10.22059/IJVM.2021.329603.1005191

Iranian Journal of Veterinary Medicine
Volume 16- Issue 2

Original Article
Online ISSN: 2252-0554

Evaluation of Soy Lecithin Efficacy in Comparison with Egg Yolk on Freezing of Epididymal Sperm in Dogs

Ahmad Yousef[1], Hamid Ghasemzadeh-Nava[1*], Parviz Tajik[1], Vahid Akbarinejad[1], Armin Towhidi[2]

1. *Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
2. *Department of Animal Science, College of Agriculture and Natural Resource, University of Tehran, Karaj, Iran*

Abstract

BACKGROUND: Epididymal sperm collection is allowed by using genetic material post-mortem or after orchiectomy from high-value animals or endangered species.

OBJECTIVES: The aim of this study was to improve the accessibility of the dog epididymal sperm cryopreservation system based on the appropriate dose of lecithin.

METHODS: Epididymal sperm from castrated testes of mature healthy dogs in veterinary centers were collected and divided into six groups: G_1: egg yolk 20% (control), G_2: lecithin 0.4% (L0.4), G_3: lecithin 0.8% (L0.8), G_4: lecithin 1.2% (L1.2), G_5: lecithin 1.6% (L1.6), and G_6: lecithin 2% (L2). Evaluation of Spermatozoa was done before freezing by Motility test, Eosin- Nigrosin vital staining and Hypo-Osmotic Swelling Test (HOST) and after thawing by Computer Assisted Semen Analysis (CASA), HOST, Eosin-Nigrosin vital staining, Mitochondrial Membrane Potential (MMP) and Intracellular Reactive Oxygen Species (ROS).

RESULTS: In frozen samples, total motility and proportion of sperm with intact plasma membrane integrity based on the HOS test were lesser in all groups treated with different concentrations of lecithin than in the control group ($P \leq 0.05$). However, beat cross frequency (BCF) was higher in all groups treated with different concentrations of lecithin as compared with the control group ($P \leq 0.05$). Yet progressive motility, the proportion of live sperm based on the Eosin- Nigrosin test, VAP, VSL, VCL, STR, LIN, and ALH did not differ among various experimental groups ($P > 0.05$). The proportion of sperm with morphological defects did not differ between fresh and frozen samples and among various experimental groups ($P > 0.05$). Mitochondrial membrane potential was greater in the control group than 0.4% lecithin group ($P = 0.026$). The proportion of sperm positive for ROS was lesser in the control group than 0.4% lecithin group ($P = 0.049$).

CONCLUSIONS: Egg yolk was superior to the lecithin-based extenders to cryopreserve epididymal sperm of dogs.

KEYWORDS: Cryopreservation of epididymal sperm, Dog, Lecithin, Mitochondrial membrane potential, Reactive oxygen spices

Correspondence

Hamid Ghasemzadeh-nava, Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran. Tel: +98 (021) 66923510, Fax: +98 (021) 6693222, Email: hghasem@ut.ac.ir

Received: 2021-08-02

Accepted: 2021-09-28

How to Cite This Article

Yousef, A, Ghasemzadeh-Nava, H, Tajik, P, Akbarinejad V, Towhidi, A. (2022). Evaluation of Soy Lecithin Efficacy in Comparison with Egg Yolk on Freezing of Epididymal Sperm in Dogs. *Iranian Journal of Veterinary Medicine, 16*(2), 166-177.

Introduction

High economic value, zootechnical, and affective character of some individuals increase the advances of reproductive biotechnologies for future preservation (Thomassen & Farstad 2009). In the case of azoospermia or when a donor male accidentally dies or undergoes orchiectomy, the retrieval of epididymal spermatozoa opens new possibilities to generate progeny. Spermatozoa can be collected by different techniques from ex vivo or in vivo testicles and cryopreserved for future use (Luvoni & Morselli, 2017).

Collecting sperm from the epididymis allows the use of genetic material post-mortem or after orchiectomy from high-value animals or endangered species (Ortiz *et al.*, 2017), and there are several situations in which epididymal sperm for artificial insemination may be used. The most obvious reason for AI is the perceived inability of the male and female to breed (for example: weakness, arthritis, back pain, premature ejaculation, etc.). For many years Egg yolk was widely used as a cryoprotectant in dog semen extenders. Still, there are some concerns and risks with the use of egg yolk, including the risk of bacterial contamination and the potential risk of causing disease (Hermansson, Johannisson & Axnér, 2021). Soy lecithin is a promising option for egg yolk substitutes due to ease of component standardization, availability, and a reduced potential risk of contamination. It has a similar composition (i.e., low-density lipoprotein) as egg yolk and may provide protection to the sperm plasma membrane during cold shock (Dalmazzo *et al.,* 2018). Some of the studies have shown the effect of soy lecithin as a suitable alternative to egg yolk on ejaculated sperm in dogs (Beccaglia, Anastasi & Luvoni, 2009[a]; Beccaglia *et al.* 2009[b]; Kmenta *et al.*, 2011; Kasimanickam *et al.*, 2012), and so far, its effect on epididymal semen in dogs has not been studied. The aim of the present study was to compare five different concentrations of soya bean lecithin, with egg yolk as a control, in tris extender for cryopreservation epididymal sperm of dogs.

Materials and Methods

Soybean lecithin (L-a-phosphatidylcholine (product number: P3644)) in this study was prepared from Sigma (St. Louis, MO, USA), and other chemicals were purchased from Merck (Darmstadt, Germany). Straws used were from IMV Co. (France).

Animals

Ten intact male dogs aged between 1 - 8 years old, with different breeds (Golden Retriever, Pomeranian, Terrier, Shitzu) and body weights (between 3 kg and 25 kg were used for this study. The dog testes were temporarily preserved at 4°C in a plastic can filled with a physiological solution (0.9% saline solution) supplemented with gentamycin at a concentration of 10mg/mL, a physiological solution. The testes were processed within 2 hours of castration.

