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.

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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.
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 prior to freezing. This medium also requires the addition of 20% chicken egg yolk. SDS has a deleterious effect when contact is too long. 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 spermatozoon). 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 × 10⁶ spermatozoa per milliliter of the mixture of the spermatic and prostatic fractions. Below this concentration, it is considered to be unsuitable for freezing.

### Table 1

<table>
<thead>
<tr>
<th>Tris (g)</th>
<th>Citric acid (g)</th>
<th>Fructose (g)</th>
<th>Penicillin–Streptomycin (IU/g)</th>
<th>Distilled water (ml)</th>
<th>EY (ml)</th>
<th>Glycerol (ml)</th>
<th>LDL (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EY</td>
<td>3.026</td>
<td>1.7</td>
<td>1.25</td>
<td>10⁶–1</td>
<td>100</td>
<td>20</td>
<td>3.2</td>
</tr>
<tr>
<td>6% LDL</td>
<td>3.026</td>
<td>1.7</td>
<td>1.25</td>
<td>10⁶–1</td>
<td>100</td>
<td>3.2</td>
<td>16.5</td>
</tr>
</tbody>
</table>

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).

### 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.

#### 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 × 10⁶ spermatozoa/ml, each of the extenders was added in a sufficient quantity, i.e. in a volume greater than...
Table 2
Composition of the 3 diluents of the Equex STAMP semen extender: diluent 1: pre-freezing dilution medium. Diluent 2: freezing medium containing glycerol. Thawing diluent: thawing medium without glycerol or Equex.

<table>
<thead>
<tr>
<th></th>
<th>Diluent 1</th>
<th>Diluent 2</th>
<th>Thawing diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.025 g</td>
<td>3.025 g</td>
<td>3.025 g</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.7 g</td>
<td>1.7 g</td>
<td>1.7 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.25 g</td>
<td>1.25 g</td>
<td>1.25 g</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06 g</td>
<td>0.06 g</td>
<td>0.06 g</td>
</tr>
<tr>
<td>DHS</td>
<td>0.1 g</td>
<td>0.1 g</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>77 ml</td>
<td>72 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3 ml</td>
<td>7 ml</td>
<td>–</td>
</tr>
<tr>
<td>Equex STM</td>
<td>–</td>
<td>1 ml</td>
<td>–</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>20 ml</td>
<td>20 ml</td>
<td>–</td>
</tr>
</tbody>
</table>

or equal to 100 μl, as a function of the concentration of the sample, to obtain a final volume of 400 μl or more.

Preparation of the samples with the Equex extender required additional steps: a first dilution with performed with diluent 1 to obtain a final concentration of 200 × 10^6 spz/ml.

2.3.2. Equilibration

The cryotubes were then maintained at a temperature of +4 °C for 1 h for the EY and 6% LDL extenders, and for 2 h for the Equex medium.

Two 0.25 ml straws were filled manually and the straws sealed with polyvinyl alcohol at +4 °C after 1 h with the EY and 6% LDL extenders, and after 2 h with the Equex extender.

The straws were placed horizontally on a metal ramp and maintained at +4 °C for 30 min with the EY and 6% LDL extenders.

With Equex®, after 2 h of refrigeration at +4 °C, the same volume of diluent 2 was added to diluent 1, giving a final concentration of 100 × 10^6 spz/ml; the straws were filled and frozen immediately thereafter.

2.3.3. Freezing process

After 1 h 30 min with EY and 6% LDL and after 2 h with Equex®, the canine semen was frozen in liquid nitrogen vapours (4 cm above the liquid nitrogen) at −110 °C for 10 min.

The straws were then immersed vertically in liquid nitrogen at −196 °C prior to storage in vats.

2.4. Semen thawing

The straws with the EY and 6% LDL extenders were immersed directly in water at +37 °C for 30 s, and then wiped with absorbent paper.

The ends were then cut to release the contents into a plain tube maintained at +37 °C.

The straws containing Equex® were thawed in a standard manner: the contents of the straws were collected into test tubes containing 250 μl of warmed Tris–EY diluent and maintained at +37 °C in a water bath.

Each batch of the various diluents (EY, Equex®, and 6% LDL) was analysed 10 min later.

