Refrigeration of canine sperm at + 4°C: Comparative study of four different extenders for the refrigeration of canine sperm at +4°C: LDL, Tris egg yolk, Equex®, and INRA96®

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SUMMARY

The aim of this study was to evaluate the effects of LDL-based extenders in comparison with other media used for the refrigeration of canine semen: Tris egg yolk (EY), Equex®, and INRA96®. The test extender was composed of 6% LDL. The percentage of mobile spermatozoa after 4 days storage in a domestic refrigerator at +4°C was 52.2%, 44.2%, 17.2%, and 24.5% for the 6% LDL, Tris Egg Yolk (20%), Equex®, and INRA 96® extenders respectively for 100% of the dogs. After 7 days of storage, these percentages fell to 33.6%, 26.4%, 5.5%, and 14.8 % in the same extenders for 50% of the dogs. In vitro fertility tests were performed with the two extenders that gave the best mobility results, i.e. 6% LDL and Tris egg yolk (20%). These tests were conducted on the day of sampling (D0), 48 hours, and 96 hours after sampling. The results of the HOS test were 81.2% and 85.8% for egg yolk and 6% LDL respectively at D0, 74.1% and 78.5% two days later (D2), and 71% and 76.1% at D4. The results of the FITC/PSA test were as follows: 70.2% and 84.8% on D0, 69.3% and 84.2% on D2, and 59.6% and 73.7% on D4 for the EY and 6% LDL respectively. The acridine orange test was positive; in nearly 100% of cases none of the spermatozoa had been denatured on D0, D2, and D4. The 6% LDL extender was capable of preserving spermatozoa that had been stored in a domestic refrigerator at +4°C for at least 4 days. This meant that the spermatozoa retained good cytoplasmic membrane integrity, had not capacitated, and contained intact DNA in comparison with the other extenders tested. The duration of storage is a very important consideration when faced with the problem of sending semen over ever-greater distances. Equex® and INRA 96® gave results that were 30% inferior; these extenders therefore seem ill-adapted for the refrigeration of canine sperm.

Keywords : Sperm, dog, refrigeration at +4C, mobility, in vitro fertility tests.

RESUME

Réfrigération du sperme canin à +4°C : Etude comparative de 4 milieux de réfrigération de la semence canine à +4°C : LDL, Jaune d'œuf, Equex® et INRA 96®

L'objectif de cette étude est d'étudier les effets de l'association LDL par comparaison avec d'autres milieux de réfrigération de la semence canine : Tris jaune d'œuf (JO), l'Equex® et l'INRA96®. Le milieu testé était à base de 6% de LDL. Le pourcentage des spermatozoïdes mobiles après 4 jours de conservation à + 4°C dans un réfrigérateur domestique, est de 52,2% vs 44,2% vs 17,2% vs 24,5% pour les dilueurs à base de 6% LDL, Tris jaune d'œuf (20%), Equex® et INRA 96® respectivement pour 100% des chiens. Alors que le taux des spermatozoïdes mobiles 7 jours après le prélèvement est de 33,6%, 26,4%, 5,5% et 14,8% dans les mêmes dilueurs pour 50% des chiens. Les tests de fertilités in vitro ont été réalisés sur deux milieux suite aux résultats de mobilités à savoir le dilueur à base de 6% LDL et le Tris jaune d'œuf à 20%. Ces tests ont été réalisés le jour du prélèvement (J0), 48 heures et 96 heures plus tard. Les résultats du test HOS sont de 81,2% et 85,8% pour le jaune d'œuf et le 6% LDL respectivement à J0, 74,1% et 78,5% deux jours après (J2) et 71% et 76,1% à J4. Pour le FITC/PSA, 70,2% vs 84,8% à J0, 69,3% vs 84,2% à J2 et 59,6% vs 73,7% pour le JO vs 6% LDL respectivement. Le test à l'acridine orange s'est révélé positif puisque tous les spermatozoïdes n'étaient pas dénaturés à J0, J2 et J4 pratiquement dans 100 % des cas. Le dilueur à base de 6% LDL est capable de préserver les spermatozoïdes conservés dans un réfrigérateur domestique à + 4°C, pendant au moins 4 jours ; se traduisant au niveau des spermatozoïdes par une bonne intégrité de la membrane cytoplasmique, l'absence d'activation acrosomique et la présence d'un ADN intact par rapport aux autres dilueurs testés. Cette durée de conservation est très importante à considérer pour palier au problème de l'envoi de semence sur des distances plus ou moins longues. L'Equex® et l'INRA96® obtiennent des résultats inférieurs à 30% et semble inadaptés pour la réfrigération de sperme canin.

Mots-clés : Sperme, chien, Réfrigération à +4°C, Mobilité, Tests de fertilité in vitro.

INTRODUCTION

The first successful use of chilled semen in dogs dates back to 1954 [10]. Its use has become increasingly widespread over the past 20 years, notably thanks to the development of dog breeding on an international level. It is easier to transport than frozen semen, which requires liquid nitrogen, and easy to inseminate (intravaginal vs. intrauterine insemination) [19].

