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

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20 **Abstract:**

BACKGROUND : Epididymal sperm collection is allowed the use of 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
25 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 as compared with 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 than 0.4% lecithin group ($P = 0.026$). The proportion of sperm positive for ROS was lesser in the control than 0.4% lecithin group ($P = 0.049$). **CONCLUSIONS:** egg yolk was superior to the lecithin-based extenders to cryopreserve epididymal sperm of dogs.

Key words: cryopreservation of epididymal sperm, dog, lecithin, mitochondrial membrane potential, reactive oxygen spices

Introduction

high economic value, zoo technical, and affective character of some individuals increases the advances of reproductive biotechnologies for future preservation (Thomassen and 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

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progeny. Spermatozoa can be collected by different techniques from ex vivo or in vivo testicles and can be cryopreserved for future use (Luvoni and 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, Urbano *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 but 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 *et al.*, 2021). Soy lecithin is a promising option as a substitute for egg yolk, due to ease of component standardization, readily availability, and reduced the 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, Losano *et al*. 2018). Some of studies have shown the effect of soya lecithin as a suitable alternative to egg yolk on ejaculated sperm in dogs (Beccaglia, Anastasi et al. 2009, Beccaglia, Anastasi et al. 2009, Kmenta, Strohmayer *et al*., 2011, Kasimanickam, 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.

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70 **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).

a. Animals

75 Ten intact male dogs aged between 1 - 8 years old, with different breeds (Golden Rertriever, Pamranin, Terrier, Shitzu) and body weights (between 3 kg and 25 kg were used for this study. Testes of the dog 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, physiological solution. The testes were processed within 2 hours of castration.

80 **b. 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, thereafter 85 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 90 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 mixer of the extender and

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spermatozoa were transferred to beaker (contains 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.

c. 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 micro gram/ml, which 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%). Glycerol 7% was added to Tris buffer at one step.

d. Freezing and thawing

After two hours of refrigeration, the samples reached to 4°C. They 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. For thawing, straws were placed in a warm water bath at 37.5 ° C for 30 seconds. 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 done on freeze-thawed spermatozoa.

e. Tests on spermatozoa

i. Eosin-nigrosin staining

Eosin- nigrosin staining was performed to determine vitality and morphology of sperm. Briefly, an aliquot of semen (5 μL) was placed on 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 lens (×100) of Nikon –Japan microscope.

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ii. Hypo-Osmotic swelling Test (HOST)

115 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 micro tube 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 cover slip. Sperm (n=200) were evaluated using a phase-contrast microscope and sperm with coiled tails were recorded (Ramu 120 and Jeyendran 2013).

iii. 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) 125 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).

iv. Mitochondrial Membrane Potential (MMP):

130 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 removal of 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 135 dissolved in DMSO; Sigma-Aldrich, MO, USA) was added to 1 mL of the diluted sample, which

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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, Fathi et al. 2018).

v. Evaluation of intracellular ROS (Reactive Oxygen Species)

Frozen–thawed semen was re-suspended with phosphate-buffered saline (PBS) at a final
140 concentration of 1-3×106 spermatozoa ml. The intracellular ROS was determined by 2,7-dichlorodihydro fluorescein diacetate (DCFH-DA) (25 µm) which were separately added to 1–3×106 sperm/ml fractions and incubated at 25°C for 40 min, respectively, in the dark room. 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) were evaluated
145 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 for the distinction of dead sperm. Data were expressed as the percentage of fluorescent spermatozoa.

f. Statistical Analysis

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

Results

g. Fresh epididymal sperm assessment

The maximum and minimum limit of initial motility and progressive motility of spermatozoa
155 were 90-95 and 85-80 respectively. The average percentage of total and progressive motility were 93.3 ± 2.9, 83.3 ± 2.9 respectively. Initial values for the HOST and Eosin- Nigrosin were 88.1

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± 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.

h. Freeze-thawed epididymal sperm assessment

160 i. 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 as compared with the fresh semen specimen ($P < 0.001$). The best total and progressive motility of sperm after thawing among groups was in the egg yolk group (46 ± 8.5, 26.3 ± 15.8 respectively). Although, among the soy-lecithin groups, 165 the higher total and progressive motility was seen in L0.4. In frozen samples, total and progressive motility of spermatozoa were lesser in all groups treated with different concentrations of lecithin as compared with the control group ($P \leq 0.05$). Table 4 shows the results of the evaluation of the motility of spermatozoa between groups after freezing and thawing.

170 ii. 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 as compared with the fresh semen specimen ($P < 0.001$). The percentage of sperm with the integrity of the plasma membrane between groups is shown in Table 5. The egg yolk extender was able to preserve the integrity of 175 the plasma membrane of the spermatozoa better than the other groups (65.1 ± 8.1), although 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.