Sample Collection

Sperm samples were collected by repeated incision of the epididymal tail and proximal vas deferens in extruded (tris-based) medium without glycerol at 37°C. Sperm motility (total and progressive) was assessed by microscopic examination. Briefly, 5 µL of each sample was deposited on microscope slides previously warmed at 37°C and covered by coverslips, after that Eosin-Nigrosin staining and HOST (Hypo Osmotic Swelling Test) were performed. Sperm concentration was assessed using a standard counting chamber (Neubauer Lam); the final concentration of sperm was (50×10^{6} /mL). The extenders (Tris-egg yolk and tris-lecithin) with glycerol were prepared and put in the fridge at 4°C. The extender tris-lecithin with glycerol was vortexed for 30 minutes. Sperm extracted from the testes was divided into six groups: G_1: egg yolk 20% (control), G_2: lecithin 0.4% (L0.4), G_3: lecithin 0.8% (L0.8), G_4: lecithin 1.2% (L1.2), G_5: lecithin 1.6% (L1.6), and G_6: lecithin 2% (L2). Then, the extender and spermatozoa mixer were transferred to the beaker (containing 500 mL of water at 25°C) and stored in the refrigerator for 2 hours at 4°C for cooling. Then extended sperm was loaded into 0.5 mL straws. Samples with motility <50% were removed from the study.

Extender Preparation

The basic extender was made of TRIS-buffer (3.025 g + citric acid 1.7 g + fructose 1.25 g + Penicillin 100IU/ mL +Streptomycin 100 µg /mL, which was added to 100 mL of distilled water). The pH was set at 6.8-7. Then egg yolk 20% or different concentrations of lecithin (0.4, 0.8, 1.2, 1.6, and 2%) was

added to the medium. Glycerol 7% was added to Tris buffer at one step.

Freezing and Thawing

After two hours of refrigeration, the samples reached 4°C. They were placed in the vapor of liquid nitrogen (4 cm above the liquid nitrogen) for 10 minutes to reach -102°C. Straws were subsequently plunged into liquid nitrogen and stored until thawing. Straws were placed in a warm water bath at 37.5°C for 30 seconds for thawing. The sperm released from each straw was stored in a test tube for 5 minutes at 37.5°C. Then, all tests (CASA, Eosin- Nigrosin staining, HOST, MMP, ROS) were carried out on freeze-thawed spermatozoa.

Tests on Spermatozoa

Eosin-Nigrosin Staining

Eosin- nigrosin staining was performed to determine the vitality and morphology of sperm. Briefly, an aliquot of semen (5 µL) was placed on a slide and mixed well with (5 µL) eosin stain 1% for 30 seconds. Then, 5 µL of nigrosin stain 10% was added for 30 seconds on a 37°C heat plate, and a smear was made on a microscope slide. Finally, slides were examined under oil by a lens (×100) of Nikon –Japan microscope.

Hypo-Osmotic Swelling Test (HOST)

The hypo-osmotic swelling (HOS) is prepared by mixing 75 mmol of fructose and 25 mmol of sodium citrate with distilled water. Briefly, 25 µL sperm thawed + 25 µL (HOS) solution in a microtube and incubate semen/HOS solution mixture for at least 30 min, at 37°C. Then 5 µL of the mixture was placed on a warm slide and mounted with a coverslip. The sperms (n=200) were evaluated using a phase-contrast microscope, and sperms with coiled tails were recorded (Ramu & Jeyendran, 2013).

Computer Assisted Semen Analysis (CASA)

Sperm motility was evaluated with a Sperm analyzer CEROS II™ Hamilton Thorne. Five microliters of semen samples were pipetted onto a warm microscope slide and a coverslip placed on top. Sperm motility was analyzed in eight fields using a software program (Sperm Vision) with settings adjusted for dog spermatozoa. Total motility and the following parameters were evaluated: VCL (track velocity), VAP (path velocity), VSL (straight line velocity), LIN (linearity), STR (straightness), BCF (beat cross frequency), and ALH (amplitude of lateral head displacement).

Mitochondrial Membrane Potential (MMP):

MMP was evaluated using JC-1, a lipophilic cationic dye. JC-1 in spermatozoa with high MMP forms aggregates emitting red fluorescence, while in spermatozoa with low MMP remains as monomers emitting green fluorescence. Initially, semen samples were centrifuged for 5 minutes at 500 × g. After removing the supernatant, the spermatozoa were diluted with phosphate-buffered saline at the concentration of 1×10^6 sperm per mL. Then, 1 mL of JC-1 (200 mmol dissolved in DMSO; Sigma-Aldrich, MO, USA) was added to 1 mL of the diluted sample, which was further incubated at 38°C for 40 minutes. Green and red fluorescence of JC-1 was monitored with FL1 (530 nm) and FL2 (585 nm) detectors, respectively (Akbarinejad *et al.* 2018).

Evaluation of intracellular ROS (Reactive Oxygen Species)

Frozen–thawed semen was re-suspended with phosphate-buffered saline (PBS) at a final concentration of 1-3×106 mL spermatozoa. The intracellular ROS was determined by 2,7-dichlorodihydro fluorescein diacetate (DCFH-DA) (25 µm), separately added to 1–3×106 sperm/mL fractions and incubated at 25°C for 40 min, respectively, in the darkroom. Each sample was analyzed using a flow cytometer with a 488 nm argon laser (Becton Dickinson FACScan, San Jose, CA, USA). Green fluorescence of DCFH-DA (500–530 nm) was evaluated with excitation wavelength at 488 nm and emission wavelength at 525–625 nm in the FL-2 channel. Propidium iodide PI was used as a counterstain dye for DCFH to distinguish dead sperm. Data were expressed as the percentage of fluorescent spermatozoa.

Statistical Analysis

All data were evaluated using GLM procedure. The LSMEANS statement was used to perform multiple comparisons. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., Carry, NC, USA). Differences at P-value< 0.05 were considered statistically significant.

Results

Fresh Epididymal Sperm Assessment

The maximum and minimum limits of spermatozoa's initial and progressive motility were 90-95 and 85-80, respectively. The average percentage of total and progressive motility were 93.3 ± 2.9 and 83.3 ± 2.9, respectively. Initial values for the HOST and Eosin-Nigrosin were 88.1 ± 2.9, 82.7 ± 2.5 respectively. Table 2 shows the evaluation of motility, HOST and Eosin-Nigrosin tests. Table 3 shows the morphology of the epididymal sperm.