Furthermore, the gestation rate is higher after artificial insemination using chilled semen than with frozen semen [18].

A certain number of phenomenon occur during the chilling process, some of which are not yet fully understood such as "cold shock"; the latter leads to a reduction in spermatozoa motility and metabolism and altered membrane permeability, which results in a disruption in cellular

exchanges [6, 18]. These changes cannot be reversed by warming the semen. Several parameters can be used to limit this phenomenon: extenders (some of which play a direct role as cell membrane protectants), andother factors such as storage temperature and the speed of refrigeration can help to improve the preservation of chilled semen.

Bouchard et *al.*, 1990, compared three storage temperatures (+35°C, +22°C, and +4°C), and demonstrated that the best results are obtained at +4°C [6]. However, the speed of chilling from +37°C to +4°C should not be too slow. Three refrigeration speeds were tested by Bouchard et *al.* 1990 [6], -1°C/min, -0.3°C/min, -0.1°C/min, the first two (fast and moderate) resulted in superior preservation of motility parameters.

For a long time, the standard semen extenders used in dogs were made with chicken egg yolk. However, egg yolk presents numerous disadvantages. It is associated with a health risk as egg yolk is an excellent microbial culture medium and can promote the onset of uterine infection following insemination of the bitch. Egg yolk also poses problems for analyses using a CERROS type image analyser or during biochemical assays [21]. Lastly, the components of egg yolk can vary as a function of the diet of the chicken, and are not necessarily all beneficial for spermatozoa; the granules are actually deleterious for the respiration and motility of spermatozoa [11, 16].

In this article, the authors explored the advantages of an extender made with purified LDL for the refrigeration of canine sperm for a minimum period of 4 days in comparison with a chicken egg yolk based extender, Equex® (canine semen extender), and INRA 96® (equine semen extender).

In-vitro motility tests were subsequently conducted to determine the most appropriate extender for chilling canine semen.

First experiment:

MATERIAL AND METHOD

ANIMALS

Sampling

During this experiment, 20 ejaculates were harvested from 7 dogs: one Doberman, 1 golden retriever, and 5 beagles, aged between 2 and 7 years. The 5 beagles belonged to the Department of Reproductive pathology at ONIRIS: The National Veterinary, Food agriculture, and food hygiene school of Loire Atlantique, Nantes, France, the Doberman and golden retriever belonged to breeders.

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 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 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.

Evaluation of sperm quality

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, because 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 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 [5, 9].

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

Another drop was placed between the slide and coverslip and observed at high magnification to assess 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.

Spermatozoal concentration

The spermatozoa were counted using a Malassez cell.

An ejaculate should present a minimal concentration of around 200×10^6 spermatozoa per millilitre of the mixture of spermatic and prostatic fractions. Below this concentration, it is considered to be unsuitable for cooling.

EXTENDERS

Extraction of LDL

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.

Two extenders for cooling spermatozoa were prepared using a basic diluent (Table 1) with the addition of 20% egg yolk (control) or 6% LDL; the other extenders used were Equex® STAMP, INRA 96®, INRA 96® + EY 20%, INRA 96® + LDL 6% (Table 2).

TRIS	3.025 g
Citrate	1.7 g
Glucose	1.25 g
Penicillin	0.06 g
DHS	0.1 g
Distilled water	77 ml

Table 1: Composition of the TRIS extender (basic extender)

Extenders (QSP 30 ml)	Basic extender (ml)	EY (ml)	Equex® (ml)	LDL (g)	INRA 96® (ml)
Egg yolk (EY)	24	6		-	-
Equex® STAMP	23.7	6	0.3		
6% LDL	25.5	-		4.95	-
INRA 96®	-	-		-	30
INRA 96® + 20% EY	-	6		-	24
INRA 96® + 6% LDL	-	-		4.95	25.5

Table 2: Composition of the various semen extenders for refrigeration.

Day	Survival extender	EY	Equex®	6% LDL	INRA 96®	INRA96® 6% LDL	INRA96® 20% EY	N° of dogs
0	82	85.4	87.9	89.9	82.4	71.8	60.1	20
1	-	72	55.3	74.3	56.1	41.3	34.1	20
2	-	61.9	34.8	64.2	42.7	36.3	28.8	20
3	-	53.5	22.7	58.5	32.9	25.6	23.1	20
4	-	**44.2**	**17.2**	**52.2**	**24.5**	**20.5**	**19**	**20**
5	-	37.6	13.3	46.5	18.6	9.8	11.1	15
6	-	31.4	9.5	37.1	14	4.2	9.5	14
7	-	**26.4**	**5.5**	**33.6**	**14.8**	**5.4**	**9.4**	**10**
8	-	22.3	7.4	27.5	17.4	4.1	13.9	8
9	-	18.8	1	16.1	21.8	2.2	8.1	3
10	-	16.2	0.6	10	17.9	1.9	8.4	3

Table 3: Percentage of mobile spermatozoa in chilled canine semen at +4°C as a function of the extender and time (in days).