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iii. 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 which there was no significant difference among them. 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

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Table 7.

iv. CASA

210 CASA analysis was performed on frozen-thawed semen samples to determine the effect of soy lecithin and egg yolk on motility parameters (Table 8, Table 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 as compared with the control group ($P \leq 0.05$).

215 v. MMP

The MMP level of spermatozoa in EY or LS 0.4 after the post-thawing were presented in Figure1. A comparison was made between the control and LS 0.4 groups, which revealed better results in the control than lecithin groups. Depending on the dye JC-1, the results showed there was a significant difference between the control and LS 0.4 group ($P = 0.026$).

220 vi. ROS (Superoxide) Production

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

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225 **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 to find a suitable alternative to egg yolk from a non-animal
230 source for the purpose of global trade. Since, lecithin in egg yolk plays a key role in preventing cold shock, and the latter can be obtained from vegetable sources such as soy and sunflower, the researchers focused their attention on finding the ideal type and concentration of lecithin which achieves protection of sperm from cold shock. Egg Yolk extender has been shown its efficiency to maintain a good level of total and progressive motility of sperm as well as for the integrity of
235 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
240 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
245 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

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cryopreservation was, at least partly, due to excessive elevation of ROS and impairment of mitochondrial function in sperm treated with lecithin as 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, Sharma *et al*., 2006). However, when the ROS production is excessive, an imbalance occurs between the ROS-generating system and enzymatic and non-enzymatic antioxidants that are responsible for ROS removal, and this, in turn, leads to oxidative stress. This type of stress causes structural damage to biomolecules, DNA, lipids, carbohydrates, and proteins, as well as to other cellular components, including mitochondria (Dalmazzo, Losano *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.Louis, MO, EUA), (General Nutrition Corporation, Pitts burgh, 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. If the presence of long-chained polyunsaturated fatty acids contributes to increased membrane fluidity, 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).

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270 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, Losano *et al*., 2018, Hermansson, Johannisson *et al*., 2021) and opposed to some of other studies which also studied the effect of lecithin on the ejaculated semen of dogs (Beccaglia, Anastasi et al. 2009, Beccaglia, Anastasi et al. 2009, Kmenta, Strohmayer et al. 2011, Kasimanickam, Kasimanickam *et al*.,
275 2012, Sánchez-Calabuig, Maillo *et al*., 2017, Zakošek Pipan, Casal *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 shock such as in cats (Vick, Bateman *et al*., 2012, Vansandt, Bateman *et al*., 2021), in goat (Salmani, Towhidi *et al*., 2014), in rams (Forouzanfar,
280 Sharafi *et al*., 2010), in bulls (Aires, Hinsch *et al*., 2003). Also, some other studies have shown the negative effects of Lecithin such as in buck (Sarıözkan, Bucak *et al*., 2010, Roof, Bowley *et al*., 2012, Salmani, Nabi *et al*., 2013, Tabarez, García *et al*., 2020), in black rhinoceros and Indian rhinoceros (Wojtusik, Stoops *et al*., 2018), in Japanese white rabbits (Nishijima, Kitajima *et al*., 2015), in brown bear (Alvarez-Rodríguez, Alvarez *et al*., 2013). For epididymal sperm in
285 dog Nothling et al., 2007 studied the effect of adding prostate fluids to frozen epididymal sperm in dogs and compared it with commercial 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, as well as after thawing because such addition results in better motility, longevity, and
290 sperm morphology (Nöthling, Gerber *et al*., 2007). Lopez *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

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extender AndroMed (Lopes, Soares *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 and it had a positive effect, for instance, in bulls (Mousavi, Towhidi *et al.*, 2019), in goats (Nadri, Towhidi *et al*., 2019), and in human (Mutalik, Salian *et al*., 2014). Other investigators have suggested adding enhancers to lecithin extenders, such as adding bovine serum albumin (Alcay, Toker *et al*., 2019) which had a positive effect, or the antioxidant glutathione (Zhandi and 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, Ezeh *et al*., 2009). The same result was obtained by Jenyedran, 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, Acosta *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 the use of 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 completely replace the EY and protect the sperm membranes, but it may be difficult to obtain the benefits of the entire EY with all its compounds by adding single substances.

