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Freezing canine sperm: Comparison of semen extenders containing Equex® and LDL (Low Density Lipoproteins)

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ABSTRACT

Chicken egg yolk is held as an excellent cryoprotective agent for freezing canine semen. Recent advances have enabled the extraction of low density lipoproteins from egg yolk, which are responsible for the cryoprotective abilities of the latter. The objective of this article was to compare 3 semen extenders for freezing canine semen: 2 containing egg yolk (Tris egg yolk and Equex STAMP) and one containing 6% LDL. After freezing and thawing 20 ejaculates from 5 different dogs, the 6% LDL extender produced 50% mobile spermatozoa, compared with 48% with the Equex® extender and 27.7% with the extender containing egg yolk alone (EY). *In vitro* functional tests demonstrated that the integrity of the plasma membrane (hypoosmotic test) was respected in 65–66% of spermatozoa as a function of the extender; DNA integrity was respected in more than 97% of the spermatozoa. The Equex® extender provided superior acrosome integrity (FITC/PSA test): 68.4% compared with 55.1% with LDL and 53.3% with egg yolk. However, the 6% LDL extender resulted in fewer spermatozoal anomalies (Spermac® test), with 54.6% normal spermatozoa compared to 53.6% for Equex® and 53.3% with the egg yolk. All six of the bitches inseminated artificially via the intra-uterine route (Scandinavian technique) using semen frozen in the 6% LDL extender became pregnant. The LDL extender resulted in percentages of mobile spermatozoa and movement characteristics that were as good if not better than those obtained with the reference extenders following thawing. The 6% LDL extender appears to have the same cryoprotective qualities as the reference diluent, Equex® STAMP.

1. Introduction

Chicken egg yolk has long been the only cryoprotective substance used in canine semen extenders, but the complexity of its composition makes it difficult to produce identical results every time, it also contains substances that inhibit the respiration of spermatozoa thus reducing their mobility (Kampschmidt et al., 1953; Pace and Graham, 1974; Watson and Martin, 1975). It would therefore appear advantageous to replace it with the molecules that are responsible for the cryoprotective effect of egg yolk, i.e. the low density lipoproteins (LDL), and to study their effects on the survival of spermatozoa during the freeze – thaw process.

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Equex STM Paste® (Nova Chemical Sales, Scituate Inc., MA, USA) is the reference extender for canine semen. It contains a water-soluble anionic detergent, SDS (Sodium Dodecyl Sulfate), which gives good post-thaw fertility (Arriola and Foote, 1987; Penfold and Moore, 1993; Rota et al., 1997), increased membrane permeability, and reduces osmotic stress (Arriola and Foote, 1987). Furthermore, the protective effect of SDS on canine semen is greater when the spermatozoa are exposed to the detergent immediately prior to freezing rather than during the equilibration period (Pena and Linde-Forsberg, 2000), which suggests that SDS has a deleterious effect when contact is too long. This medium also requires the addition of 20% chicken egg yolk prior to freezing.

LDL, at a concentration of 6%, results in improved spermatozoal survival following the freezing and thawing of canine semen in comparison with chicken egg yolk alone (Bencharif et al., 2008); these results prompted the commercialisation of this product under the name of CANIFREEZE by IMV (IMV, Aigle, France).

The aim of this article was to compare the effects of extenders containing LDL, chicken egg yolk, and Equex on the survival and mobility of spermatozoa after freezing and thawing.

Functional *in vitro* tests will then be conducted on the frozen-thawed spermatozoa in the different extenders tested: FITC/PSA, HOS test, Acridine Orange; their *in vivo* efficacy will be assessed via artificial inseminations in bitches.

2. Materials and methods

2.1. Semen collection and processing

Twenty ejaculates were used from 5 different dogs aged from 2 to 7 years: 4 beagles from the Department of Reproductive Pathology, and 1 privately owned golden retriever. The owners of the dogs included in the study provided written consent. All experimental procedures were carried out in compliance with the ethical committee of the ONIRIS, National Veterinary School of Nantes.

The samples were taken every 2 days by the same operator, to eliminate any variations in semen quality resulting from the collection technique.

The dogs were sampled in the presence of a bitch, preferably in heat, to provide stimulation, and three people: the operator, an assistant to immobilise the bitch in front of the male, and another assistant to change the tubes for each of the different fractions of the ejaculate.

The semen was collected using a rubber artificial vagina, lubricated with glycerine, with a sterile glass tube attached to the end. The entire unit was stored in an oven at +37 °C until use.

The 3 fractions of the ejaculate in the dog were collected into 3 different plain tubes: the transparent urethral fraction, milky white spermatic fraction, and the slightly opaque to transparent prostatic fraction, and stored in a water bath at +37 °C.

Only the spermatic and prostatic fractions were assessed.

A total of 1.5 ml of spermatic phase was required for this test, due to the fact that 3 different extenders were being tested; when there was not enough, it was topped up with liquid from the prostatic phase. One drop of the mixture of the spermatic and prostatic fractions was placed on a slide and observed at low magnification on a microscope with a heated stage at +37 °C. The mobility was scored using the MILOVANOV scale from 0 to 5 (Fontbonne and Badinand, 1993; Bencharif et al., 2008).

Only ejaculates with a mass motility of 3 or more were frozen.

Another drop was placed between the slide and coverslip and observed at high magnification to assess the motility in comparison with all of the spermatozoa, which should be greater than or equal to 50%. It was attributed a score from 0 to 5 (0: spermatozoa are dead and 5: progressive motile spermatozoa). The ejaculates used in the experiment had a score of 3 or more.

The spermatozoa were counted using a Malassez cell.

An ejaculate should present a minimal concentration of around 300×10^6 spermatozoa per milliliter of the mixture of the spermatic and prostatic fractions. Below this concentration, it is considered to be unsuitable for freezing.

2.2. Preparation of the extenders

2.2.1. Extraction of LDL from chicken egg yolk

The technique used to extract the LDL is protected by a patent that was submitted jointly by the Veterinary School of Nantes and the INRA of Nantes.

2.2.2. Preparation of the extenders

Two extenders for freezing spermatozoa were prepared using a basic diluent with the addition of 20% egg yolk (control) or 6% LDL (Table 1); the third extender used was Equex® STAMP (Table 2).

2.3. Freezing

2.3.1. Dilution

One hundred microliters each of the egg yolk (EY) and LDL extenders were placed in different test tubes in a water bath at +37 °C, 200 μl of semen were then added to each of these tubes. To obtain a final concentration of 100×10^6 spermatozoa/ml, each of the extenders was added in a sufficient quantity, i.e. in a volume greater than

Table 1
Detailed composition of the Tris egg yolk and 6% LDL semen extenders.

	Tris (g)	Citric acid (g)	Fructose (g)	Penicillin–Streptomycin (IU/g)/100 ml	Distilled water (ml) QSP (100 ml)	EY (ml)	Glycerol (ml)	LDL (g)
EY	3.026	1.7	1.25	10^6–1	100	20	3.2	–
6% LDL	3.026	1.7	1.25	10^6–1	100	–	3.2	16.5