Day	Survival extender	EY	Equex®	6% LDL	INRA 96®	INRA96® 6% LDL	INRA96® 20% EY	N° of dogs
0	42	46	49	55	41	36	35	20
1		26	3	24	18	11	10	20
2		19	1	16	12	6	11	20
3		13	0	10	7	5	6	20
4		10	0	8	6	3	5	20
5		34	0	4	4	1	2	15
6		12	0	2	2	0	1	14
7		1	0	2	2	0	1	10
8		1	0	1	3	0	1	8
9		5	0	0	5	1	1	3
10		4	0	0	4	1	2	3

Table 4: Percentage of progressively motile spermatozoa in the chilled semen as a function of the extender and the time.

REFRIGERATION

Dilution

100 µl of each extender studied were placed in separate test tubes maintained in a water bath at +37°C, then 200 µl of semen were added to each of the tubes, i.e. a total of 7 cryotubes.

To obtain a final concentration of 100 x 10^6 spermatozoa/ml, each of the extenders was added in sufficient quantities, i.e. more or less than 100 µl depending on the concentration of the sample, to obtain a final volume of 400 µl or more (Figure 1).

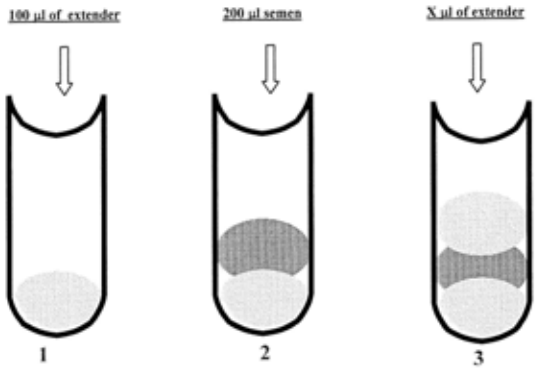

Figure 1 : Exemple of semen dilution with different media for obtained the final concentration 100 X 10^6 spz/ml.

Refrigeration

Once the concentrations had been adjusted, the different cryotubes were placed in a domestic refrigerator at +4°C.

Reheating

Every 24 hours over the 10 days of analysis, around 30µl were taken from each tube after the spermatozoa had been resuspended using a vortex. Each of the extenders was placed in a 2 ml test tube that was placed in a water bath at +37°C for 10 minutes.

AUTOMATED ANALYSIS OF THE CHILLED SEMEN

The sperm from each of the 20 dogs was analysis every day for the duration of the 10-day study, i.e. 11 analyses including D0: the day of sampling. Normally, analyses are discontinued once the concentration of mobile spermatozoa falls below 30%, which is the threshold for performing artificial insemination, however, in our study we continued the analyses until all of the spermatozoa had died or ceased to show progressive movement.

The automated semen analysis was performed in the department of Reproductive Pathology at the National Veterinary School of Nantes, France. A HAMILTON THORNE analyser was used, with the CEROS 12 software program.

We used a micrometric pipette to take 2 µl of semen, which were warmed to +37°C for 10 minutes, and placed in the chamber of Leja® cell (IMV, Aigle, France), the latter was then placed on a heated stage at +37°C.

Their content was observed at a magnification of x200.

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 following parameters were studied:

- Motility (as a percentage).
- % of progressive spermatozoa (as a percentage)
- VCL: curvilinear line velocity (expressed in m/s).
- VSL: straight-line velocity (expressed in m/s).
- VAP: average path velocity (expressed in m/s).
- ALH: lateral head displacement (amplitude of the lateral movements of the head expressed in µm).

STATISTICAL ANALYSES

The equality of variances on paired observations statistical test was applied. This enables the comparison of the results obtained for the 6% LDL extender versus the other extenders. The comparison was performed up to the 4th day post-collection, where the percentage of spermatozoa was greater than 30% in 100% of the animals sampled; from then on, the number of dogs decreased, making some of the results anomalous. We particularly studied the parameter p for P (T<=t), the difference was considered as significant when $p<0.05$.

To perform this statistical test, a function of Excel was used.

RESULTS

In this study, spermatozoal motility and velocity parameters were compared in six different extenders on the day of the sampling and daily until the tenth day after sampling (table 1). Statistical tests were conducted to determine whether the differences were significant for the results obtained up to the 4th day.

MOTILITY

This corresponds to the mean percentage of motile spermatozoa for the 20 dogs over the 10 days (Table 3). The extender containing 6% LDL preserved the motility of more than 30% of the spermatozoa up to the 7th day post-sampling.

Motility in the Equex® extender decreased rapidly over 48 hours. The INRA 96® extender alone or supplemented with LDL and egg yolk presented lower means than those of the extenders containing LDL alone. The difference in motility between the egg yolk extender and the LDL extenders was not deemed significant according to our statistical analysis; however, the LDL extender gave slightly superior results.