Table 1. Components of extender Tris – egg yolk, Tris- lecithin

Composition	Tris-EY	Tris- lecithin
Tris (g)	3.025	3.025
Citric acid (g)	1.7	1.7
Fructose (g)	1.25	1.25
Penicillin (IU/ ml)	100	100
Streptomycin (µg /ml)	100	100
Glycerol (%)	7	7
Egg Yolk (%)	20	0
Lecithin	0	0.4-0.8- 1.2- 1.6- 2
DW (100 mL)	DW (100 mL)	DW (100 mL)

DW: Distilled water

Table 2. Assessment of fresh epididymal spermatozoa

Total Motility (%)	Progressive Motility (%)	Positive HOST (%)	Live Eosin-Nigrosin (%)
93.3 ± 2.9	83.3 ± 2.9	88.1 ± 2.9	82.7 ± 2.5

Table 3. Morphology of epididymal sperm before freezing.

Group	Initial (%)
Detached heads	0.97 ± 0.9
detached acrosome	0.5 ± 0.9
Double tail	0.13 ± 0.2
Coiled tails	4.5 ± 1.3
Proximal droplet	1.5 ± 0.9
Distal droplet	22.7 ± 3.7
Abnormal head	0.3 ± 0.2
Bent midpiece	0.8 ± 0.3
Thickened middle piece	0.4 ± 0.4
Relocation middle piece	0.3 ± 0.3
Double middle piece	0

Freeze-thawed Epididymal Sperm Assessment

Motility of freeze-thawed spermatozoa

In general, the values of total and progressive motility decreased post-freezing-thawing in cryopreserved samples in all treatment groups compared with the fresh semen specimen (P<0.001). The best total and progressive motility of sperm after thawing among groups were in the egg yolk group (46 ± 8.5, 26.3 ± 15.8, respectively). However, among the soy-lecithin groups, the higher total and progressive motility were seen in L0.4. In frozen samples, total and progressive motility of spermatozoa were lesser in all groups treated with different concentrations of lecithin than in the control group (P≤0.05). Table 4 shows the evaluation results of the motility of spermatozoa between groups after freezing and thawing.

Table 4. Evaluation of the motility of sperm among groups before freezing and after thawing

Motility	Time	EY	LS 0.4	LS 0.8	LS 1.2	LS 1.6	LS 2
T M (%)	B F	93.3 ± 2.9	93.3 ± 2.9	93.3 ± 2.9	93.3 ± 2.9	93.3 ± 2.9	93.3 ± 2.9
	A F	46 ± 8.5 [a]	31 ± 16.4[b]	22 ± 7.2[b]	12.3 ± 6.8[b]	16.7 ± 7.6[b]	10 ± 0[b]
P M (%)	B F	83.3 ± 2.9	83.3 ± 2.9	83.3 ± 2.9	83.3 ± 2.9	83.3 ± 2.9	83.3 ± 2.9
	A F	26.3 ± 15.8[a]	19.3 ± 14.4[b]	13 ± 6.1[b]	6.3 ± 3.2[b]	7.7 ± 6.4[b]	4 ± 1.7[b]

BF: Before freezing, AF: After Freezing, TM: Total Motility, PM: Progressive Motility. [ab] Significant differences within column for each parameter.

HOST After Thawing

The proportion of sperm with intact plasma membrane integrity based on the HOS test decreased the following cryopreservation in all treatment groups compared with the fresh semen specimen (P<0.001). The percentage of sperm with the plasma membrane integrity between groups is illustrated in Table 5. The egg yolk extender was able to preserve the integrity of the plasma membrane of the spermatozoa better than the other groups (65.1 ± 8.1). However, L 0.4 achieved better results than the different concentrations of lecithin in other groups (18.4 ± 3.4), the egg yolk was the best.

Table 5. Hypo Osmotic Swelling Test after thawing

HOST	Time	EY	LS 0.4	LS 0.8	LS 1.2	LS 1.6	LS 2
Positive (%)	B F	88.1 ± 2.9	88.1 ± 2.9	88.1 ± 2.9	88.1 ± 2.9	88.1 ± 2.9	88.1 ± 2.9
	A F	65.1 ± 8.1[a]	18.4 ± 3.4[b]	9.2 ± 2.2[b]	10.9 ± 5.6[b]	6 ± 3[b]	9.1 ± 5.9[b]

BF: Before freezing, AF: After Freezing. [ab] Significant differences within a row for each parameter.

Eosin- Nigrosin Staining After Thawing

The percentage of live sperm among groups was listed in Table 6. The results showed the percentages of live sperm in groups L 0.4, L 0.8, egg yolk was 52.5±16.2, 46.1±11.3, 43.2±10.4, respectively; there was no significant difference among them. The proportion of sperm with morphological defects did not differ between fresh and frozen samples and among various experimental groups (P>0.05). The results are listed in Table 7.

Table 6. Eosin-Nigrosin test after thawing.

EN	Time	EY	LS 0.4	LS 0.8	LS 1.2	LS 1.6	LS 2
Live (%)	B F	82.7 ± 2.5	82.7 ± 2.5	82.7 ± 2.5	82.7 ± 2.5	82.7 ± 2.5	82.7 ± 2.5
	A F	43.2 ± 10.4[a]	52.5 ± 16.2[a]	46.1 ± 11.3[a]	40.1 ± 13.2[a]	27.1 ± 7.6[b]	31.3 ± 7.3[b]

BF: Before freezing, AF: After Freezing. [ab] Significant differences within row for each parameter.

Table 7. Morphology of spermatozoa before freezing and after thawing

Groups	Initial (before freezing)	EY	LS 0.4	LS 0.8	LS 1.2	LS 1.6	LS 2
detached heads	0.97 ± 0.9[a]	1.3 ± 1.1 [a]	1.5 ± 1.9 [a]	2.9 ± 4.1 [a]	4.6 ± 7 [a]	1.1 ± 0.8 [a]	4.9 ± 3.6 [a]
detached acrosome	0.5 ± 0.9 [a]	1.6 ± 1.9 [a]	1.6 ± 1.7 [a]	2 ± 2.7 [a]	0.6 ± 0.3 [a]	1.7 ± 2 [a]	1.8 ± 1.9 [a]
double tail	0.13 ± 0.2 [a]	0 [a]	0 [a]	0 [a]	0.2 ± 0.3 [a]	0 [a]	0 [a]
coiled tails	4.5 ± 1.3 [a]	5.9 ± 1.8 [a]	4.4 ± 1.6 [a]	3.2 ± 2.3 [a]	3.4 ± 1.1 [a]	3.7 ± 1.1 [a]	2.5 ± 2.2 [a]
proximal droplet	1.5 ± 0.9 [a]	1.4 ± 1.2 [a]	0 [a]	0 [a]	0.5 ± 0.9 [a]	0.7 ± 1.2 [a]	0.5 ± 0.4 [a]
distal droplet	22.7 ± 3.7 [a]	28.3 ± 14.9[a]	26.3 ± 1.7 [a]	33.2 ± 2.3 [a]	26.4 ± 6.6 [a]	32.9 ± 11.3 [a]	32.1 ± 3.4 [a]
abnormal head	0.3 ± 0.2 [a]	0 [a]	0.4 ± 0.7 [a]	0.3 ± 0.5 [a]	0 [a]	0 [a]	0.3 ± 0.6 [a]
bent mid piece	0.8 ± 0.3 [a]	3.9 ± 5.6 [a]	4.1 ± 7.1 [a]	2.9 ± 1.1 [a]	0.7 ± 0.6 [a]	1.5 ± 0.4 [a]	1.5 ± 0.5 [a]
thickened middle piece	0.4 ± 0.4 [a]	0 [a]	0.5 ± 0.5 [a]	0 [a]	0 [a]	0.3 ± 0.6 [a]	0.7 ± 0.3 [a]
elocation middle piece	0.3 ± 0.3 [a]	0.9 ± 1.5 [a]	0.4 ± 0.7 [a]	0 [a]	0.3 ± 0.5 [a]	0 [a]	0.6 ± 0.4 [a]
double middle piece	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]	0.3 ± 0.6 [a]	0 [a]

