Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 26 No. 3 Ser. No. 92 2025

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Assessment of lecithin nanoliposomes addition to fresh low-density lipoprotein-based diluent for cold storage of ram semen

Nahri, R.[1]**; Eslami, M.**[2*] **and Farrokhi-Ardabili, F.**[3]

[1]Graduated from Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2]Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3]Department of Animal Sciences, Faculty of Agriculture, Urmia University, Urmia, Iran

[*]***Correspondence:*** *M. Eslami, Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. E-mail: m.eslami@urmia.ac.ir*

10.22099/ijvr.2025.51361.7626

(Received 6 Oct 2024; revised version 3 Jun 2025; accepted 14 Jul 2025)

Abstract

Background: Maintaining the quality of collected semen is a crucial goal in assisted reproductive technologies. **Aims:** The present study aimed to evaluate the effect of lecithin nanoliposomes (LNLs) addition to purified low-density lipoproteins (LDL)-based extender on cold preservation of ram semen for up to 72 h. **Methods:** Ejaculates were collected from fertile rams, assessed, diluted with tris-citric acid-fructose-purified LDL extender, pooled and used within the experiment. Experimental groups supplemented with 0 (control), 1, 2, 4, and 8 mM, LNLs and stored at 4°C for 72 h. Kinematics, viability, DNA fragmentation index (DFI), membrane integrity (MI), malondialdehyde (MDA) and total nitrate nitrite (TNN) were assessed at 0, 24, 48 and 72 h. **Results:** Total motility, forward progressive motility, curvilinear velocity, average path velocity and straightness variables were improved by 1-8 mM LNLs addition ($P<0.05$). Greater viability (at 48 and 72 h) and MI (at 24, 48 and 72 h), and lower DFI (at 24, 48 and 72 h) were detected in LNLs treated groups compared with the control group ($P<0.05$). LNLs addition at 2-8 mM resulted in lower amounts of MDA and TNN at 48 and 72 h ($P<0.05$). **Conclusion:** LNLs were recommended as a protectant in ram semen tris-LDL-based diluent because they reduce peroxidative/nitrosative damage, fragmented DNA indices, and improve kinematics during cold preservation.

Key words: DNA fragmentation, Lecithin nanoliposomes, Malondialdehyde, Ram, Spermatozoa

Introduction

After semen collection and during cooling process (from 37 to 4°C), the lipids of spermatozoa membrane converted from fluid state to gel or crystalline like state. The alteration in composition and organization of spermatozoa membrane lipids during colling process was reported in detail (Holt, 2000). These events indices the irreversible damages, and reduce the fertilization potential of spermatozoa (Yeste, 2016). Due to lesser cytoplasmic contents of spermatozoa compared with somatic cells, the reactive oxygen/nitrogen species scavenging capacity of spermatozoa is very low. On the other hand, the presence of high amounts of polyunsaturated fatty acids (PUFA) and/or lower ration of cholesterol/phospholipids in small ruminants compared with bull or stallion, their spermatozoa were reported highly sensitive to cooling or freezing processes (Alvarez and Storey, 1995). One of the goals in the small ruminant theriogenology, is the maintaining or elevation of spermatozoa quality during cold or frozen preservation. Therefore, reducing the damages due to oxidative/peroxidative or nitrosative reactions is mandatory during storages as short or long term (Holt, 2000).

The main ingredients of lecithin are the phosphatidylcholines, which has capability to protect spermatozoa membrane during cooling/freezing process (Nadri *et al*., 2019). Furthermore, the antioxidative function, and reactive oxygen/nitrogen species scavenging potential of lecithin were reported when added to diluent before cooling (Nadri *et al*., 2019). The effects of lecithin on semen quality of different animal and avian species during chilled or frozen preservation were reported (Kmenta *et al*., 2011; Alvarez-Rodríguez *et al*., 2013; Singh *et al*., 2017; Dalmazzo *et al*., 2019; Nadri *et al*., 2019; Sun *et al*., 2020; Sun *et al*., 2021). However, the negative effects were reported on mitochondrial function and post-thawed kinematics of ram spermatozoa by adding lecithin to diluent (Del Valle *et al*., 2012; Mata-Campuzano *et al*., 2015). Although the capability of lecithin as a suitable substitute for egg yolk in ram semen diluent were proposed (Forouzanfar *et al*., 2010). Due to this controversy, the present study was conducted to assess the ram semen diluent supplementation with small amounts of lecithin, as nanoliposomes, concurrent with LDL during cold preservation up to 72 h. To do so, varying markers such as malondialdehyde (as peroxidative index), total nitrate-nitrite (as nitrosative index), acridine orange staining (as DNA fragmentation index), membrane integrity were evaluated along with the motion characteristics of ram spermatozoa during chilled preservation up to 72 h.

Materials and Methods

Experimental location, animals and semen collection

The studied animals were maintained at the facilities belong to the Animal Sciences Department of the Faculty of Agriculture, Urmia University, Urmia, Iran. Fertile Qezel rams (n=5) with the age of 2-3 years were allocated to semen collection using an artificial vagina in the presence of estrus ewes. Semen was gathered within breeding season (n=35 samples). All collected samples were accepted to enter the experiment due to having total and forward progressive motility greater than 85% and 75%, respectively.