PERCENTAGE OF PROGRESSIVE SPERMATOZOA

The results obtained for progressive spermatozoa seem to confirm those obtained for motility. The results of the LDL and egg yolk extenders were similar. These two extenders gave better results than the others (table 4). The INRA96® extender gave better results when used alone than when supplemented with LDL and egg yolk. Finally, the Equex® extender gave the worst results.

MOVEMENT CHARACTERISTICS

The parameters for VAP, VSL, VCL, and ALH were studied.

The results for the VAP are shown in table 5. Up until D8 inclusive, the results obtained for the extenders containing 6% LDL were generally superior to the other extenders. Up until D4, the 6% LDL extender gave results that were statistically comparable to those with the egg yolk extender. Equex® proved once again to be the least effective for chilling. The extender made with LDL alone resulted in superior VAP than the INRA96® extender alone or supplemented with LDL/egg yolk.

The results for the VSL are displayed in table 6. The difference was significant throughout the experiment compared with Equex®, and only for the first three days in comparison with INRA96®; the difference was weak with the extenders composed of INRA96® supplemented with egg yolk and LDL. There was no significant difference with egg yolk and INRA 96® + 20% EY, which gave the best results.

The results for the VCL are given in table 7; the differences were always significant in comparison with the egg yolk, Equex®, and INRA96® extenders. The results of the 6% LDL extender were also better than those of the egg yolk extender.

Lateral head of displacement: ALH

The means obtained for this parameter are given in table 8; the differences were significant throughout the experiment in comparison with both the INRA 96® and Equex® extenders. They were overall significantly better for the other extenders, with the exception of the pure egg yolk extender on D3.

Second experiment

The results of the first experiment demonstrated the superiority of the 6% LDL extender for the chilling of canine semen over the other extenders. In this second experiment, we wanted to determine whether a motile spermatozoon retains its fertilising capacity after a few days of refrigeration.

In Vitro functional tests were thus undertaken: HOS, FITC/PSA, and Acridine Orange (AO).

MATERIAL AND METHOD

PREPARATION OF THE REFRIGERATED SEMEN

Sperm collection

The animals

During this experiment, 10 ejaculates were collected from 4 beagles, aged between 2 and 7 years. They belonged to the ONIRIS department of Reproductive Pathology: National Veterinary, Agrofood, and Food Hygiene School of Loire Atlantique, Nantes, France.

The samples were collected as described previously.

Only the spermatic and prostatic fractions were assessed.

Mass and individual motility were assessed as described in the previous experiments.

PREPARATION OF THE EXTENDERS

Following the motility results obtained in the previous experiment, two extenders were tested: Egg yolk extender (EY) and the 6% LDL (Table 9).

Refrigeration

Dilution

After collection, the spermatic and prostatic phases were mixed to obtain a final volume of at least 900 µl of sperm; the mixture was placed in a water bath and divided into two cryotubes of the same volume, containing 200 µl of each extender to be tested.

After counting in a Malassez cell and the determination of spermatozoal concentration, we added the required volume of the various extenders to obtain a final concentration of 100 x 10^6 spermatozoa per ml. Each cryotube was attributed a different extender.

Refrigeration

Once the concentrations had been adjusted, the different cryotubes were placed in a domestic refrigerator at +4°C.

Reheating

On each day of the analysis, the spermatozoa in each of the 2 extenders were re-suspended after agitation in a vortex for a few seconds; 20 µl of the extenders were sampled and placed in a 2 ml test tube, and heated for 10 min in a water bath at +37°C.

Day	Survival extender	EY	Equex®	6% LDL	INRA 96®	INRA 96® + 6% LDL	INRA 96® + +20% EY	N° of dogs
0	121	111	112	131	109	101	120	20
1	-	106	66	107	86	82	94	20
2	-	98	57	98	75	75	90	20
3	-	89	52	89	72	65	70	**20**
4	-	80	48	82	54	60	68	20
5	-	**66**	**44**	**68**	**39**	**48**	**50**	**15**
6	-	**63**	**35**	**56**	**45**	**45**	**47**	**14**
7	-	54	41	59	37	39	51	10
8	-	**55**	28	56	45	37	39	8
9	-	63	18	37	66	43	44	3
10	-	48	12	35	46	42	45	3

Table 5: Smoothed path velocity (VAP) (µm/s) of spermatozoa stored at +4°C as a function of the extender and time

Day	Survival extender	EY	Equex®	6% LDL	INRA 96®	INRA96® 6% LDL	INRA96® 20% EY	N° of dogs
0	**103**	**101**	**98**	**116**	100	97	116	20
1	-	85	47	83	75	74	85	20
2	-	78	42	75	65	67	**84**	**20**
3	-	**70**	**39**	**67**	**64**	**59**	**64**	**20**
4	-	62	36	61	49	54	61	20
5	-	**50**	**35**	**49**	**35**	**42**	**44**	**15**
6	-	**49**	**29**	**41**	**40**	**39**	**42**	**14**
7	-	41	33	43	32	34	45	10
8	-	**41**	**23**	**42**	**40**	**32**	**35**	**8**
9	-	**50**	**16**	29	59	39	39	3
10	-	36	11	29	41	38	39	3

Table 6: Straight line velocity (VSL) (µm/s) of spermatozoa stored at +4°C as a function of the extender and time.