[aa] No significant differences within column for each parameter.

CASA

CASA analysis was performed on frozen-thawed semen samples to determine the effect of soy lecithin and egg yolk on motility parameters (Tables 8 and 9). Parameters of VAP, VSL, VCL, STR, LIN, and ALH did not differ among various experimental groups (P>0.05). However, BCF was higher in all groups treated with different concentrations of lecithin compared with the control group (P≤0.05).

Table 8. Assessment of motility in freeze-thawed spermatozoa by CASA

Group	T Motility (%)	P motility (%)
EY	46 ± 8.5[a]	28 ± 16.6[a]
LS 0.4	32 ± 14.8[b]	20 ± 13.2 [a]
LS 0.8	22.3 ±6.8 [b]	12.7 ± 6.4 [a]
LS 1.2	14.3 ± 8.7 [b]	8.3 ± 4.9 [a]
LS 1.6	18.3 ± 5.8 [b]	7.7 ± 4.6 [a]
LS 2	10 ± 0 [b]	4 ± 1.7 [a]

[ab] Significant differences within column for each parameter.

Table 9. Evaluation of motility in freeze-thawed spermatozoa by CASA

Group	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
EY	83.4 ± 5.2 [a]	66.6 ± 3.7 [a]	141.7 ± 5.5 [a]	9.4 ± 0.7 [a]	21.2 ± 1.6[a]	78.3 ± 0.6 [a]	48.7 ± 2.5 [a]
LS 0.4	70 ± 15.8 [a]	55.2 ± 9.6 [a]	131 ± 48.6 [a]	9.5 ± 2.1 [a]	30.3 ± 3.4[b]	80.3 ± 8.7 [a]	51.3 ± 17.6 [a]
LS 0.8	60.4 ± 28.4 [a]	49.6 ± 21.2 [a]	101.4 ± 52.9 [a]	10.1 ± 1.9 [a]	31.5 ± 0.8 [b]	85 ± 6.9 [a]	59.7 ± 12.1 [a]
LS 1.2	83 ± 14.6 [a]	63.3 ± 5.6 [a]	147.9 ± 43.6 [a]	9.5 ± 1.2 [a]	30.4 ± 2.9 [b]	79.7 ± 9.3 [a]	50 ± 15.7 [a]
LS 1.6	72 ± 32.6 [a]	57.6 ± 23.2 [a]	133.9 ± 73.1	9.6 ± 2 [a]	27.4 ± 4.8 [b]	82 ± 8.9 [a]	52 ± 14.7 [a]
LS 2	58.3 ± 18.4 [a]	48.2 ± 11.6 [a]	100 ± 43.7	9.5 ± 1.4 [a]	27.2 ± 1.6 [b]	85.3 ± 10.5 [a]	59.7 ± 19.4 [a]

[aa] No significant differences within column for each parameter.

MMP

The MMP level of spermatozoa in EY or LS 0.4 after the post-thawing was presented in Figure 1. A comparison was made between the control and LS 0.4 groups, which revealed better results in the control group than in the lecithin groups. Depending on the dye JC-1, the results showed a significant difference between the control and LS 0.4 group (P=0.026).

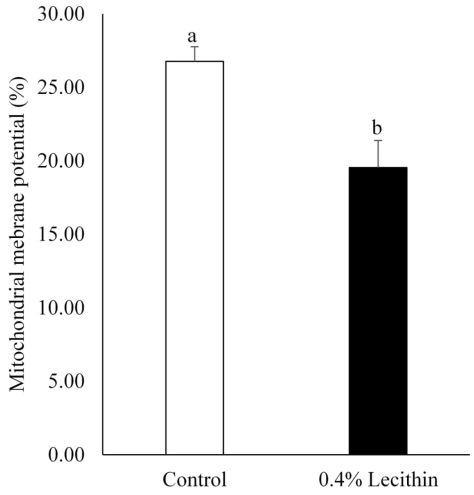

Figure 1. Mitochondrial membrane potential after thawing

ROS (Superoxide) Production

The proportion of sperm positive for ROS in EY or LS 0.4 after thawing was presented in Figure 2. The level of H_2O_2 was higher in LS 0.4 than in the control group (P=0.049). Production of hydrogen peroxide decreased in the EY group after freezing-thawing compared with LS 0.4.