Semen dilution and experimental groups

After semen collection from the rams (every sampling day), an equal volume of each semen sample (0.5-0.7 ml) was taken, pooled with other samples, diluted with Tris-citric acid-fresh LDL-based diluent at the 400 × 10^6 spermatozoa/ml, and used for the experiment. The mentioned diluent was prepared according to the previous research (Bahmani *et al*., 2023). The number of spermatozoa was counted using Neubauer slide. The diluted semen was divided into equal five experimental groups. Group 1, was served as control and did not receive any other additive. Experimental groups 2, 3, 4, and 5 received the prepared lecithin nanoliposomes at 1, 2, 4, and 8 mM, respectively. Experimental samples were put in the refrigerator (set at 4°C) using a water jacket (the initial temperature of water was at 35°C) for up to 72 h. Using slow colling method, it takes about 3 h to reach 4°C.

Preparation of fresh LDL-based diluent

The standard protocol (MacBee and Cotterill, 1979; Mortazavi *et al*., 2020) was used to extract and prepare the egg yolk plasma. Ammonium sulfate (20%, w/v) were added gradually to egg yolk plasma, and stirred at 4°C for 1 h. In order to omit the livetin, the solution was centrifuged (Hettich, Germany) for two times (9,000 ×g, 4°C, for 60 min). The upper portion of recovered solution was dialyzed using a cellulose membrane (Cut off: 1200 Da, D7884-5FT, Sigma-Aldrich) for 2 days. The obtained solution was centrifuged for additional two times, and the supernatant used as fresh pure LDL for preparation of Tris-citric acid-fresh LDL-based diluent as previously described (Bahmani *et al*., 2023).

Preparation of lecithin nanoliposomes

The lecithin nanoliposomes was prepared by dissolving 1.55 g soybean lecithin in 50 ml ethanol within a flask (Najafi *et al*., 2019). The ethanol was evaporated using a rotary evaporator (Heidolph, Germany). The dehydrated lecithin biofilm, was dissolved in 5 ml distilled water within the rotary evaporator without vacuum. The obtained solution was homogenized at 11,000 g using a cell grade homogenizer (T10 Basic, IKA®, Werke GmbH & Co. KG, Staufen, Germany). The homogenized solution was sonicated using a probe sonicate for 20 min at the highest power (FAPAN Co. Ltd., Iran). The prepared stock solution (400 mM) of lecithin nanoliposomes was stored at 4°C and used within a week. The nano particle analyzer (NPA) system (HORIBA, Ltd., SZ-100, Version 2.20, Japan) was used to assess different indices of prepared LNLs.

Assessment of spermatozoa parameters

Kinematics

The kinematics of spermatozoa were assessed under a phase-contrast microscope (Labomed Inc., Culver City, CA, USA) equipped with a digital warm stage, connected to the computer-assisted sperm analysis (CASA) software (Test Sperm 3.2, Videotest, St. Petersburg, Russia). Before assessment, the examined samples were diluted with the extender at 20 × 10^6 spermatozoa/ml, and at least 400 spermatozoa were analyzed for every evaluation. Different kinematics variable such as total motility (TM), forward progressive motility (FPM), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), linearity (LIN), and straightness (STR) were assessed at 0, 24, 48, and 72 h.

Acridine orange staining

The staining with acridine orange (Chohan *et al*., 2004) was used to evaluate the injury to DNA of ram spermatozoa at studied time points. Before staining, the extender was removed by washing using centrifugation at 550 ×g for three times. The washing media contained the ingredient of extender except than LDL and antibiotics. After washing, the smear was prepared, dried, and fixed using the Carnoy's solution (composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid, 1 g of ferric chloride). After fixation, the slides wear soaked in the acridine orange prepared stain (Polysciences®, Warrington, PA, USA) solution, for 5 min. The stain residual of smears was deleted by washing using distilled water. The stained smears were assessed under a fluorescent microscope (Nikon, Model GS7, Tokyo, Japan) with wavelength set at 490 nm. The spermatozoa with green or yellow-red fluorescence were classified as intact or damaged DNA, respectively (Fig.

1). The injury to genome of spermatozoa, was presented as DNA fragmentation index (DFI %) at 0, 24, 48, and 72 h.

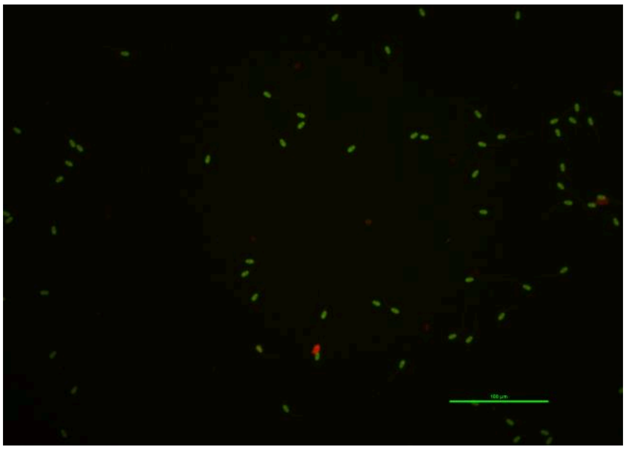

Fig. 1: Ram spermatozoa stained with acridine orange and assessed using florescent microscope. The spermatozoa with green or yellow-red fluorescence were classified as intact or damaged DNA, respectively (scale bar, 100 µm)

Eosin-nigrosine staining

The live/dead spermatozoa were assessed using eosin-nigrosine staining (Evans and Maxwell, 1987). By counting pink and white spermatozoa (at least 200 cells) under an optic microscope, the viability was reported as percentage at above-mentioned time points.