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

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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 می دهد. **هدف:** هدف از این مطالعه بهبود دسترسی به سیستم انجماد اسپرم اپیدیدیم سگ بر اساس دوز مناسب لسیتین بود. **روش کار:** اسپرم اپیدیدیمی از بیضه های سگ های بالغ و سالم پس از اخته در مراکز درمانی دامپزشکی جمع آوری شد و به شش گروه تقسیم شدند: (گروه اول لسیتین ٪0/4 L0.4 ، گروه دوم لسیتین ٪0/8 L0.8 ، و گروه سوم لسیتین ٪ 1/2 L1.2 ، گروه چهارم لسیتین ٪ 1/6 L1.6 ، گروه پنجم لسیتین ٪2 L2 و گروه کنترل زرده تخم مرغ ٪20 EY 20). قبل از انجماد، ارزیابی تحرک، رنگ آمیزی حیاتی ائوزین نیگروزین و HOST انجام شد و پس از یخ گشایی آزمایشات روی اسپرم به کمک روش های CASA، HOST ، ائوزین نیگروزین، پتانسیل غشای میتوکندری (MMP) ، رادیکال‌های آزاد اکسیژن داخل سلولی (ROS). انجام شدند. **نتایج:** در نمونه های پس از یخ گشایی، تحرک کلی و نسبت اسپرم با یکپارچگی غشای پلاسما دست نخورده بر اساس آزمون HOS در همه گروه های تحت غلظت های مختلف لسیتین در مقایسه با گروه کنترل کمتر بود ($P \leq 0.05$). BCF در همه گروه های تحت غلظت های مختلف لسیتین در مقایسه با گروه کنترل بیشتر بود ($P \leq 0.05$). با این وجود، تحرک پیش رونده، نسبت اسپرم زنده بر اساس آزمایش ائوزین-نیگروسین، VAP، VSL، VCL، STR، LIN و ALH در گروه های مختلف آزمایشی تفاوتی ندارند ($P> 0.05$). نسبت اسپرم با عیب مورفولوژیکی بین نمونه های تازه و منجمد و در بین گروه های مختلف آزمایشی تفاوت نداشت ($P <0.05$). پتانسیل غشای میتوکندری در گروه شاهد بیشتر از گروه لسیتین ٪0/4 بود (P = 0.026). نسبت اسپرم مثبت برای ROS در گروه شاهد کمتر از گروه لسیتین ٪0/4 بود (P = 0.049). **نتیجه گیری نهایی:** زرده تخم مرغ نسبت به اکستندر های مبتنی بر لسیتین برای انجماد اسپرم اپیدیدیمی سگ برتری داشت، و لستین سویا به عنوان جایگزین مناسب برای انجماد اسپرم اپیدیدمی سگ مناسب نبود.

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

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 (100ml)	DW (100ml)	DW (100ml)

DW: Distilled water

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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

340 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

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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.

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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}

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

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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

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(%)	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.

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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.

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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.

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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]

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

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

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Mitochondrial mebrane potential (%)

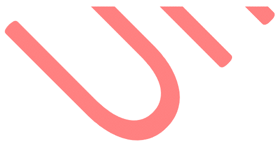
30.00
25.00
20.00
15.00
10.00
5.00
0.00

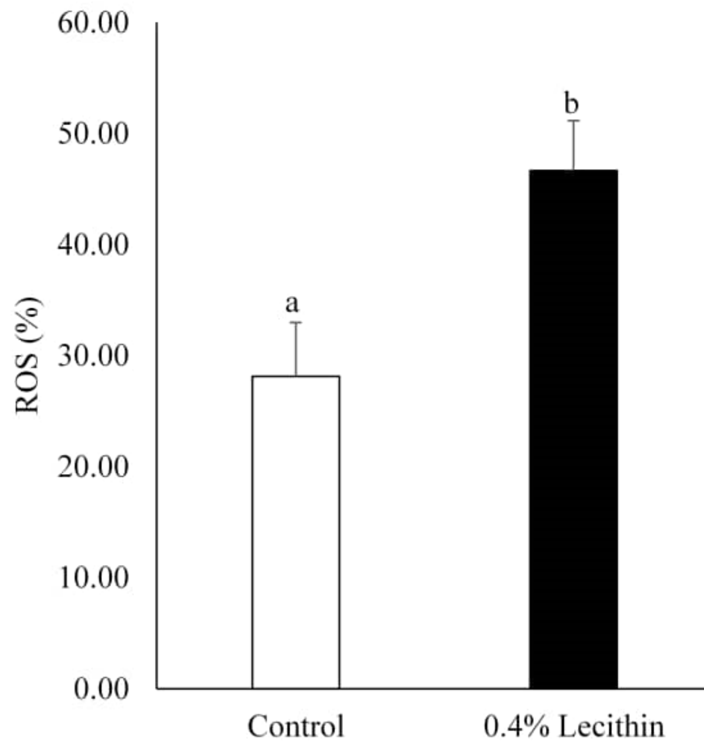
a

b

Control

0.4% Lecithin





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