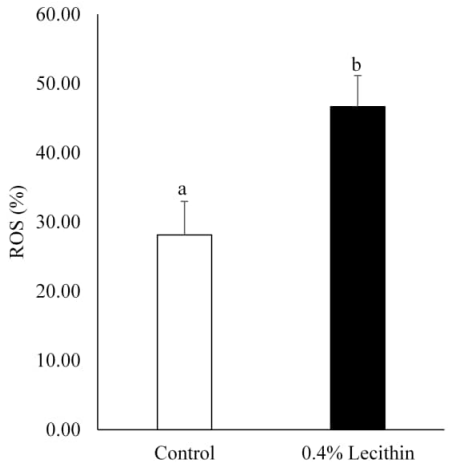

Figure 2. Reactive oxygen species (ROS) *after thawing*

Discussion

Egg Yolk is not a defined entity but a complex biological compound containing proteins, vitamins, phospholipids, glucose, and antioxidants which are all potentially useful for cell membrane integrity. Unfortunately, it is also a biologically hazardous compound (Farstad, 2009). Research in recent years has focused on finding a suitable alternative to egg yolk from a non-animal source for global trade. Since lecithin in egg yolk plays a crucial role in preventing cold shock, and the latter can be obtained from vegetable sources such as soy and sunflower, the researchers focused on finding the ideal type and concentration of lecithin to protect sperm from cold shock. Egg Yolk extender has been shown efficient for maintaining a good level of total and progressive motility of sperm as well as the integrity of the plasma membrane, while soy lecithin did not provide the same level of efficacy. When analyzing the movement parameters (VAP, VSL, VCL, STR, LIN, and ALH) according to CASA, there was no significant difference between the experimental and control groups except for the BCF values, which were high in all the experimental groups compared to the control group. This indicates severe damage to the plasma membrane of the sperm, which results in severe tail injuries. Soy lecithin 0.4 and 0.8, respectively, had the highest total and progressive movement ratios (31 ± 16.4, 19.3 ± 14.4/ 22 ± 7.2, 13 ± 6.1) among the rest of the different concentrations of lecithin. Soy lecithin, in different concentrations, was able to maintain a good percentage of sperm life; in contrast, it had a very negative impact on the integrity of the plasma membrane, as the percentage of sperm with a healthy plasma membrane was very low. In general, the results of lecithin 0.4 % were superior to the rest of the lecithin concentrations, and the groups L 1.6 and L2 had the worst results. Assessment of ROS level and mitochondrial activity in the present study revealed that the adverse effect of lecithin on canine sperm during cryopreservation was at least partly due to excessive elevation of ROS and impairment of mitochondrial function in sperm treated with lecithin compared with sperm treated with egg yolk. Sperm generate the physiological amounts of reactive oxygen species (ROS) that are important for sperm capacitation, acro-some reaction, and the ability to

fertilize the oocyte (Agarwal *et al.*, 2006). However, when the ROS production is excessive, an imbalance occurs between the ROS-generating system and enzymatic and non-enzymatic antioxidants responsible for ROS removal, which leads to oxidative stress. This type of stress causes structural damage to biomolecules, DNA, lipids, carbohydrates, proteins, and other cellular components, including mitochondria (Dalmazzo *et al.*, 2018), and it may also compromise both the genetic integrity and the fertilizing capacity. There are several companies that extract lecithin, whether from soybeans or sunflowers, and there are several concentrations of it, and some of it is used for research and other nutritional purposes. For example, Sigma company has soy lecithin in many forms: (P3644, P5638, P7443, P3782), and other companies such as Swanson Health Products, Fargo, ND, USA, Minitube®, Tiefenbach, G), Solae Company, St.Lo-uis, MO, EUA, General Nutrition Corporation, Pittsburgh, PA. Differences have been found among canid species in the ability of their spermatozoa to withstand freezing. There are differences in sperm membrane fatty acid composition among species, which may explain part of these differences. Suppose the presence of long-chained polyunsaturated fatty acids contributes to increased membrane fluidity. In that case, this relationship may be biphasic, i.e., either too much membrane fluidity or too little could compromise successful sperm cryopreservation. An increase in fluidity of the outer leaflet of the plasma membrane has been shown in frozen-thawed dog spermatozoa (Farstad, 2009).

The results of this study were consistent with several studies which studied the effect of lecithin on the ejaculated semen of dogs (Axnér and Lagerson 2016; Dalmazzo *et al.*, 2018; Hermansson, Johannisson & Axnér, 2021) and opposed to some of other studies which also studied the effect of lecithin on the ejaculated semen of dogs (Beccaglia, Anastasi & Luvoni, 2009[a]; Beccaglia *et al.,* 2009b; Kmenta *et al.,* 2011; Kasimanickam *et al.*, 2012; Sánchez-Calabuig *et al.*, 2017; Zakošek Pipan *et al.*, 2020). Researchers have studied the role of soy lecithin in protecting against cold shock among different animals and found different results. The results of some researchers' studies have shown the positive effects of soy lecithin on protecting sperm from cold shocks, such as in cats (Vick *et al.*, 2012; Vansandt *et al.*, 2021), in goats (Salmani *et al.*, 2014), in rams (Forouzanfar *et al.*, 2010), in bulls (Aires *et al.*, 2003). Also, some other studies have shown the negative effects of Lecithin such as in buck (Sarıözkan *et al.*, 2010; Roof *et al.*, 2012; Salmani *et al.*, 2013; Tabarez, García & Palomo, 2020), in black rhinoceros and Indian rhinoceros (Wojtusik, Stoops & Roth, 2018), in Japanese white rabbits (Nishijima *et al.*, 2015), in brown bears (Alvarez-Rodríguez *et al.*, 2013). For epididymal sperm in dog Nöthling *et al.* (2007) studied the effect of adding prostate fluids to frozen epididymal sperm in dogs and compared it with two prepared extenders (BilEq & Andromed) and found that BilEq extender was more suitable than Andromed as a freezing medium for epididymal sperm in dogs. Also, prostate fluids should be added before freezing of epididymal spermatozoa extended in BilEq and after thawing because such addition results in better motility, longevity, and sperm morphology (Nöthling *et al.*, 2007). Lopes *et al.* (2015) compared the Tris-egg-yolk-glycerol extender with the commercial extender AndroMed for freezing of epididymal sperm in bulls and they found that the egg yolk extender was superior to the commercial extender AndroMed (Lopes *et al.*, 2015). Other researchers applied nanotechnology on soy lecithin to reduce the size of its particles and used it as a nano extender for cryopreservation of animal semen; it had a positive effect, for instance, in bulls (Mousavi *et al.,* 2019), in goats (Nadri *et al.*, 2019), and humans (Mutalik *et al.*, 2014). Other investigators have suggested adding enhancers to lecithin extenders, such as adding bovine serum albumin (Alcay *et al.*, 2019) which had a positive effect, or the antioxidant glutathione (Zhandi & Sharafi, 2015) which had bad effects. In human semen, Reed *et al.* (2009) concluded that soy lecithin can successfully replace egg yolk as a supplement for cryopreservation medium, without adverse effects on sperm post-thawing, warranting further research into this and other phospholipids (Reed *et al.*, 2009). The same result was obtained by Jeyendran (2008), who concluded that an effective medium for freezing human sperm that does not involve the supplementation of animal products may be developed by using phospholipids derived from soybean oil, along with DMSO and glycerol (Jeyendran *et al.*, 2008).