Spermatozoa membrane integrity

The hypo-osmotic swelling (HOS) assay was used to examine membrane integrity. The fresh HOS solution (Correa and Zavos, 1994) was prepared, added to the sample (8:1), and put in the water bath (37°C) for 60 min. Using a phase contrast microscope (Olympus, BX41, Tokyo, Japan) coiled-tailed spermatozoa within a total 200 cells were counted, and the intact membrane spermatozoa were calculated and reported as percentage at 0, 24, 48, and 72 h.

After performing the above-mentioned tests at every time point, the remainder of the sample was homogenized using a cell grade homogenizer (T10 Basic, IKA®, Werke GmbH & Co. KG, Staufen, Germany) at 7000 g for 3 min. The homogenate of spermatozoa-diluent stored at -20°C until malondialdehyde (MDA) measurement.

Malondialdehyde (MDA) measurement

The quantity of MDA in homogenate of spermatozoa-diluent was assessed using the described method by Stern *et al*. (2010). In brief, one part of sample was mixed with two parts of prepared solution, and incubated at 100°C for 15 min. By completing the reaction, centrifugation was done (1500 ×g for 12 min), and the optical density of supernatant was recorded at 535 nm using a visible spectrophotometry. The quantity of MDA was reposted as µmol/L.

Quantity of total nitrate-nitrite (TNN)

The quantity of TNN was measured as an indicator of nitrosative stress using the Griess reaction (Green *et al*., 1982). The prepared Griess solution was mixed with the samples, after incubation at darkness within the laboratory for 10 min, the absorbances were recorded at 540 nm using an ELISA reader (DANA, Iran). Using an standard plot, the quantity of TNN was expressed as nmol/L at 0, 24, 48, and 72 h.

Statistical analysis

Two-way ANOVA was used to analyze the effect of lecithin nanoliposomes and time (treatment × time) on kinematics, viability, spermatozoa membrane integrity, nitrate-nitrite, acridine orange staining and the MDA variables. The arcsine transformation was done on percent variables. The Holm-sidak was used as post hoc test. Analysis was performed by SigmaStat software (ver. 3.5, Chicago, IL). Probability value less than 0.05 was considered to be statistically significant. Results were presented as least-squares mean values.

Results

The reports of NPA system showed that the mean, median, mode, standard deviation and molecular weight indices for the prepared LNLs were 89.7 nm, 83.6 nm, 86.4 nm, 23.3 nm and 1.112×10^4 kDa, respectively. The minimum and maximum count rate among different prepared samples were 2169 and 2475 kCPS, respectively. The average count rate was calculated 2320 kCPS.

Greater TM were observed in LNL treated groups compared with the control group at 48, and 72 h ($P<0.001$; Table 1). Furthermore, FPM was significantly higher in LNL received groups compared with the control group at 24, 48, and 72 h time points ($P<0.001$, Table 1). Significant decrease in TM and FPM were detected at 48 and 72 h compared with 0 h in all groups ($P<0.001$, Table 1).

Higher VCL was observed in LNL treated groups compared with the control group at 48 and 72 h ($P<0.001$, Table 2). The 2, 4, and 8 mM received LNL groups showed higher VAP compared with the control group at 24, 48 and 72 h ($P<0.001$, Table 2). The STR variable was improved by all concentrations of LNL compared with the control group at 72 h ($P<0.001$, Table 2). LNL enrichment at 2 and 4 mM resulted in greater LIN compared with the control group at 24, 48 and 72 h ($P<0.001$, Table 2).

Addition of LNL at mentioned concentrations, improved the percentage of viable spermatozoa at 48 and 72 h ($P<0.001$, Table 3). Furthermore, the differences among LNL received groups with the control group in the membrane integrity variable were detected at 24, 48 and 72 h ($P<0.001$, Table 3). The lower DFI were observed in LNLs treated groups compared with the control group at 24, 48 and 72 h ($P<0.001$ Table 3).

Diluent enrichment with 2-8 mM LNL decreased the amounts of lipid peroxidation index (MDA) and TNN values at 48 and 72 h (P<0.01, Table 4).

Table 1: Percentage of total and forward progressive motility of ram spermatozoa (least square means) supplemented with lecithin nanoliposomes (LNL; 0, 1, 2, 4, and 8 mM) in the LDL-based diluent, and stored for various time points at 4°C

Parameter	Experimental groups	Time of storage (h)			
		0	24	48	72
Total motility (%)	Control	94.81 ^{Aa}	86.86 ^{Ab}	72.78 ^{Bc}	65.14 ^{Bd}
	LNL 1 mM	93.57 ^{Aa}	90.02 ^{Aa}	83.55 ^{Ab}	73.18 ^{Ac}
	LNL 2 mM	96.44 ^{Aa}	88.08 ^{Ab}	85.35 ^{Ab}	74.20 ^{Ac}
	LNL 4 mM	94.90 ^{Aa}	88.48 ^{Ab}	84.96 ^{Ab}	75.53 ^{Ac}
	LNL 8 mM	95.49 ^{Aa}	89.35 ^{Ab}	82.20 ^{Ac}	73.80 ^{Ad}
Forward progressive motility (%)	Control	77.01 ^{Aa}	57.28 ^{Bb}	45.53 ^{Bbc}	40.03 ^{Bc}
	LNL 1 mM	78.29 ^{Aa}	67.38 ^{Ab}	55.62 ^{Abc}	47.42 ^{Ac}
	LNL 2 mM	76.63 ^{Aa}	70.12 ^{Ab}	63.30 ^{Abc}	51.51 ^{Ac}
	LNL 4 mM	76.48 ^{Aa}	70.46 ^{Ab}	61.52 ^{Ac}	49.83 ^{Ac}
	LNL 8 mM	76.78 ^{Aa}	68.78 ^{Ab}	60.55 ^{Ab}	50.22 ^{Ac}

