3 experiments exploring the possible benefits and mechanism of action of liposomes for chilling (4 °C) canine sperm over a period of 4 days.

In the first experiment, 20 ejaculates collected from 5 Beagle dogs were chilled in an extender containing 6% low density lipoproteins (LDL) (Control), or one of 7 extenders containing different concentrations (2, 4, 6, 8, 10, 15, 20%) of liposomes (LIPO). These ejaculates were chilled over 4 days and motility was assessed daily using a Hamilton Thorne analyzer (HTM-IVOS, 14.0). The 2% LIPO obtained the best results ($p = 0.038$) after four days (72.55% motile spermatozoa and 31.4% progressive spermatozoa).

In experiment 2, 10 ejaculates were collected from same 5 dogs and chilled in 6% LDL or 2% LIPO-based extenders. Sperm integrity characteristics were assessed prior to refrigeration and every 48 h for four days (D0, D2, and D4). Acrosome integrity was assessed using the FITC-PSA test (Fluorescein IsoThiocyanate-Pisum Sativum Agglutinin), plasma membrane (PM) integrity using both the hypo-osmotic swelling test (HOSt) and SYBR14/Propidium Iodide test (SYBR14/PI), and DNA integrity using the Acridine-Orange test (AO). The 2% LIPO extender provided equivalent preservation of sperm integrity parameters to the reference extender (6% LDL).

In experiment 3, a Langmuir-Blodgett trough was used to evaluate the mechanistic interactions between LDL, LIPO, prostatic fluid, and the canine spermatozoal membrane during chilling. Results indicate that LDL and LIPO interact differently with the biomimetic membrane. The most likely conclusion of these findings is that LDL and liposomes employ different protective mechanisms during the chilling (4 °C) of canine spermatozoa.

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1. Introduction

Chilled canine semen was successfully used for the first time in 1954 (Harrop, 1954). It has become increasingly

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popular over the few last decades, especially with the development of artificial insemination and dog breeding on an international level.

However, refrigeration exposes sperm to cold shock, which induces irreversible (structural and functional) damage to spermatozoa thus impairing the fertility of chilled semen (Watson and Morris, 1987).

Extenders are used to protect chilled sperm by preventing or reducing the effects of cold shock. This protective action is mainly due to membrane stabilizing compounds (Graham and Foote, 1987) i.e. cooling protectants (Farstad, 1996), which are traditionally represented by egg yolk (EY) in canine sperm preservation (Farstad, 2009).

EY presents several disadvantages, such as the risk of microbial contamination and the deleterious effects of the high density lipoproteins found in EY; whole EY has therefore been replaced by its cryoprotective fraction i.e. low density lipoproteins (LDL) (Foulkes, 1977; Pace and Graham, 1974; Quinn et al., 1980).

The latter were extracted with 97% purity according to the method described by Moussa et al. (2002) and have proven effective in chilling bull (Vera-Munoz et al., 2011), ram (Gharibi et al., 2014), stallion (Moreno et al., 2013), and dog (Bencharif et al., 2013) semen.

Nevertheless, LDL present two major technological and bio-sanitary limitations. They cannot be produced on an industrial scale or sterilized in compliance with bio-health regulations (unpublished data). A suitable substitute is therefore warranted.

Liposomes (LIPO) are artificial vesicles composed of one or several concentric lipid bi-layers, which have the ability to encapsulate molecules (Elhissi et al., 2015). They are composed of phospholipids (PL), i.e. the active principle of LDL in sperm membrane protection and stabilization (Bergeron and Manjunath, 2006). Unlike LDL, they can be produced industrially and incorporated into clear, semi-synthetic, chemically defined, easily sterilized and ready-to-use extenders (Elhissi et al., 2015).

In recent years, LIPO have been shown to be a viable alternative to EY for the cryopreservation of human (Mutalik et al., 2014), equine (Pillet et al., 2012), bull (Röpke et al., 2011), and buffalo (Kumar et al., 2015) semen. Le Guillou et al. (2015) demonstrated the possible replacement of LDL by liposomes in freezing bull semen and explored the mechanistic interactions of these extenders with the sperm plasma membrane using a biomimetic model, the Langmuir Blodgett trough. The latter technique mimics the outer leaflet of the spermatozoal membrane in the form of a monolayer of surfactant molecules placed over a liquid sub-phase similar to the basic extender (Le Guillou et al., 2013). This model membrane is thus used to simulate interactions between spermatozoa membranes and molecules injected into the liquid sub-phase (Le Guillou et al., 2015).

Liposomes have yet to be used to chill dog semen and there are no studies into their mechanism of action for protecting canine spermatozoa against cold shock.

This study therefore aims to determine the possible benefits of using LIPO instead of LDL to chill canine sperm (over a period of four days) and to explore their mechanism of sperm protection using the Langmuir Blodgett trough.

2. Materials and method

2.1. Experiment 1: refrigeration of canine semen

2.1.1. Semen collection and initial evaluation

Twenty-three ejaculates were collected at intervals of 48–72 h from five Beagles, aged between 3 and 7 years, and belonging to the department of reproductive pathology at Oniris: The National Veterinary, Food agriculture, and food hygiene school of Loire Atlantique, Nantes, France.

The samples were all collected manually by the same person in a fractioned manner and in the presence of a female on heat. Only the sperm-rich fraction was analyzed (Kutzler, 2005).

Immediately after collection, each ejaculate was evaluated for quality and ability to be chilled (mass motility and concentration).

Mass motility was assessed using a microscope with a heated stage at +37 °C, and scored using the MILOVANOV scale from zero to five (0: immobile spermatozoa and 5: presence of waves). Spermatozoa concentration was measured using a photometer calibrated for dog semen (SpermaCu®, Minitube, Tiefenbach, Germany). Only ejaculates with a minimal concentration of 300×10^6 spermatozoa/ml and a mass motility of 4 or more were included in this study (Bencharif et al., 2008).

2.1.2. Preparation of extenders

Extraction of LDL was performed according to the technique described by Moussa et al. (2002) (patented number 0100292). Liposomes were produced according to the technique described by Le Guillou et al. (2015), from an extract of EY-phospholipids composed of ~70% phosphatidylcholine (PC) and ~30% phosphatidylethanolamine (PE), purchased from Avanti Polar Lipids (Alabaster, AL).

Eight extenders for refrigerating spermatozoa were prepared using a basic diluent (composition shown in Table 1) with the addition of 6% LDL (Control) or one of the 7 different concentrations of liposomes (2%, 4%, 6%, 8%, 10%, 15%, and 20%). The composition of the extenders is given in Table 1.

The 6% LDL-based extender was used as a single control in our experiment following the results of Bencharif et al. (2013), who used it successfully to preserve canine sperm at +4 °C for four days in 100% of the dogs used in their study.

Table 1
Composition of the liposome-based extenders.

Extender	2%	4%	6%	8%	10%	15%	20%
Phospholipid powder (in g)	1.82	3.64	5.46	7.28	9.10	13.65	18.20
TRIS-Base (in g)	30.26						
Citric acid (in g)	17.00						
Fructose (in g)	12.50						
Glutathione (in g)	1.44						
Streptomycin (in mg)	1						
Baytril 10% (in ml)	0.8						
Distilled water (in l)	1						

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