2.5. Automated analysis of the thawed semen

The automated analysis of the semen was performed in the reproductive pathology laboratory of the department of in vitro fertilisation of the CHU of Nantes, France. A HAMILTON THORNE analyser was used with the CEROS 12 software program.

After thawing, 2 μl of semen were sampled using a micrometric pipette and placed in the chamber of a Leja® cell (IMV, Aigle, France).

To prevent thermal shock, the cells were first placed on a heated stage at +37 °C.

Their content was observed at a magnification of 200×. Five fields were chosen at random over the chamber and analysed at a time, a mean was then taken.

Twenty images are taken per second. The objects present in the cell are recognised as being spermatozoa as a function of their size and brightness.

The image analyser was used to study the following parameters during thawing:

### Motility: (as a percentage).

- VCL: curvilinear line velocity (expressed in μm/s).
- VSL: straight line velocity (expressed in μm/s).
- VAP: average path velocity (expressed in μm/s).
- ALH: lateral head displacement (amplitude of the lateral movements of the head expressed in μm).

2.6. Assessment of semen integrity

Following the motility study, the in vitro functional parameters of the semen were assessed as a function of the integrity of the plasma membrane, the acrosome, and the absence of significant anomalies of the spermatozoa. Artificial insemination tests in bitches were also performed with sperm frozen in the 6% LDL extender.

Ten ejaculates from 4 dogs, 3 beagles and one golden retriever, were frozen as described previously in the three media: egg yolk, Equex®, and 6% LDL.

Ten minutes following thawing, three analyses were performed to assess the integrity of

- The flagellar plasma membrane: HOS test.
- The acrosome: FITC/PSA test (acrosomal reaction).
- The DNA: Acridine Orange test (AO).
- The acrosome status: Spermac® test (acrosomal malfunction).

2.6.1. Assessment of the integrity of the flagellar plasma membrane: HOS test: hypoosmotic test

Twenty five microlitres of thawed semen were placed in an Eppendorf tube; 25 μl of a hypoosmotic solution (100 mOsmol/l) prepared with 75 mM of fructose and 25 mM of sodium tricarbonate mixed with distilled water, were then added.

After 60 min of incubation at +37 °C, 15 μl of the solution were placed between a slide and coverslip and observed using an optical microscope (Olympus CK2, ULWCD 0.30) at a magnification of 40× (100 spermatozoa are counted per slide).
This simple test is used to identify living spermatozoa that react to the hypooosmotic solution by swelling and curling of the flagella in comparison with dead spermatozoa, which remain unchanged.

2.6.2. Assessment of the integrity of the acrosome: FITC/PSA test

The integrity of the acrosome was analysed using the FITC–PSA test (\textit{Pisum sativum agglutinin}) in accordance with the technique described by Mendoza et al. (1992). Five microliters of thawed semen were placed on the extremity of a glass slide and spread with another slide to produce a smear. Following air-drying, the slides were immersed for 15 min in a solution of 100\% methanol. After drying, they were incubated with a solution of 200\,\mu l of FITC–PSA (50\,\mu g/ml) in PBS for 10 min at room temperature in a dry, humid chamber. The slides were then rinsed with distilled water to remove excess stain then immersed for 15 min in distilled water. After air-drying, they were stored at $+4\,^\circ\text{C}$ prior to observation with a fluorescence microscope (LEIKA DM-IRB).

The spermatozoa were classified as follows ($n=200$ spermatozoa/slide):

- \textbf{Spermatozoa +}: If the acrosome is fluorescent, the spermatozoon is considered as being alive.
- \textbf{Spermatozoa −}: If the acrosome is not fluorescent, the spermatozoon is considered as being dead.

2.6.3. Assessment of DNA integrity: Acridine Orange test

For this test, 150\,\mu l of semen were placed in a plain tube containing 4ml of PBS warmed in a water bath at $+37\,^\circ\text{C}$. The tubes were centrifuged at 500 $\times$ g for 10 min. Then 100\,\mu l of the pellet were sampled and re-suspended in 100\,\mu l of TNE solution with a pH of 7.2 (TNE = Tris–HCl 0.01 M, NaCl 0.15 M, EDTA 1 mM). Four hundred microliters of Triton solution were then added to the mixture. Thirty seconds later, 1.2 ml of Acridine Orange were added to the tube. After 5 min, a smear was made using 5\,\mu l of the final solution fixed between a slide and coverslip. The result is read as a function of the fluorescent emission of the spermatozoa. Green fluorescence signifies fixation to native DNA (double strand), and red fluorescence signifies fixation to denatured DNA (single stranded).