Standard error of the mean (SEM) of least square mean values for total and forward progressive motility are 2.77 and 1.94, respectively. $^{A, B}$ Values with different superscripts indicate significant differences (P<0.05) among treated groups for each variable at each time points. $^{a, b, c, d}$ Values with different superscripts indicate significant differences (P<0.05) between the data at the same row (among different time points within a group)

Table 2: Different CASA variables of ram spermatozoa (least square means) supplemented with lecithin nanoliposomes (LNL; 0, 1, 2, 4, and 8 mM) in the LDL-based diluent, and stored for various time points at 4°C

Parameter	Experimental groups	Time of storage (h)			
		0	24	48	72
VCL (µm/s)	Control	120.07 ^{Aa}	100.10 ^{Bb}	75.53 ^{Cc}	66.87 ^{Cd}
	LNL 1 mM	122.96 ^{Aa}	108.18 ^{ABb}	85.59 ^{Bc}	76.08 ^{Bd}
	LNL 2 mM	121.44 ^{Aa}	103.22 ^{ABb}	95.89 ^{Ab}	83.29 ^{ABc}
	LNL 4 mM	122.61 ^{Aa}	107.32 ^{ABb}	98.45 ^{Ac}	83.50 ^{ABd}
	LNL 8 mM	120.69 ^{Aa}	114.28 ^{Aa}	97.21 ^{Ab}	88.38 ^{Ac}
VSL (µm/s)	Control	35.38 ^{Aa}	26.26 ^{Aa}	30.81 ^{Aa}	29.16 ^{Aa}
	LNL 1 mM	35.57 ^{Aa}	39.84 ^{Aa}	32.65 ^{Aa}	28.27 ^{Aa}
	LNL 2 mM	37.68 ^{Aa}	43.66 ^{Aa}	36.33 ^{Aa}	35.35 ^{Aa}
	LNL 4 mM	36.42 ^{Aa}	42.32 ^{Aa}	36.52 ^{Aa}	33.19 ^{Aa}
	LNL 8 mM	36.63 ^{Aa}	40.02 ^{Aa}	34.68 ^{Aa}	34.58 ^{Aa}
VAP (µm/s)	Control	55.65 ^{Aa}	41.64 ^{Bb}	34.53 ^{Bc}	28.12 ^{Cd}
	LNL 1 mM	55.05 ^{Aa}	46.91 ^{ABb}	36.13 ^{ABc}	32.28 ^{Bc}
	LNL 2 mM	57.83 ^{Aa}	50.71 ^{Ab}	41.02 ^{Ac}	40.17 ^{Ac}
	LNL 4 mM	55.93 ^{Aa}	49.49 ^{Ab}	40.60 ^{Ac}	38.00 ^{ABc}
	LNL 8 mM	56.90 ^{Aa}	48.15 ^{Ab}	40.99 ^{Ac}	39.44 ^{ABc}
STR (%)	Control	81.92 ^{Aa}	80.17 ^{Aa}	80.81 ^{Aa}	74.80 ^{Bb}
	LNL 1 mM	80.19 ^{Aa}	80.64 ^{Aa}	78.82 ^{Aa}	80.01 ^{Aa}
	LNL 2 mM	82.90 ^{Aa}	83.55 ^{Aa}	81.74 ^{Aa}	83.55 ^{Aa}
	LNL 4 mM	83.17 ^{Aa}	81.68 ^{Aa}	80.03 ^{Aa}	79.83 ^{Aa}
	LNL 8 mM	82.65 ^{Aa}	79.55 ^{Aa}	79.29 ^{Aa}	80.69 ^{Aa}
LIN (%)	Control	42.92 ^{Aa}	33.67 ^{Bb}	33.01 ^{Bb}	31.31 ^{Bb}
	LNL 1 mM	42.58 ^{Aa}	37.18 ^{ABb}	35.88 ^{ABb}	32.73 ^{ABb}
	LNL 2 mM	43.97 ^{Aa}	41.60 ^{Aa}	39.24 ^{Aa}	38.70 ^{Aa}
	LNL 4 mM	44.19 ^{Aa}	41.03 ^{Aab}	38.81 ^{Ab}	37.92 ^{Ab}
	LNL 8 mM	41.92 ^{Aa}	35.33 ^{ABb}	35.23 ^{ABb}	35.44 ^{ABb}

Standard error of the mean (SEM) of least square mean values for VCL, VSL, VAP, STR, LIN are 2.81, 3.44, 1.58, 1.34, 1.48, respectively. $^{A, B, C}$ Values with different superscripts indicate significant differences (P<0.05) among treated groups for each variable at each time points. $^{a, b, c, d}$ Values with different superscripts indicate significant differences (P<0.05) between the data (among different time points within a group) at the same row