Conclusion

The results of our current study showed that lecithin, which was used in different concentrations, was

not a suitable substitute for egg yolk extenders for preserving epididymal sperm in dogs. The need to develop a specific medium without animal proteins is obvious. Lipids or lipoproteins in natural or synthetic form may be able to substitute standard whole EY-based diluents in preserving sperm survival during cooling and freezing. Some lipids of lipoproteins may be able to replace the EY and protect the sperm membranes completely. Still, it may be challenging to obtain the benefits of the entire EY with all its compounds by adding single substances.

Acknowledgments

The authors gratefully acknowledge the support provided by the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Conflict of Interest

The authors declared no conflict of interest.

References

Agarwal, A., Sharma, R. K., Nallella, K. P., Thomas Jr, A. J., Alvarez, J. G., & Sikka, S. C. (2006). Reactive Oxygen Species as an Independent Marker of Male Factor Infertility. *Fertility and Sterility*, *86*(4), 878-885. [DOI:10.1016/j.fertnstert.2006.02.111] [PMID]

Aires, V. A., Hinsch, K. D., Mueller-Schloesser, F., Bogner, K., Mueller-Schloesser, S., & Hinsch, E. (2003). In Vitro and in Vivo Comparison of Egg Yolk-Based and Soybean Lecithin-Based Extenders for Cryopreservation of Bovine Semen. *Theriogenology*, *60*(2), 269-279.

Akbarinejad, V., Fathi, R., Shahverdi, A., Esmaeili, V., Rezagholizadeh, A., & Ghaleno, L. R. (2018). Activator of Mitochondrial Aldehyde Dehydrogenase (Alda-1) Could Enhance Quality of Equine Cooled Semen by Ameliorating Loss of Mitochondrial Function Over Time. *Journal of Equine Veterinary Science*, *70*, 63-70. [DOI:10.1016/j.jevs.2018.08.004]

Alcay, S., Toker, M. B., Gökçe, E., Önder, N. T., Üstüner, B., & Zekariya, N. U. R. (2019). Long Term Incubation Resilience of Post-Thaw Ram Semen Diluted with Lecithin-Based Extender Supplemented with Bovine Serum Albumin. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *25*(3).

Alvarez-Rodríguez, M., Alvarez, M., Anel-López, L., Martínez-Rodríguez, C., Martínez-Pastor, F., Borragan, S., ... & de Paz, P. (2013). The Antioxidant Effects of Soybean Lecithin-or Low-Density Lipoprotein-Based Extenders for the Cryopreservation of Brown-Bear (Ursus Arctos) Spermatozoa. *Reproduction, Fertility and Development*, *25*(8), 1185-1193. DOI:10.1071/RD12181] [PMID]

Axnér, E., & Lagerson, E. (2016). Cryopreservation of Dog Semen in a Tris Extender with 1% or 2% Soya Bean Lecithin as a Replacement of Egg Yolk. *Reproduction in Domestic Animals*, *51*(2), 262-268. [DOI:10.1111/rda.12675] [PMID]

Beccaglia, M., Anastasi, P., & Luvoni, G. C. (2009[a]). Freezing of Canine Semen in an Animal-Free Protein Extender. *Veterinary Research Communications*, *33*(1), 77-80. [DOI:10.1007/s11259-009-9249-9] [PMID]

Beccaglia, M., Anastasi, P., Chigioni, S., & Luvoni, G. C. (2009[b]). Tris-lecithin Extender Supplemented with Antioxidant Catalase for Chilling of Canine Semen. *Reproduction in Domestic Animals*, *44*, 345-349. [DOI:10.1111/j.1439-0531.2009.01410.x] [PMID]

Dalmazzo, A., Losano, J. D. A., Rocha, C. C., Tsunoda, R. H., Angrimani, D. D. S. R., Mendes, C. M., ... & Barnabe, V. H. (2018). Effects of Soy Lecithin Extender on Dog Sperm Cryopreservation. *Animal Biotechnology*, *29*(3), 174-182. [DOI:10.1080/10495398.2017.1334662] [PMID]

Farstad, W. (2009). Cryopreservation of Canine Semen–New Challenges. *Reproduction in Domestic Animals*, *44*, 336-341. [PMID] [DOI:10.1111/j.1439-0531.2009.01418.x]

Forouzanfar, M., Sharafi, M., Hosseini, S. M., Ostadhosseini, S., Hajian, M., Hosseini, L., ... & Nasr-Esfahani, M. H. (2010). In Vitro Comparison of Egg Yolk–Based and Soybean Lecithin–Based Extenders for Cryopreservation of Ram Semen. *Theriogenology*, *73*(4), 480-487. [DOI:10.1016/j.theriogenology.2009.10.005] [PMID]

Hermansson, U., Johannisson, A., & Axnér, E. (2021). Cryopreservation of Dog Semen in a Tris Extender with two Different 1% Soybean Preparations Compared with a Tris Egg Yolk Extender. *Veterinary Medicine and Science*, *7*(3), 812-819. [DOI:10.1002/vms3.445] [PMID] [PMCID]

Jeyendran, R. S., Acosta, V. C., Land, S., & Coulam, C. B. (2008). Cryopreservation of Human Sperm in a Lecithin-Supplemented Freezing Medium. *Fertility and Sterility*, *90*(4), 1263-1265. [DOI:10.1016/j.fertnstert.2007.10.068] [PMID]

Kasimanickam, V. R., Kasimanickam, R. K., Memon, M. A., & Rogers, H. A. (2012). Effect of Extenders on Sperm Mitochondrial Membrane, Plasma Membrane and Sperm Kinetics During Liquid Storage of Canine Semen at 5 C. *Animal Reproduction Science*, *136*(1-2), 139-145. [DOI:10.1016/j.anireprosci.2012.10.024] [PMID]

Kmenta, I., Strohmayer, C., Müller-Schlösser, F., & Schäfer-Somi, S. (2011). Effects of a Lecithin and Catalase Containing Semen Extender and a Second Dilution with Different Enhancing Buffers on the Quality of Cold-Stored Canine Spermatozoa. *Theriogenology*, *75*(6), 1095-1103. [DOI:10.1016/j.theriogenology.2010.11.018] [PMID]