Day	Survival extender	EY	Equex®	6% LDL	INRA96®	INRA96® 6% LDL	INRA96® 20% EY	N° of dogs
0	**188**	**137**	**155**	**179**	**138**	**116**	**140**	**20**
1	-	**175**	**127**	**191**	**132**	**113**	131	20
2	-	163	104	179	115	107	126	20
3	-	155	88	165	105	94	100	20
4	-	141	76	156	75	87	98	20
5	-	**121**	**62**	**133**	**56**	**74**	**74**	**15**
6	-	**115**	**47**	**111**	**64**	**75**	**69**	**14**
7	-	101	56	118	60	62	77	10
8	-	**103**	**35**	100	71	55	59	8
9	-	109	24	72	91	64	68	3
10	-	87	15	58	71	62	65	3

Table 7: Track velocity (VCL) (µm/s) of spermatozoa stored at +4°C as a function of the extender and time.

Day	Survival extender	EY	Equex*	6% LDL	INRA 96®	INRA 96® + 6% LDL	INRA 96® + 20% EY	N° of dogs
0	**9**	**6**	**7**	**7**	**5**	**4**	**4**	20
1	-	8	8	9	7	6	6	20
2	-	8	7	9	7	6	5	20
3	-	**8**	**7**	**9**	**6**	**5**	**4**	**20**
4	-	8	6	9	4	4	5	20
5	-	**7**	**6**	**9**	**3**	**3**	**3**	**15**
6	-	**8**	**2**	**8**	**3**	**2**	**2**	**14**
7	-	8	3	9	3	3	2	10
8	-	**8**	**3**	**9**	**3**	**1**	**2**	**8**
9	-	**6**	**0**	**6**	**3**	**3**	5	3
10	-	5	0	5	4	3	4	3

Table 8: Amplitude of lateral head displacement (ALH) (µm) of spermatozoa stored at +4°C as a function of the extender and time.

Extenders (QSP 30 ml)	Basic extender (ml)	EY (ml)	LDL (g)
Egg yolk (EY)	24	6	-
6% LDL	25.5	-	4.95

Table 9: Composition of the various semen extenders for refrigeration.

STUDY OF SPERMATOZOAL MEMBRANE INTEGRITY

Due to the low volume of the spermatic phase in the dog and the fact that the chilled semen must survive for a minimum of 48 hours and if possible 4 days in order to be used for artificial insemination, we tested the extenders on the day of sampling (D0), 48 hours later (D2), and 96 hours (D4) after chilling.

After re-suspending the spermatozoa in the vortex, 200 µl of the semen were sampled and placed in a test tube, which was placed in a water bath at +37°C for 10 minutes, three tests were conducted to assess the integrity of:

- The flagellar plasma membrane: **HOS test**
- The acrosome: **FITC/PSA test**
- The DNA: **Acridine Orange test (AO)**

Assessment of flagellar plasma membrane integrity

HOS test: Hypo-osmotic

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

After 60 minutes incubation at 37°C, 15µl of the solution are placed between a slide and coverslip and observed using an optical microscope (Olympus CK2, ULWCD 0.30) at a magnification of x40 (100 spermatozoa are counted per slide).

This simple test is used to identify living spermatozoa whose flagella react to the hypooosmotic solution by swelling and curling, whilst any dead spermatozoa remain unchanged.

Assessment of acrosome integrity

FITC/PSA test

The integrity of the acrosome was analysed using the FITC-PSA test (pisum sativum agglutinin) in accordance with the technique described by Mendoza et *al.,* (1992) [13]. Five microlitres of thawed semen were placed on the extremity of a glass slide and then spread with another slide to produce a smear. Following air-drying, the slides were immersed for 15 minutes in a solution of 100% methanol. After drying, they were incubated with a solution of 200 µl of FITC-PSA (50 µg/ml) in PBS for 10 minutes at room temperature in a dark, humid chamber. The slides were then rinsed with distilled water to remove excess stain and immersed for 15 minutes in distilled water. After air-drying, they were stored at +4°C prior to observation with a fluorescence microscope (LEIKA DM-IRB).

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

* **Spermatozoa +:** If the acrosome is fluorescent, the spermatozoon is considered alive.

* **Spermatozoa -:** If the acrosome is not fluorescent, the spermatozoon is considered dead.

Assessment of DNA integrity

Acridine Orange test

For this test, 150 µl of semen were placed in a plain tube containing 4 ml of PBS warmed in a water bath at 37°C. The tubes were centrifuged at 500 g for 10 minutes. Then 100 µl of the pellet were sampled and re-suspended in 100 µl of TNE solution with a pH of 7.2 (TNE = Tris HCl 0.01 M, NaCl 0.15 M, EDTA 1 mM). Four hundred microlitres 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 minutes, a smear was made using 5 µl 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 in comparison with the proportion of spermatozoa that emit red fluorescence and the total population of spermatozoa (green + red fluorescence).