Table 3: Percentage of viability, membrane integrity (evaluated by HOST) and DNA fragmentation index (DFI) of ram spermatozoa (least square means) supplemented with lecithin nanoliposomes (LNL; 0, 1, 2, 4, and 8 mM) in the LDL-based diluent, and stored for various time points at 4°C

Parameter	Experimental groups	Time of storage (h)			
		0	24	48	72
Viability (%)	Control	94.81 ^{Aa}	86.86 ^{Ab}	72.78 ^{Bc}	65.14 ^{Bd}
	LNL 1 mM	93.57 ^{Aa}	90.02 ^{Aa}	83.55 ^{Ab}	73.18 ^{Ac}
	LNL 2 mM	96.44 ^{Aa}	88.08 ^{Ab}	85.35 ^{Ab}	74.20 ^{Ac}
	LNL 4 mM	94.90 ^{Aa}	88.48 ^{Ab}	84.96 ^{Ab}	75.53 ^{Ac}
	LNL 8 mM	95.49 ^{Aa}	89.35 ^{Ab}	82.20 ^{Ac}	73.80 ^{Ad}
Membrane integrity (%)	Control	77.01 ^{Aa}	57.28 ^{Bb}	45.53 ^{Bbc}	40.03 ^{Bc}
	LNL 1 mM	78.29 ^{Aa}	67.38 ^{Ab}	55.62 ^{Abc}	47.42 ^{Ac}
	LNL 2 mM	76.63 ^{Aa}	70.12 ^{Ab}	63.30 ^{Abc}	51.51 ^{Ac}
	LNL 4 mM	76.48 ^{Aa}	70.46 ^{Ab}	61.52 ^{Ac}	49.83 ^{Ac}
	LNL 8 mM	76.78 ^{Aa}	66.78 ^{Ab}	60.55 ^{Ab}	48.22 ^{Ac}
DFI (%)	Control	1.55 ^{Aa}	5.73 ^{Bb}	11.60 ^{Bbc}	17.12 ^{Bc}
	LNL 1 mM	1.76 ^{Aa}	3.21 ^{Ab}	5.34 ^{Abc}	8.81 ^{Ac}
	LNL 2 mM	1.41 ^{Aa}	2.74 ^{Ab}	4.51 ^{Abc}	6.39 ^{Ac}
	LNL 4 mM	1.63 ^{Aa}	2.89 ^{Ab}	4.20 ^{Ac}	7.06 ^{Ac}
	LNL 8 mM	1.49 ^{Aa}	2.67 ^{Ab}	4.78 ^{Ab}	6.13 ^{Ac}

Standard error of the mean (SEM) of least square mean values for viability, membrane integrity and DFI are 2.43, 2.15, and 0.24, respectively. $^{A, B}$ Values with different superscripts indicate significant differences (P<0.05) among treated groups for each variable at each time points. $^{a, b, c}$ Values with different superscripts indicate significant differences (P<0.05) between the data at the same row

Table 4: The quantities (least square means) of malondialdehyde (MDA) and total nitrite-nitrate (TNN) of ram semen supplemented with lecithin nanoliposomes (LNL; 0, 1, 2, 4, and 8 mM) in the LDL-based diluent, and stored for various time points at 4°C

Parameter	Experimental groups	Time of storage (h)			
		0	24	48	72
MDA (μmol/L)	Control	1.86 ^{Aa}	2.42 ^{Aab}	3.21 ^{Bb}	3.48 ^{Bb}
	LNL 1 mM	1.69 ^{Aa}	2.12 ^{Ab}	2.86 ^{ABb}	3.08 ^{ABb}
	LNL 2 mM	1.88 ^{Aa}	2.04 ^{Aab}	2.31 ^{Ab}	2.53 ^{Ab}
	LNL 4 mM	1.75 ^{Aa}	1.97 ^{Aab}	2.14 ^{Ab}	2.63 ^{Ab}
	LNL 8 mM	1.79 ^{Aa}	2.11 ^{Aab}	2.43 ^{Ab}	2.71 ^{Ab}
TNN (nmol/L)	Control	169.32 ^{Aa}	182.29 ^{Aa}	267.30 ^{Bb}	311.83 ^{Bc}
	LNL 1 mM	158.19 ^{Aa}	186.31 ^{Aa}	238.25 ^{ABb}	279.21 ^{ABc}
	LNL 2 mM	176.75 ^{Aa}	193.48 ^{Aa}	207.53 ^{Ab}	227.44 ^{Ab}
	LNL 4 mM	166.24 ^{Aa}	178.90 ^{Aa}	198.40 ^{Ab}	236.97 ^{Ac}
	LNL 8 mM	171.57 ^{Aa}	180.69 ^{Aa}	202.63 ^{Ab}	220.39 ^{Ab}

Standard error of the mean (SEM) of least square mean values for MDA and TNN values are 0.1 and 5.57, respectively. $^{A, B}$ Values with different superscripts indicate significant differences (P<0.05) among treated groups for each variable at each time points. $^{a, b, c}$ Values with different superscripts indicate significant differences (P<0.05) between the data at the same row

Discussion

The main purpose of the present study was to evaluate the effectiveness of lecithin, as nanoliposomes, together with the fresh LDL-based diluent on the quality of chilled preserved ram semen. The present study indicates the reducing effects of LNLs, at proper amounts, on the peroxidative/nitrosative and DNA fragmentation indices concurrent with improvement in kinematics of spermatozoa during cold storage, especially at 48 and 72 h.