Lopes, G., Soares, L., Ferreira, P., & Rocha, A. (2015). Tris–Egg Yolk–Glycerol (TEY) Extender Developed for Freezing Dog Semen is a Good Option to Cryopreserve Bovine Epididymal Sperm Cells. *Reproduction in Domestic Animals*, *50*(1), 97-103. [DOI:10.1111/rda.12454] [PMID]

Luvoni, G. C., & Morselli, M. G. (2017). Canine Epididymal Spermatozoa: A Hidden Treasure with Great Potential. *Reproduction in Domestic Animals*, *52*, 197-201. [DOI:10.1111/rda.12820] [PMID]

Mousavi, S. M., Towhidi, A., Zhandi, M., Amoabediny, G., Mohammadi-Sangcheshmeh, A., Sharafi, M., & Hussaini, S. M. H. (2019). Comparison of Two Different Antioxidants in a Nano Lecithin-Based Extender for Bull Sperm Cryopreservation. *Animal Reproduction Science*, *209*, 106171. [DOI:10.1016/j.anireprosci.2019.106171] [PMID]

Mutalik, S., Salian, S. R., Avadhani, K., Menon, J., Joshi, H., Hegde, A. R., ... & Adiga, S. K. (2014). Liposome Encapsulated Soy Lecithin and Cholesterol Can Efficiently Replace Chicken Egg Yolk in Human Semen Cryopreservation Medium. *Systems Biology in Reproductive Medicine*, *60*(3), 183-188. [DOI:10.3109/19396368.2014.902521] [PMID]

Nadri, T., Towhidi, A., Zeinoaldini, S., Martínez-Pastor, F., Mousavi, M., Noei, R., ... & Sangcheshmeh, A. M. (2019). Lecithin Nanoparticles Enhance the Cryosurvival of Caprine Sperm. *Theriogenology*, *133*, 38-44. [DOI:10.1016/j.theriogenology.2019.04.024] [PMID]

Nishijima, K., Kitajima, S., Koshimoto, C., Morimoto, M., Watanabe, T., Fan, J., & Matsuda, Y. (2015). Motility and Fertility of Rabbit Sperm Cryopreserved Using Soybean Lecithin as an Alternative to Egg Yolk. *Theriogenology*, *84*(7), 1172-1175. [DOI:10.1016/j.theriogenology.2015.06.018] [PMID]

Nöthling, J. O., Gerber, D., Colenbrander, B., Dijkstra, M., Bakker, T., & De Cramer, K. (2007). The Effect of Homologous Prostatic Fluid on Motility and Morphology of dog Epididymal Spermatozoa Extended and Frozen in Biladyl with Equex STM Paste or Andromed. *Theriogenology*, *67*(2), 264-275. [DOI:10.1016/j.theriogenology.2006.07.018] [PMID]

Ortiz, I., Urbano, M., Dorado, J., Morrell, J. M., Al-Essawe, E., Johannisson, A., & Hidalgo, M. (2017). Comparison of DNA Fragmentation of Frozen-Thawed Epididymal Sperm of Dogs Using Sperm Chromatin Structure Analysis and Sperm Chromatin Dispersion test. *Animal Reproduction Science*, *187*, 74-78. [DOI:10.1016/j.anireprosci.2017.10.007] [PMID]

Ramu, S., & Jeyendran, R. S. (2013). The Hypo-Osmotic Swelling Test for Evaluation of Sperm Membrane Integrity. In *Spermatogenesis* (pp. 21-25). Humana Press, Totowa, NJ. [DOI:10.1007/978-1-62703-038-0_3] [PMID]

Reed, M. L., Ezeh, P. C., Hamic, A., Thompson, D. J., & Caperton, C. L. (2009). Soy Lecithin Replaces Egg Yolk for cryopreservation of Human Sperm Without Adversely Affecting Postthaw Motility, Morphology, Sperm DNA Integrity, or Sperm Binding to Hyaluronate. *Fertility and Sterility*, *92*(5), 1787-1790. [DOI:10.1016/j.fertnstert.2009.05.026] [PMID]

Roof, D. J., Bowley, S., Price, L. L., & Matsas, D. J. (2012). Comparison of Two Commercial Extenders for Cryopreservation of Goat Semen Without Sperm Washing. *Theriogenology*, *77*(2), 412-420. [DOI:10.1016/j.theriogenology.2011.08.015] [PMID]

Salmani, H., Nabi, M. M., Vaseghi-Dodaran, H., Rahman, M. B., Mohammadi-Sangcheshmeh, A., Shakeri, M., ... & Zhandi, M. (2013). Effect of Glutathione in Soybean Lecithin-Based Semen Extender on Goat Semen Quality After Freeze-Thawing. *Small Ruminant Research*, *112*(1-3), 123-127. [DOI:10.1016/j.smallrumres.2012.12.015]

Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M., & Sharafi, M. (2014). In Vitro Assessment of Soybean Lecithin and Egg Yolk Based Diluents for Cryopreservation of Goat Semen. *Cryobiology*, *68*(2), 276-280. [DOI:10.1016/j.cryobiol.2014.02.008] [PMID]

Sánchez-Calabuig, M. J., Maillo, V., Beltrán-Breña, P., de la Fuente Martínez, J., Galera-Carrillo, S., Pérez-Gutiérrez, J. F., & Pérez-Cerezales, S. (2017). Cryopreservation of Canine Sperm Using Egg Yolk and Soy Bean Based Extenders. *Reproductive Biology*, *17*(3), 233-238. [DOI:10.1016/j.repbio.2017.05.007] [PMID]

Sarıözkan, S., Bucak, M. N., Tuncer, P. B., Taşdemir, U., Kinet, H., & Ulutaş, P. A. (2010). Effects of Different Extenders and Centrifugation/Washing on Postthaw Microscopic-Oxidative Stress Parameters and Fertilizing Ability of Angora Buck Sperm. *Theriogenology*, *73*(3), 316-323. [DOI:10.1016/j.theriogenology.2009.09.015] [PMID]

Tabarez, A., García, W., & Palomo, M. J. (2020). Soy Lecithin as a Potential Alternative to Powdered Egg Yolk for Buck Sperm Cryopreservation Does not Protect Them from Mitochondrial Damage. *Animal Reproduction Science*, *217*, 106473. [DOI:10.1016/j.anireprosci.2020.106473] [PMID]