The rate of denaturation is assessed by comparing the proportion of spermatozoa that emit red fluorescence with the total population of spermatozoa (green + red fluorescence).

2.6.4. Assessment of the acrosome status: Spermac® test

2.6.4.1. Description of the reagents. The test requires the use of 3 reagents (A: red liquid, B: yellow liquid, C: green liquid), and a fixative (colourless).

2.6.4.2. Protocol. A smear is made using diluted sperm and dried for 5 min. The slide is immersed in the fixative for 5 min and dried for 1 h. It is then rinsed with 7 successive immersions in a beaker containing distilled water. The slide is then immersed for 2 min in solution A, rinsed, then immersed for 1 min in solution B and rinsed in a stream of distilled water; finally the slide is immersed in solution C before being rinsed again. It is then left to dry before being observed with the oil immersion microscope (100 $\times$ lens).

2.6.4.3. Interpretation. The various parts of the spermatozoa take up the stain differently, thus facilitating the detection of any anomalies of each part (Photo 1).

2.7. Fertility test

2.7.1. Follow-up of bitches

Six beagle bitches from the Department of Reproduction Pathology at the ONIRIS, Ecole Nationale Vétérinaire et Agroalimentaire de Nantes, aged from 5 to 9 years, were used for this study. They were housed in kennels, in pairs,
and all received the same feed and prophylactic treatment (vaccination, etc.).

2.7.2. Monitoring oestrus

Bitches coming into heat were monitored daily with vaginal smears stained using the Harris-Schorr technique (Boue et al., 2000; Aiudi et al., 2006; Bulterijs, 1953). When the vaginal smears indicated an eosinophilic index of 60–70%, a blood sample was taken into a heparinised tube. The progesterone was assayed semi-quantitatively (Ovulation-Test1, Seyne-Sur-Mer, France) following centrifugation and the following procedure applied:

- For a concentration of less than 2.5 ng/ml, a further blood test was taken 48 h later.
- For a concentration of between 2.5 and 8 ng/ml, the bitches were tested daily.
- For a concentration close to 8 ng/ml, a quantitative assay was performed daily until the concentration exceeded 8 ng/ml.

2.7.3. Artificial insemination (A.I.)

Bitches with a serum progesterone level greater than 8 ng/ml were considered as being in the ovulatory period. They were inseminated 48 h later, which corresponds to the duration of maturation of the oocytes. The bitches were inseminated via intra-uterine AI, using the Scandinavian technique, under sedation with Xylazine (ROMPUN N.D.V.) (Bayer Pharma Division Santé animale, Puteaux Cedex, France) at a dose of 0.2 mg/kg IV. The bitches then received an alpha-2-antagonist, Atipamezole (ANTISEDAN N.D.V.) (PFIZER Santé animale, Paris, France), at a dose of 0.4 mg/kg IM immediately after the artificial insemination, to neutralise the effect of the alpha-2-agonist. The procedure was repeated 24 h later with semen that had been frozen in a medium containing 6% LDL, with a total of 200 million spermatozoa (eight straws of 0.25 ml).

2.8. Statistical analysis

We used the ANOVA model with repeated data according to a linear model with mixed effects, analysed using the S-plus® statistics software program, to determine the influence of the various treatments on the motility at T0 + 10 min as well as the various motility parameters, these same tests were applied for the vital stains. However, although the ANOVA with repeated data did not demonstrate any overall significant effect, we thought it was interesting to compare the batches of the various extenders between themselves using a comparison of means test for unpaired samples.

Each value corresponds to the result “p of P (T ≤ t) unilateral”, if P < 0.05 then the difference is statistically significant.

3. Results

3.1. Motility

The motility was 49.9% with the 6% LDL extender and 47.9% with the Equex® extender. The egg yolk extender gave a motility of less than 30% (the threshold for the use of artificial insemination in a bitch) (Fig. 1).

There was a significant difference between the 6% LDL and Equex® extenders in comparison with the egg yolk extender; however, there was no statistically significant difference between the 6% LDL and Equex® extenders, but the values tended to be superior with the 6% LDL.