Egg yolk has been used as cold shock protectant and a main component of ram semen diluent for many years (Salamon and Maxwell, 1995). In recent decades, soybean lecithin was introduced as egg yolk replacement, due to its lower biological risks, and examined in the extender of different animal species (Aurich *et al*., 2007; Zhang *et al*., 2009; de Paz *et al*., 2010; Singh *et al*., 2017; Dalmazzo *et al*., 2019). However, literature indicated that substitution of egg yolk with lecithin within the extender, resulted in decreased (Wojtusik *et al*., 2018) or similar (Forouzanfar *et al*., 2010; Nadri *et al*., 2019) semen quality compared with egg yolk during cold or frozen/thawed preservation. Furthermore, a previous study revealed that mitochondrial inner membrane surface (MIMS), mitochondrial inner membrane potential (MIMP), and consequently total and progressive frozen/thawed motile ram spermatozoa were significantly reduced following replacement of egg yolk with 3.5% soybean lecithin compared with egg yolk prepared diluent (Del Valle *et al*., 2012). They indicated that sublethal damages induced by lecithin reduced ram sperm functionality, which could not be detected by conventional analysis tests for spermatozoa (confirmed by changes in MIMS). Therefore, we aimed to enrich the fresh LDL prepared diluent with small amounts of LNL (0.075%-0.6%) for cold storage of ram semen. Our results revealed that, using small doses of lecithin (especially 2-8 mM) improved the kinematics (varying CASA indices), viability and membrane integrity variables of cold stored ram semen. Using 0.5-3.5% soybean lecithin did not improve ram semen total and progressive motility compared with egg yolk-based diluent during liquid storage (de Paz *et al*., 2010). Furthermore, by replacement of lecithin (2-4%) with egg yolk, motility of goat spermatozoa was reduced compared with egg yolk extender (Nadri *et al*., 2019). However, replacing the egg yolk with nano-lecithin (at 2% w/v) improved some CASA variables of frozen/thawed goat spermatozoa (Nadri *et al*., 2019). In the present study, lecithin was used as nano-liposome particles. The particle size of used lecithin within the diluent could potentially affect the semen quality during cold or frozen storage (Mousavi *et al*., 2019; Nadri *et al*., 2019). The smaller particle size, introduced as nanoparticles or LNLs, has greater solubility in aqueous based diluent (extender) compared with conventional forms (Nadri *et al*., 2019). The another reported influencing factor is the concentration of added or used lecithin. The optimum concentrations of lecithin for cold or frozen storage of boar (Wojtusik *et al*., 2018), goat (Nadri *et al*., 2019), ram (Forouzanfar *et al*., 2010), buffalo bull (Singh *et al*., 2017), rooster (Sun *et al*., 2021), and canine (Dalmazzo *et al*., 2019) semen were reported >2%, 2%, 1%, 1.5%, 1%, and 0.1%, respectively. In the present experiment LNL used amounts were 1 mM, 2 mM, 4 mM, and 8 mM which is equivalent to 0.075%, 0.15%, 0.3%, and 0.6% within the diluent, respectively. We used LNL concurrent with fresh derived LDL of egg yolk, while the mentioned optimum reported amounts of liposomal lecithin-based diluent (Forouzanfar *et al*., 2010; Nadri *et al*., 2019) omitted the egg yolk or its derivatives from their study. Therefore, reported optimum amounts of our study was remarkably lesser compared with those reported in other studies. Furthermore, these wide range of variety may also due to species-specific differences (Sun *et al*., 2021).

During extended liquid-cold storage of semen, the semen quality was reduced due to peroxidation of spermatozoa membrane. Higher presence of polyunsaturated fatty acids in ram spermatozoa membrane, causes more susceptibility of them to peroxidative reaction (Ashrafi *et al*., 2011). Our experiment indicates the protective role of LNLs against destructive effects of peroxidative reaction, using measure of MDA, as the main indicator of lipid peroxidation index. It was proposed that, nano-particles of lecithin might prevent the depletion of phospholipids, and then more stabilize the membrane permeability of spermatozoa during cooling/freezing processes (Röpke *et al*., 2011). It seems that LNLs could facilitate the mentioned process, due to their larger surface-to-volume ratio on spermatozoa (Nadri *et al*., 2019). Furthermore, LNLs could protect the spermatozoa via coating of the plasma membrane (Ricker *et al*., 2006) and/or interaction with damaging components of seminal plasma such as seminal plasma proteins. However, we did not measure the anti-oxidant capacity of store samples, but an interesting finding revealed an association of increasing anti-oxidant capacity of frozen-thawed sample with decrease in the particle size of the used lecithin (Mousavi *et al*., 2019). Lower lipid peroxidation in treated groups with 2-8 mM LNLs, may also due to higher amounts of anti-oxidant capacity concurrent with coating properties or capability to interaction with seminal plasma proteins during cold storage.