STATISTICAL TEST

The equality of variances statistical test was applied: paired observations. The latter enables a comparison extender per extender. It is therefore possible to determine whether there was a significant difference between the post-chilling motilities of the different extenders.

If $p<0.05$, the difference is statistically significant.

This test is a function of the Microsoft Excel software program.

RESULTS

HYPOOSMOTIC TEST

More than 70% of the spermatozoa were alive 48 and 96 hours after collection in the various extenders tested (Figure 2).

The best results were obtained with the 6% LDL extender.

There was a statistically significant difference with the Tris egg yolk (20%) extender ($p<0.05$).

FITC-PSA

Spermatozoa with a fluorescent acrosome were considered as being operational, and therefore alive, the others were dead (Figure 3).

The 6% LDL extenders gave the best results.

There again, the difference between the 6% LDL and the 20% egg yolk extenders was statistically significant ($p<0.005$).

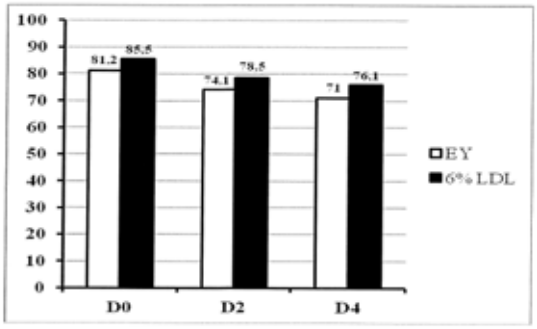

Figure 2: Percentage of spermatozoa with an intact flagellar membrane counted during the hypoosmotic test for each extender at D0, D2, and D4.

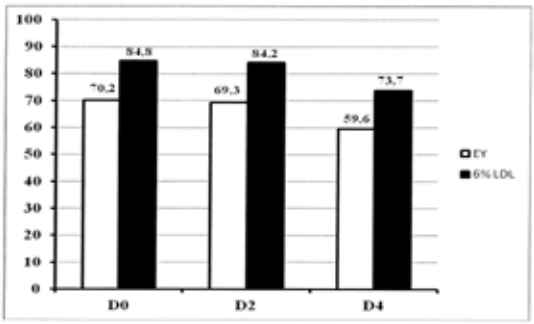

Figure 3: Percentage of spermatozoa with a fluorescent acrosome after treatment with FITC-PSA for each extender at D0, D2, and D4.

ACRIDINE ORANGE TEST

This is used to reveal damage to the cellular DNA. The results are given in Table 10.The results of the various extenders were very close to 100% over the 4 days of the experiment; the results for each dog were comprised between 99.7% and 100%, and there was no statistically significant difference between the two extenders tested ($p<0.005$).

	D0	D2	D4
EY	**100**	**99.8**	**99.7**
LDL	**99.7**	**99.7**	**99.7**

Table 10: Percentage of positive spermatozoa (native DNA) counted during the acridine orange test for each extender at D0, D2, and D4.

DISCUSSION

MOTILITY

Comparison with Equex®

Equex® STM Paste (Nova Chemical Sales, Scituate Inc., MA, USA) is commonly used for freezing canine sperm with excellent results. However, this extender is not polyvalent; it is not adapted to chilling spermatozoa at +4°C. Spermatozoal survival is limited to 48 hours, compared to 4 days or even 7 days for half of the samples chilled using the 6% LDL extender. There is therefore no safety margin. The percentage of progressive spermatozoa falls below 1% after the second day after sampling. The remainder of the parameters is for the most part always inferior to those of the other extenders. In 2000, Pena and Linde demonstrated poor spermatozoal preservation when the semen is brought into contact with Equex® some time prior to freezing rather than immediately before, which suggests that this extender has a negative effect on unfrozen semen [17]. Equex® is not suitable for storing semen at +4°C.

Comparison with the INRA 96® extender

This extender is widely used for chilling stallion semen. It contains the protective fraction found in milk (equivalent to LDL in egg yolk), NPPC (Native Phosphocaseinate). This fraction has been shown to improve spermatozoal survival during storage [2]. This extender gives good results for storing equine sperm at +15°C and +4°C [3]. A study demonstrated that the storage of caprine semen at +4°C with INRA 96® gives good results up to 7 days after sampling [12]. We therefore decided to test this extender for canine semen.

The results for the 6% LDL extender were superior to those of INRA 96®. For example, the percentage of motile spermatozoa remained above 30% until the third day, whilst this parameter remained stable up to the seventh day for the 6% LDL extender. On the last two days, the means increased for INRA 96®, this is explained by the fact that only three dogs were left in the study at this stage. These were the dogs of the largest breeds (notably the Doberman and golden retriever), who had very good sperm analysis results on the day of sampling. It would appear that for high quality sperm, and above all, sperm that is free from urethral and prostatic fluids, the INRA 96® extender enables good preservation of chilled semen, which last longer than for sperm of mediocre quality. Its efficacy therefore seems to vary as a function of the dog and the initial quality of the sperm.