3.2. Study of the movement characteristics of the spermatozoa

The means of all of the parameters obtained for each extender after thawing are given in Table 3 and Fig. 2; the values have been rounded up or down to the nearest whole number wherever possible: the extender contain-

### Table 3

<table>
<thead>
<tr>
<th>Bitch</th>
<th>Age of the bitch (years)</th>
<th>AI method with frozen semen</th>
<th>Number of puppies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>2 AI 24 h apart</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>2 AI 24 h apart</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>2 AI 24 h apart</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>2 AI 24 h apart</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>2 AI 24 h apart</td>
<td>Artifically aborted at 30 days</td>
</tr>
<tr>
<td>F</td>
<td>7</td>
<td>2 AI 24 h apart</td>
<td>Artifically aborted at 30 days</td>
</tr>
</tbody>
</table>
ing 6% LDL gave the best results. For all of the motility parameters studied, there was no statistically significant difference between the EY, Equex®, and 6% LDL extenders, although the values tended to be superior with the 6% LDL, with the exception of the curvilinear velocity (VCL) and the lateral head displacement (ALH) where the 6% LDL produced results that were superior to the others, this latter difference being statistically significant.

3.3. Functional tests of the spermatozoa

3.3.1. Integrity of the flagellar plasma membrane: HOS test

The mean numbers of swollen spermatozoa (intact flagellar membranes) obtained were to the order of 66% for the LDL and Equex® and 65% for the EY (Fig. 3).

There is no statistically significant difference between the different extenders tested.

3.3.2. Integrity of the acrosome: FITC/PSA test

The mean numbers of green spermatozoa (i.e. those with an intact stained acrosome) obtained for each extender after thawing are given in Fig. 4.

Fig. 3. Percentage of spermatozoa with an intact flagellar membrane following thawing as a function of the different extenders using the HOS test. The HOS test was used to reveal the cytoplasmic membranes of spermatozoa that have resisted the freeze – thaw cycle in the 3 different extenders studied (egg yolk, Equex, and 6% LDL) (n = 10). The 6% LDL and Equex extenders gave superior results to those obtained with chicken egg yolk (p > 0.05).

Fig. 4. The FITC/PSA test was used to demonstrate the preservation of the fertilising power of the spermatozoa over the course of the freeze – thaw cycle in the 3 extenders used in the study. This test reveals spermatozoa with an intact acrosome. The Equex extender gave the best results (p < 0.05) (n = 10).
The Spermac® test involves the demonstration of various anomalies that the spermatozoa may suffer during the freeze–thaw cycle; 53.3%, 53.6%, and 54.6% of the spermatozoa were intact following freezing and thawing in the egg yolk, Equex, and 6% LDL extenders (P > 0.05) (n = 10).

The Equex® extender gave the best percentage of green spermatozoa, i.e. with an intact acrosome; the difference is statistically significant (p < 5%).

The EY and 6% LDL extenders gave similar values.

3.3.3. Integrity of the DNA: Acridine Orange test
The mean numbers of green spermatozoa (those with double-stranded DNA, i.e. intact) obtained for each extender after thawing are greater than 97% with all of the extenders (98.4% with EY, 97.5% with Equex, and 97.9% with the 6% LDL), the integrity of the DNA is therefore maintained.

There is no statistically significant difference between the different extenders tested.

3.3.4. Integrity of the acrosome status: Spermac® test
After thawing, 54.6% of the spermatozoa did not present any abnormalities with the 6% LDL extender, compared with 53.3% for the EY, and 53.6% with Equex®. The most common type of anomaly was the loss of the acrosome. There was no statistically significant difference between the 3 extenders tested (Fig. 5).

3.4. In vivo fertility test

Six bitches were inseminated with sperm that had been frozen in the medium containing 6% LDL, 48 and 72 h after they had attained a serum progesterone concentration of 8 ng or more; all of the bitches tested positive for gestation (Table 3).

4. Discussion

4.1. Motility

4.1.1. Motility and movement parameters of the spermatozoa
The results obtained during the motility study show that the percentage of mobile spermatozoa following the freeze–thaw process was higher with the 6% LDL than the other two extenders; this difference was statistically significant in comparison with the EY, however the difference was not statistically significant with Equex®, but the values tended to be better with the 6% LDL medium.