Excessive presence of reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, can quickly react with different macromolecules and consequently alters the stability of membrane lipids, proteins, various enzymes involved in critical metabolic pathways such as replication, transcription, and translation machinery (Molodtsov *et al*., 2013; Meng *et al*., 2018). During semen preservation, excessive produced RNS could reduce motility and viability of spermatozoa via interact with membrane lipids, proteins and DNA (Moran *et al*., 2008; Santiso *et al*., 2012; Uribe *et al*., 2015). Increased lipid peroxidation was verified previously by exposure of spermatozoa to RNS species (Oztezcan *et al*., 1999). Furthermore, a positive correlation had been reported between RNS and DNA fragmentation of human spermatozoa (Khosravi *et al*., 2014). Research on human indicated higher amounts of tyrosine nitration in asthenospermic infertile men as compared with normospermic men (Vignini *et al*., 2006). The results of the present study revealed that by adding LNLs (especially at 2-8 mM), the indices of lipid peroxidation (MDA) and nitrosative reaction (TNN) were significantly reduced, and finally the percent of spermatozoa with intact DNA fragmentation were improved, verifying the previous reports (Hou *et al*., 2008; Serrano *et al*., 2020).

In conclusion, positive role of lecithin concurrent with LDL in Tris-based diluent during liquid-cold storage of ram semen was evidenced in our study. It seems that, LNLs were act via protection against peroxidative/nitrosative reactions and consequently well-being of membrane integrity. Furthermore, our study revealed the LNLs role on the reduction of DNA fragmentation index, which is influence the fertility of spermatozoa. Due to the mentioned positive roles, the LNLs, especially at 8 and 16 mM, was proposed as a protectant concurrent with egg yolk LDL during ram semen cold preservation.

Acknowledgements

The authors wish to thanks the personnel's of Dam Zist Kara Company for their cooperation during semen collection.

Conflict of interest

The authors had no conflict of interest.

References

Alvarez, JG and Storey, BT (1995). Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol. Reprod. Dev., 42: 334-346.

Alvarez-Rodríguez, M; Alvarez, M; Anel-Lópe, L; Martínez-Rodríguez, C; Martínez-Pastor, F; Borragan, S; Anel, L and 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. Reprod. Fertil. Dev., 25: 1185-1193.

Ashrafi, I; Kohram, H; Naijian, H; Bahreini, M and Poorhamdollah, M (2011). Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5°C. Afr. J. Biotechnol., 10: 6670-6674.

Aurich, C; Seeber, P and Muller-Schlosser, F (2007). Comparison of different extenders with defined protein composition for storage of stallion spermatozoa at 5°C. Reprod. Domest. Anim., 42: 445-448.

Bahmani, S; Eslami, M; Farrokhi-Ardabili, F; Imani, M and Batavani, RA (2023). Evaluation of chicken egg yolk plasma and low-density lipoprotein alone or enriched with ewe or cow skim milk in tris-citric acid-based diluent for cryostorage of ram semen. Biopreserv. Biobank., 21: 346-354.

Chohan, K; Griffin, J and Carrell, D (2004). Evaluation of chromatin integrity in human sperm using acridine orange staining with different fixatives and after cryopreservation. Andrologia. 36: 321-326.

Correa, JR and Zavos, PM (1994). The hypoosmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology. 42: 351-360.

Dalmazzo, A; Daniel, DSRA; Losano, JDA; Rocha, C; Sobrinho, CAB; Chinait Gurgel, JR; Monteiro Pacheco, PI; Minazaki, CK; Crusco, SE and Nichi, M (2019). Insights into soy lecithin and egg yolk-based extenders for chilling canine spermatozoa. Zygote. 1: 17-24.

Del Valle, I; Gómez-Durán, A; Holt, WV; Muiño-Blanco, T and Cebrián-Pérez, JA (2012). Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. J. Androl., 33: 717-725.

de Paz, P; Esteso, MC; Alvarez, M; Mata, M; Chamorro, CA and Anel, L (2010). Development of extender based on soybean lecithin for its application in liquid ram semen. Theriogenology. 74: 663-671.

Evans, G and Maxwell, WMC (1987). Frozen storage of semen. In: *Salamon's artificial insemination of sheep and goats*. (1st Edn.), Butterworths, Wellington, Butterworth-Heinemann. PP: 122-141.

Forouzanfar, M; Sharafi, M; Hosseini, S; Ostadhosseini, S; Hajian, M; Hosseini, L; Abedi, P; Nili N; Rahmani, H and Nasr-Esfahani, M (2010). *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology. 73: 480-487.

Green, LC; Wagner, DA; Glogowski, J; Skipper, PL; Wishnok, JS and Tannenbaum, SR (1982). Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal. Biochem., 126: 131-138.

Holt, WV (2000). Basic aspects of frozen storage of semen. Anim. Reprod. Sci., 62: 3-22.

Hou, ML; Huang, SY; Lai, YK and Lee, WC (2008). Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. Anim. Reprod. Sci., 104: 56-68.

Khosravi, F; Valojerdi, MR; Amanlou, M; Karimian, L and Abolhassani, F (2014). Relationship of seminal reactive nitrogen and oxygen species and total antioxidant capacity with sperm DNA fragmentation in infertile couples with normal and abnormal sperm parameters. Andrologia. 46: 17-23.

Kmenta, I; Strohmayer, C; Müller-Schlösser, F and 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: 1095-1103.

MacBee, LE and Cotterill, OJ (1979). Ion-exchange chromatography and electrophoresis of egg yolk proteins. J. Food Sci., 44: 656-660.

Mata-Campuzano, M; Álvarez-Rodríguez, M; Álvarez, M; Tamayo-Canul, J; Anel, L; Paz, PD and Martínez-Pastor, F (2015). Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. Theriogenology. 83: 520-528.