Comparison with LDL

LDL, which are extracted from egg yolk, gave better results than unrefined egg yolk. This confirms the results of previous studies [15, 22], in which the authors demonstrated the existence of elements that antagonise the protective action of egg yolk. The LDL were then isolated as the protective fraction [22]. Subsequently, they were tested for the cryopreservation of semen in various species: bulls [16], Baudet de Poitou donkeys [20], and dogs [4].

The control extender was composed of 20% egg yolk, which itself contains 50% dry matter, of which 6.6%are LDL, i.e. a concentration that is very similar to that used in our 6% LDL extender, which gave superior results. Egg yolk therefore contains elements that antagonise the cryoprotective action of LDL [11, 15, 16, 22]. In 1974, Pace and Graham [16], demonstrated that it is the granules in the egg yolk that have a deleterious effect on spermatozoal motility after freezing and thawing. This was also confirmed in 1996 by Demianowicz and Strezek, on the spermatozoa of boars [7].

FUNCTIONAL TESTS

Membrane integrity

The percentage of live sperm remains greater than 70% for the two extenders on D4. With frozen canine semen, these same extenders were below 70% [4]. Chilling has a less deleterious effect on spermatozoal plasma membranes than freezing.

LDL protect the plasma membranes better than egg yolk; one hypothesis suggests that the lipoproteins in egg yolk interact with the seminal plasma proteins responsible for modifying membrane lipids, thus protecting the membranes.

Integrity of the acrosome

The acrosome integrity is determined by FITC-PSA staining; PSA (Pisum Sativum Agglutinin) is a lectin bound to a fluorescent molecule, FITC, which binds to components of the acrosome. When the fluorescence is distributed all around the acrosome, the latter is considered to be intact. The absence of fluorescence or fluorescence that is restricted to the equatorial region signifies that a spontaneous acrosome reaction has occurred. The acrosome reaction is an important step in fecundation; an intact acrosome is therefore an important component of fertility.

The results were better than in the study on frozen semen [4]: with the 6% LDL extender, over 70% of the spermatozoa had an intact acrosome at D4. Chilling therefore induces fewer acrosome reactions than freezing.

LDL induce fewer acrosome reactions than egg yolk. The extender containing 6% LDL respected the integrity of the acrosome better, maybe through direct protection via exchange or repair of phospholipids from the acrosome membrane, or simply because the extender has a lower progesterone content than egg yolk due to the elimination of progesterone by the pores of the dialysis membrane during the preparation of LDL. The progesterone found in egg yolk plays a role in the capacitation of spermatozoa in cattle [24],

horses [14], and man [1, 23]; it acts via an extragenomic action on human spermatozoa, via secondary activation of calcium channels, leading to an increase in the Ca^{2+} concentration inside the cell, which leads to capacitation of the spermatozoa [1]. Such a mechanism has also been demonstrated in the dog [24].

DNA integrity

The Acridine Orange (AO) test is used to demonstrate structural modifications such as DNA denaturation, which may occur in the cell nuclei.

This staining technique differentiates between cells with intact DNA (green stain) and cells with denatured DNA (orange).

The DNA was unaltered by the refrigeration process irrespective of the extender used (99.7% to 100% of the DNA was intact). Likewise, during freezing, very little denaturation is seen [4, 8].

CONCLUSION

The 6% LDL extender is capable of preserving chilled spermatozoa in a domestic refrigerator at +4°C for 7 days for half of the samples and 4 days for all of the dogs in the study; the mean motility of the semen remained superior to 30%.

The duration of storage is an important factor when considering the transportation of semen over ever-greater distances; given that the majority of transporters require at least 24 hours to deliver the semen and non-working days need to be taken into account.

The 6% LDL extender was capable of preserving spermatozoa that had been stored in a domestic refrigerator at +4°C for at least 4 days. This meant that the spermatozoa retained good cytoplasmic membrane integrity, had not capacitated, and contained intact DNA in comparison with the other extenders tested.