Furthermore, the majority of the motility parameters, notably the VCL and ALH, were significantly increased in comparison with the control extenders (egg yolk and Equex®). However, there was no statistically significant difference for the VSL and VAP, but the values tended to be better with the 6% LDL extender.

The various parameters studied also demonstrate that the 6% LDL extender improved the movement characteristics of the spermatozoa: an increase in the mean velocities was observed in comparison with the other extenders.

4.1.2. Comparison with Equex®
Equex® STM Paste (Nova Chemical Sales, Scituate Inc., MA, USA) is the reference extender for canine semen. Its use as an extender during freezing improves motility, post-thaw survival, acrosome integrity, and fertility rate in comparison with the chicken egg yolk extender alone (Arriola and Foote, 1987; Rota et al., 1997). Its principal active ingredient, SDS (sodium dodecyl sulfate), increases membrane permeability and therefore reduces osmotic shock (Arriola and Foote, 1987).

The results obtained in our study show that the Equex® extender produced 48% motile spermatozoa following thawing whilst the 6% LDL produced around 50%; this difference was not statistically significant, but the means tended to be better with the latter.

Furthermore, the movement characteristics of the spermatozoa were improved by the 6% LDL, with an increase in the mean velocities in comparison with the other extenders; this difference was statistically significant especially in comparison with the Equex® extender for the VCL and ALH.

The use of the Equex® extender also presents the disadvantage of the need for two successive dilutions (instead of one with the other two extenders) and the equilibration phase after the first dilution takes an hour longer than with the other two extenders. The thawing process requires the dilution of the frozen semen in a thawing diluent. The 6% LDL extender therefore gives results that are comparable to those of Equex® in terms of the percentage of mobile spermatozoa. However, it is quicker and easier to use.

The Equex medium is composed of 20% egg yolk, which results in the appearance of granules on the monitor during analysis with the HAMILTON THORNE image analyser, which could be interpreted as dead or living spermatozoa, thus falsifying the results. The 6% LDL extender does not contain any such granules, thus giving a perfectly clear medium.

4.2. Assessment of semen integrity

4.2.1. Assessment of the integrity of the flagellar plasma membrane, HOS test (hypoosmotic swelling test)
The hypoosmotic swelling test (HOS) was used to assess the integrity of the plasma membrane (PM). This test is used routinely in humans (Bernt et al., 1989). The principle
membrane protection that is as good as both the extenders the flagellar plasma membrane. The 6% LDL extender offers with frozen–thawed spermatozoa (Rota et al., 2000; Correa distilled water (100–150 mOsm/kg H₂O) gave good results demonstrated that a solution of fructose and sodium citrate in swelling without causing lysis. Preliminary studies demon-
strated that a solution of fructose and sodium citrate in (Rodriguez-Gil et al., 1994). The solution used for the test must be sufficiently hypoosmotic to enable cellular swelling without causing lysis. Preliminary studies demonstrated that a solution of fructose and sodium citrate in distilled water (100–150 mOsm/kg H₂O) gave good results with frozen–thawed spermatozoa (Rota et al., 2000; Correa and Zavos, 1994; Revell and Mrode, 1994). The results show that all 3 of the extenders used provided good protection for the flagellar plasma membrane. The 6% LDL extender offers membrane protection that is as good as both the extenders that are based on egg yolk, i.e. EY alone or Equex®.

4.2.2. Assessment of the integrity of the acrosome, FITC/PSA test

It has been proposed that egg yolk contains proges-
terone (Bowden et al., 2001); the latter is responsible for the capacitation of spermatozoa and is therefore prejudicial to the preservation of spermatozoa during freezing. This test is justified by the fact that mammalian spermatozoa are incapable of fertilising the oocyte immediately after ejaculation. It is the fixation of the spermatozoon to the zona pellucida that triggers the acrosome reaction, making it capable of fertilisation. Spermatozoa that spontaneously undergo an acrosome reaction after ejaculation or following freezing are incapable of fixing to the zona pellucida and are therefore unable to fertilise the oocyte (Yanagimachi, 1994).