Meng, Q; Yin, J; Jin, M and Gao, H (2018). Distinct nitrite and nitric oxide physiologies in *Escherichia coli* and *Shewanella oneidensis*. Appl. Environ. Microbiol., 84: e00559-18.

Molodtsov, V; Nawarathne, IN; Scharf, NT; Kirchhoff, PD; Showalter, HD; Garcia, GA and Murakami, KS (2013). X-ray crystal structure of the *Escherichia coli* RNA polymerase in complex with Benzoxazinorifamycin. J. Med. Chem., 56: 4758-4763.

Moran, JM; Madejón, L; Ortega Ferrusola, C and Peňa, FJ (2008). Nitric oxide induces caspase activity in boar spermatozoa. Theriogenology. 70: 91-96.

Mortazavi, SH; Eslami, M and Farrokhi-Ardabili, F (2020). Comparison of different carrier-compounds and varying concentrations of oleic acid on freezing tolerance of ram spermatozoa in tris-citric acid-egg yolk plasma semen diluent. Anim. Reprod. Sci., 219: 106533.

Mousavi, SM; Towhidi, A; Zhandi, M; Amoabediny, G; Mohammadi-Sangcheshmeh, A; Sharafi, M and Hussaini, SMH (2019). Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. Anim. Reprod. Sci., 209: 106171.

Nadri, T; Towhidi, A; Zeinoaldini, S; Martínez-Pastor, F; Mousavi, M; Noei, R; Tar, M and Sangcheshmeh, AM (2019). Lecithin nanoparticles enhance the cryosurvival of caprine sperm. Theriogenology. 133: 38-44.

Najafi, A; Taheri, RA; Mehdipour, M; Martínez-Pastor; M and Rouhollahi, A (2019). Improvement of post-thawed sperm quality in broiler breeder roosters by ellagic acid loaded liposomes. Poult. Sci., 98: 440-446.

Oztezcan, S; Turkoglu, UM; Kervancioglu, E; Kocak, T; Kocak-Toker, N and Aykac-Toker, G (1999). *In vitro* effects of peroxynitrite on human spermatozoa. Andrologia. 31: 195-198.

Ricker, JV; Linfor, JJ; Delfino, WJ; Kysar, P; Scholtz, EL; Tablin, F; Crowe, JH; Ball, BA and Meyers, SA (2006). Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. Biol. Reprod., 74: 359-365.

Röpke, T; Oldenhof, H; Leiding, C; Sieme, H; Bollwein, H and Wolkers, W (2011). Liposomes for cryopreservation of bovine sperm. Theriogenology. 76: 1465-1472.

Salamon, S and Maxwell, WMC (1995). Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci., 37: 185-249.

Santiso, R; Tamayo, M; Gosálvez, J; Johnston, S; Mari, A; Fernández, C; Losada, C and Fernández, JL (2012). DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. Mutat. Res-/Fund. Mol. Mech. Mut., 734: 41-49.

Serrano, R; Garrido, N; Céspedes, JA; González-Fernández, L; García-Marín, LJ and Bragado, MJ (2020). Molecular mechanisms involved in the impairment of boar sperm motility by peroxynitrite-induced nitrosative stress. Int. J. Mol. Sci., 21: 1208.

Singh, AK; Kumar, A; Honparkhe, M; Kaur, S and Brar, PS (2017). Comparison of *in vitro* and *in vivo* fertilizing potential of buffalo bull semen frozen in egg yolk-, soya bean lecithin- and liposome-based extenders. Reprod. Domest. Anim., 53: 195-202.

Stern, ST; Potter, TM and Neun, BW (2010). Hep G2 hepatocyte lipid peroxidation assay. NCL Method GTA-4. Version 1.1. National Cancer Institute's Nanotechnology Characterization Laboratory Assay Cascade Protocols [Internet]. Bethesda (MD): National Cancer Institute (US). doi: 10.17917/H65D-SJ70.

Sun, LW; Fan, WH; Wu, CF; Zhang, SS; Dai, JJ and Zhang, DF (2020). Effect of substituting different concentrations of soybean lecithin and egg yolk in tris-based extender on goat semen cryopreservation. Cryobiology. 92: 146-150.

Sun, L; He, M; Wu, C; Zhang, S; Dai, J and Zhang, D (2021). Beneficial influence of soybean lecithin nanoparticles on rooster frozen-thawed semen quality and fertility. Animals. 11: 1769.

Uribe, P; Boguen, R; Treulen, F; Sánchez, R and Villegas, JV (2015). Peroxynitrite-mediated nitrosative stress decreases motility and mitochondrial membrane potential in human spermatozoa. Mol. Hum. Reprod., 21: 237-243.

Vignini, A; Nanetti, L; Buldreghini, E; Moroni, C; Ricciardo-Lamonica, G; Mantero, F; Boscaro, M; Mazzanti, L and Balercia, G (2006). The production of peroxynitrite by human spermatozoa may affect sperm motility through the formation of protein nitrotyrosine. Fertil. Steril., 85: 947-953.

Wojtusik, J; Stoops, MA and Roth, TL (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.

Yeste, M (2016). Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 85: 47-64.

Zhang, SS; Hu, JH; Li, QW; Jiang, ZL and Zhang, XY (2009). The cryoprotective effects of soybean lecithin on boar spermatozoa quality. Afr. J. Biotechnol., 8: 6476-6480.