Thomassen, R., & Farstad, W. (2009). Artificial Insemination in Canids: A Useful Tool in Breeding and Conservation. *Theriogenology*, *71*(1), 190-199. [DOI:10.1016/j.theriogenology.2008.09.007] [PMID]

Vansandt, L. M., Bateman, H. L., Miller, A. G., Herrick, J. R., Moresco, A., González, R., ... & Swanson, W. F. (2021). Cross-Species Efficacy of a Chemically-Defined, Soy Lecithin-Based Cryomedium for Semen Banking in Imperiled Wild Felids. *Theriogenology*, *159*, 108-115. ` DOI:10.1016/j.theriogenology.2020.10.021. PMid:33130295.] [PMID]

Vick, M. M., Bateman, H. L., Lambo, C. A., & Swanson, W. F. (2012). Improved Cryopreservation of Domestic Cat Sperm in a Chemically Defined Medium. *Theriogenology*, 78(9), 2120-2128. [DOI:10.1016/j.theriogenology.2012.08.009] [PMID]

Wojtusik, J., Stoops, M. A., & Roth, T. L. (2018). Comparison of Soy Lecithin, Coconut Water, and Coconut Milk as Substitutes For Egg-Yolk in Semen Cryodiluent for Black Rhinoceros (Diceros Bicornis) and Indian Rhinoceros (Rhinoceros Unicornis). *Theriogenology*, *121*, 72-77. [DOI:10.1016/j.theriogenology.2018.07.042] [PMID]

Zakošek Pipan, M., Casal, M. L., Šterbenc, N., Virant Klun, I., & Mrkun, J. (2020). Vitrification Using soy Lecithin and Sucrose: A New Way to Store the Sperm for the Preservation of Canine Reproductive Function. *Animals*, *10*(4), 653. [DOI:10.3390/ani10040653] [PMID] [PMCID]

Zhandi, M., & Sharafi, M. (2015). Negative Effect of Combined Cysteine and Glutathione in Soy Lecithin-Based Extender on Post-Thawed Ram Spermatozoa. *Cell and Tissue Banking*, *16*(3), 443-448. [DOI:10.1007/s10561-014-9488-z] [PMID].

10.22059/IJVM.2021.329603.1005191

Iranian Journal of Veterinary Medicine

Abstracts in Persian Language

Online ISSN 2252-0554

مجله طب دامی ایران، ۱۴۰۰، دوره ۱۶، شماره ۲، ۱۶۶-۱۷۷

بررسی اثر لسیتین سویا در مقایسه با زرده تخم مرغ بر انجماد اسپرم اپیدیدیمی در سگ

احمد یوسف[۱]، حمید قاسم زاده نوا[۱*]، وحید اکبری نژاد[۱]، پرویز تاجیک[۱]، آرمین توحیدی[۲]

[۱] گروه مامایی و بیماری های تولیدمثل دام ، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران

[۲] گروه علوم دامی، دانشکده کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران

(دریافت مقاله: ۱۱ مرداد ماه ۱۴۰۰، پذیرش نهایی: ۰۶ مهر ۱۴۰۰)

زمینه مطالعه: استحصال اسپرم از ناحیه اپیدیدیم اجازه استفاده ماده ژنتیکی در پس از مرگ یا پس از برداشت بیضه از حیوانات با ارزش یا در معرض انقراض را می‌دهد.

هدف: هدف از این مطالعه بهبود دسترسی به سیستم انجماد اسپرم اپیدیدیم سگ بر اساس دوز مناسب لسیتین بود.

روش کار: اسپرم اپیدیدیمی از بیضه‌های سگ‌های بالغ و سالم پس از اخته در مراکز درمانی دامپزشکی جمع‌آوری شد و به شش گروه تقسیم شدند (گروه اول لسیتین ٪ L0.4 ، گروه دوم لسیتین ٪ L0.8 ، و گروه سوم لسیتین ٪ L1.2 ، گروه چهارم لسیتین ٪L1.6، گروه پنجم لسیتین ٪L2 و گروه کنترل زردۀ تخم مرغ ٪ EY 20). قبل از انجماد، ارزیابی تحرک، رنگ‌آمیزی حیاتی ائوزین نیگروزین و HOST انجام شد و پس از یخ‌گشایی، روی اسپرم با روش‌های CASA ، HOST، ائوزین نیگروزین، پتانسیل غشای میتوکندری (MMP) ، رادیکال‌های آزاد اکسیژن داخل سلولی (ROS) آزمایش‌ها انجام شدند.

نتایج: در نمونه‌های پس از یخ‌گشایی، تحرک کلی و نسبت اسپرم با یکپارچگی غشای پلاسما دست نخورده بر اساس آزمون HOS در همه گروه‌های تحت غلظت‌های مختلف لسیتین در مقایسه با گروه کنترل کمتر بود ($P \leq ۰/۰۵$). BCF در همه گروه های تحت غلظت های مختلف لسیتین در مقایسه با گروه کنترل بیشتر بود ($P \leq ۰/۰۵$). با این وجود، تحرک پیش‌رونده، نسبت اسپرم زنده بر اساس آزمایش ائوزین- نیگروزین، VAP، VSL، VCL، STR، LINو ALH در گروه‌های مختلف آزمایشی تفاوتی ندارند ($P \leq ۰/۰۵$). نسبت اسپرم با عیب مورفولوژیکی بین نمونه‌های تازه و منجمد و میان گروه‌های مختلف آزمایشی تفاوت نداشت ($P \leq ۰/۰۵$). پتانسیل غشای میتوکندری در گروه شاهد بیشتر از گروه لسیتین ٪۰/۴ بود ($P \leq ۰/۰۵$). نسبت اسپرم مثبت برای ROS در گروه شاهد کمتر از گروه لسیتین ٪۰/۴ بود ($P= ۰/۰۴۹$).

نتیجه‌گیری نهایی: با زرده تخم مرغ نسبت به اکستندرهای مبتنی بر لسیتین برای انجماد اسپرم اپیدیدیمی سگ برتری داشت، و لسیتین سویا به‌عنوان جایگزین مناسب برای انجماد اسپرم اپیدیدمی سگ مناسب نبود.

واژه‌های کلیدی: لسیتین، انجماد اسپرم اپیدیدمی، سگ، رادیکال‌های آزاد اکسیژن، پتانسیل غشای میتوکندری

نویسندۀ مسئول: حمید قاسم زاده نوا، گروه مامایی و بیماری های تولیدمثل دام، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران ایمیل:hghasem@ut.ac.ir