The assessment of acrosomal integrity following freezing – thawing is therefore an important parameter providing a qualitative assessment of sperm fertility. In this study, the use of *Pisum sativum* agglutinin (PSA) marked with a fluorochrome, FITC, proved effective. The 6% LDL extender gave slightly better results than the EY, but this difference was not statistically significant. However, the Equex medium appeared to give better results, with a statistically significant difference.

The medium containing 6% LDL provided good protection of acrosome integrity, possibly via a direct action through the exchange or repair of acrosomal membrane phospholipids or possibly simply because the medium has a lower progesterone content than egg yolk due to the filtering effect of the dialysis membrane. The progesterone found in egg yolk plays a role in the capacitation of sper-
matoozoa in cattle (Witte and Schäfer-Somi, 2007), horses (Aitken and McLaughlin, 2007), and man (Meyers et al., 1995; Wistrom and Meizel, 1993); it appears to act via an extragenomic action on human spermatozoa, via the secondary activation of calcium channels leading to an increase in intracellular Ca²⁺, which may be responsible for the capacitation of spermatozoa (Aitken and McLaughlin, 2007). Such a mechanism has also been demonstrated in the dog (Witte and Schäfer-Somi, 2007).

4.2.3. Assessment of DNA integrity, Acridine Orange test

The integrity of the DNA or chromatin is an impor-
tant factor when assessing spermatozoa fertility. Since the DNA carries all of the genetic information, anomalies could cause infertility, abortion, and neonatal malfor-
mations. During spermatogenesis, the chromatin of the germinal cell nucleus changes its packaging characteris-
ts. Once the diploid cells have undergone meiosis, the transition proteins that replace the histones are replaced with arginine–rich protamines as described by Dadoune (1995). The process of nuclear condensation in sperma-
toza is incomplete in the majority of ejaculates (Battoov et al., 1980; Evenson and Melamed, 1983; Engh et al., 1992). Fluorescence microscopy was one of the very first methods used to assess the structure of the chromatin in canine spermatozoa (Dadoune, 1995; Battoov et al., 1980); it was used to detect histones with aniline blue (Haidl and Schilling, 1994). However, flow cytometry (faster and safer), is currently the most widely used technique in research laboratories, enabling the demonstration of DNA with propidium iodide (Pasteur et al., 1994; Molina et al., 1995), ethidium bromide (Engh et al., 1992), or even Acrid-
ine Orange (Rota et al., 2005).

For this study, Acridine Orange (AO) proved to be effective despite the use of a fluorescence microscope. The results obtained are very good, being greater than or equal to 98%; this suggests that LDL provide good protection for cellular DNA during the freeze – thaw process of canine semen.

The 6% LDL medium therefore has a good capacity to preserve canine semen in comparison with media contain-
ing egg yolk and notably Equex. This result was found in all of the analyses performed and concurs with research undertaken in cows or horses (Moussa et al., 2002; Amirat et al., 2004; Jian-Hong et al., 2010; Rota et al., 1997, 2000).

4.2.4. Assessment of the integrity of the acrosome status, Spermac® test

After thawing, 54.6% of the spermatozoa did not present any abnormalities with the 6% LDL extender compared with 53.3% for the EY, and 53.6% with Equex®. It would appear that the 6% LDL extender provides similar protection to the spermatozoa during the freeze – thaw process as the Equex® or EY extenders.

4.3. Fertility test

4.3.1. Pregnancy diagnosis

Pregnancy was confirmed in all of the six bitches that were inseminated. The use of an extender containing 6% LDL did not interfere with the fertility of the spermatozoa or with fertilisation.

5. Conclusion

The extender containing 6% LDL appears to have the same cryoprotective qualities as the diluent containing the LDL extender.
Equex. However, the LDL and Tris egg yolk extenders are easier to use in terms of time and handling during the freeze – thaw process in comparison with Equex. There are also fewer errors with the image analyser due to the elimination of granules during the extraction of LDL, unlike the Equex and Tris egg yolk extenders, which are composed of 20% chicken egg yolk.

The LDL based extender is currently commercialised under the name of CANIXL Freeze; unlike Equex, it does not require any preliminary preparation, thus eliminating the possibility of bacterial contamination that could compromise the quality of the freezing process.

The 6% LDL and Equex extenders gave superior results to the Tris egg yolk extender.

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References