REFERENCES

1. AITKEN R.J., MCLAUGHLIN E.A. : Molecular mechanisms of sperm capacitation: progesterone-induced secondary calcium oscillations reflect the attainment of a capacitated state. *Soc Reprod Fertil Suppl.*, 2007, **63**, 273-293.
2. BATELLIER F., VIDAMENT M., FAUQUANT J., DUCHAMP G., ARNAUD G., YVON J.M., MAGISTRINI M. : Advances in cooled semen technology. *Anim. Reprod. Sci.*, 2001, **68,** 181-190.
3. BATELLIER F., DUCHAMP G., VIDAMENT M., ARNAUD G., PALMER E., MAGISTRINI M. : Delayed insemination is successful with new extender for storing fresh equine semen at 15°C under aerobic conditions. *Theriogenology.*, 1998, **50**, 229-236.
4. BENCHARIF D., AMIRAT L., PASCAL O., ANTON M., SCHMITT E., DESHERCES S., DELHOMME G., LANGLOIS M. L., BARRIERE P., LARRAT M., TAINTURIER D. : The advantages of combining LDL (Low Density Lipoproteins) with glutamine for the cryopreservation of canine semen. *Reprod. Dom. Anim.,* 2010, **45**, 189–200.
5. BENCHARIF D., AMIRAT L., ANTON M., SCHMITT E., DESHERCES S., DELHOMME G., LANGLOIS M. L., BARRIERE P., LARRAT M., TAINTURIER D. : The advantages of LDL (Low Density Lipoproteins) in the cryopreservation of canine semen. *Theriogenology.*, 2008, **70**, 1478–1488.
6. BOUCHARD G.F., MORRIS J.K., SIKES J.D., YOUNGQUIST R.S. : Effect of storage temperature, cooling rate and two different semen extenders on canine spermatozoal motility. *Theriogenology.*, 1990, **34**, 147-157.
7. DEMIANOWICZ W., STREZEK J. : The effect of lipoprotein fraction of egg yolk on some of the biological properties of boar spermatozoa during storage of the semen in liquid state. *Reprod. Dom. Anim.*, 1996, **31**, 279-280.
8. ENCISO M., LOPEZ-FERNANDEZ C., FERNANDEZ J.L., GARCIA P., GOSALBEZ A., GOSALBEZ J. : A new method to analyse boar sperm DNA fragmentation under bright-field or fluorescence microscopy. *Theriogenology.*, 2006, **65**, 308-316.
9. FONTBONNE A., BADINAND F. : Studies on freezing dog spermatozoa : effect of glycerol on mobility after thawing. *J. Reprod. Fert.*, Supplement., 1993, **47**, 531-532.
10. HARROP A.E. : Artificial insemination of a bitch with preserved semen. *Br. Vet. J.*, 1954, **110**, 424-425.
11. KAMPSHMIDT R.F., MAYER D.T., HERMAN H.A. : Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. *J. Dairy. Sci.*, 1953, **36**, 733-742.
12. LEBOEUF B., GUILLOUET P., BATELLIER F., BERNELAS D., BONNÉ J.L., FORGERIT Y., RENAUD G., MAGISTRINI M. : Effect of native phosphocaseinate on the in vitro preservation of fresh semen. *Theriogenology.*, 2003, **60**, 867-77.
13. MENDOZA C., CARRERAS A., MOOS J., TESARIK J. : Distinction between true acrosome reaction and degenerative acrosome loss by a onestep staining method using pisum sativum agglutinin. *J. Reprod. Fert.*, 1992, **95**, 755-763.
14. MEYERS S.A., OVERSTREET J.W., LIU I.K.M., DROBNIS E.Z. : Capacitation *In Vitro* of Stallion Spermatozoa: Comparison of Progesterone-Induced Acrosome Reactions in Fertile and Subfertile Males. *J. Androl.*, 1995, **16**, 47-54.
15. MOUSSA M., MARTINET V., TRIMÈCHE A., TAINTURIER D., ANTON M. : Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull semen. *Theriogenology.*, 2002, **57**, 1695–1706.

16. PACE M.M., GRAHAM E.F. : Components in egg yolk which protect bovine spermatozoa during freezing. *J. Anim. Sci.*,1974, **39**, 1144–1149.
17. PENA A.I., LINDE-FORSBERG C. : Effects of Equex, one or two Step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. *Theriogenology.*, 2000, **54**, 859-875.
18. ROTA A.B., STROM C., LINDE-FORSBERG C., RODRIGUEZ-MARTINEZ H. : Effects of Equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38°C. *Theriogenology.*, 1997, **47**, 1093-1101.
19. ROTA A., SROM B., LINDE-FORSBERG C. : Effects of seminal plasma and three extenders on canine semen stored at 4 °C. *Theriogenology.*, 1995, **44**, 885-900.
20. TRIMECHE A., RENARD P., LE LANNOU D., BARRIERE P., TAINTURIER D. : Improvement of motility of post-thaw Poitou Jackass sperm using glutamine. *Theriogenology.*, 1996, **45**, 1015-1027.
21. WALL R.J., FOOTE R.H. : Fertility of bull semen frozen and store in clarified egg yolk-Tris-glycerol extender. *J. Dairy. Sci.*, 1999, **82**, 817–821.
22. WATSON P.F., MARTIN C.A. : The influence of some fractions of egg yolk on the survival of ram spermatozoa at 5°C. Aust. *J. Biol. Sci.*, 1975, **28**, 145-152.
23. WISTROM C.A., MEIZEL S. : Evidence suggesting involvement of a unique human sperm steroid receptor/Cl[-] channel complex in the progesterone-initiated acrosome reaction. *Dev. biol.*, 1993, **159**, 679-690.
24. WITTE T.S., SCHÄFER-SOMI S. : Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Anim. Reprod. Sci.*, 2007, **102**, 181-193.