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The Effects on Post-Thaw Sperm Quality and Nuclear DNA Integrity of Supplementation of Low-Density Lipoprotein to Freezing Extender in the Mouse

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ABSTRACT Mice are an important research tool for genetic and molecular biology, allowing researchers to explore a variety of human illness models. Egg yolk is a common component of semen extenders for domestic animals and low-density lipoproteins (LDL) from egg yolk have some cryoprotective properties. This study aimed to investigate sperm quality characteristics and nuclear DNA integrity after post-thawing in an extender (18% raffinose + 3% skim milk) supplemented with different concentrations of LDL (2.5%, 5.0%, 7.5%, or 10%) in mice. 18% Raffinose+3% skim milk extender was used as a control group without LDL. CD-1 mice were used in the study, and semen was collected from the cauda epididymis and diluted with the extender. The straws were then frozen and thawed to evaluate progressive motility, viability, plasma membrane (HOST), acrosome, and nuclear DNA integrity parameters. Fresh sperm had the highest progressive motility, viability, plasma membrane integrity, and longevity (endurance) of progressive motility for 4 h in HTF solution. The greatest spermatologic results, including nuclear DNA integrity, were determined in fresh sperm ($p<0.05$). The greatest post-thaw progressive motility (56.7±1.3%), viability (11.6±2.9%), membrane integrity (63.0±2.7%), and longevity at the end of the 4 h (31.3±2.1%) were found in the group with supplementation of 2.5% LDL compared with the control group. The lowest progressive motility, viability, and plasma membrane integrity were determined in the 10% LDL group ($p<0.05$). The cryopreservation process increased the rates of fragmented DNA in all tested LDL and control groups compared with fresh sperm ($p<0.05$). There was no statistical difference between the control and experimental groups in terms of nuclear DNA damage after freezing and thawing ($p<0.05$). It was concluded that the addition of 2.5% LDL to the extender improved the spermatological quality parameters after freezing and could be used in the freezing and preservation of mouse sperm as it showed a higher protective effect against cold shock.

Keywords: *Low-density lipoprotein, Mouse sperm, Sperm analysis.*

ÖZ

Farelerde Sperma Sulandırıcısına Düşük Yoğunluklu Lipoprotein İlavesinin Dondurma Çözdürme Sonrası Sperm Kalitesi ve Nükleer DNA Bütünlüğü Üzerine Etkileri

Fareler genetik ve moleküler biyoloji için önemli bir araştırma aracıdır ve araştırmacıların çeşitli insan hastalığı modellerini keşfetmesine olanak tanır. Yumurta sarısı, evcil hayvanlara yönelik sperma sulandırıcılarının yaygın bir bileşenidir ve yumurta sarısından elde edilen düşük yoğunluklu lipoproteinlerin (LDL) bazı kriyoprotektif özellikleri vardır. Bu çalışma, farede farklı konsantrasyonlarda LDL (%2.5, %5.0, %7.5, %10) ilaveli sperma sulandırıcısında (%18 rafinoz + %3 yağsız süt) dondurma sonrası sperm kalite özelliklerini ve nükleer DNA bütünlüğünü araştırmayı amaçladı. LDL içermeyen kontrol grubu olarak %18 Raffinoz+%3 yağsız süt sulandırıcısı kullanıldı. Çalışmada CD-1 fareler kullanıldı ve kauda epididimisten sperma toplandı ve sperma sulandırıcısı ile sulandırıldı, payetlere çekilerek motilite, canlılık, plazma zarı (HOST), akrozom ve nükleer DNA bütünlüğü parametreleri dondurma sonrası değerlendirildi. Nativ sperma, HTF çözeltisinde 4 saat boyunca en yüksek motilite, canlılık, plazma zarı bütünlüğüne ve dayanıklılığa sahip bulundu. Nükleer DNA bütünlüğü taze spermada en yüksek belirlendi ($p<0.05$). Çözdürme sonrası en yüksek motilite (%56.7±1.3), canlılık (%11.6±2.9), membran bütünlüğü (%63.0±2.7) ve yaşam süresi (%31.3±2.1) %2.5 LDL takviyeli grupta, en düşük progresif motilite, canlılık ve plazma membran bütünlüğü ise %10 LDL grubunda belirlendi ($p<0.05$). Kriyoprezervasyon işlemi, taze sperm ile karşılaştırıldığında tüm LDL ve kontrol gruplarında parçalanmış DNA oranlarını arttırdı ($p<0.05$). Kontrol ve deney grupları arasında donma ve çözdürme sonrası nükleer DNA hasarı açısından istatistiksel fark yoktu ($p<0.05$). Sulandırıcıya %2.5 LDL ilavesinin dondurma sonrası spermatolojik kalite parametrelerini iyileştirdiği ve fare sperminin dondurulması ve korunmasında daha yüksek oranda soğuk şokuna karşı koruyucu etki gösterdiği için kullanılabileceği sonucuna varıldı.

Anahtar Kelimeler: *Düşük yoğunluklu lipoprotein, Fare spermi, Sperm analizi.*

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INTRODUCTION

Today, the mouse is used for many studies on mammalian genetics and early development. Mice with transgenes, disrupted genes, or mutant genes are routinely produced, resulting in many useful genomes that must be preserved. To avoid the unintended loss of this unique material, efficient and trustworthy technologies for gamete and embryo cryopreservation are required.

Cryopreservation of mouse spermatozoa has been achieved. Using raffinose and skim milk for cryoprotection, Nakagata has successfully cryopreserved spermatozoa from a range of strains and transgenic stocks (Nakagata 2000). Mouse spermatozoa are highly sensitive to several types of mechanical stress, free oxygen radicals, osmotic changes, and different temperatures (Ward et al. 2003; Yildiz et al. 2007). The most commonly described adverse effect of post-thawing on mouse sperm is the dramatic decrease in sperm motility, plasma membrane integrity, and fertility (Therien et al. 1999; Yildiz et al. 2010). Long-term storage of sperm is a valuable technique for the preservation of genetic resources. Egg yolk is a common component of semen cryopreservation extenders used in domestic animals. However, the use of egg yolk has negative effects, such as representing a potential risk of bacterial contamination for artificial insemination, and the high-density lipoproteins in egg yolk decrease the quality of semen by causing an efflux of cholesterol from the sperm plasma membrane and resulting in a change in fluidity that increases the sensitivity to cold shock (Ward et al. 2003). Aiming to overcome these risks, various studies have demonstrated the possibility of removing some parts of egg yolk by centrifugation. LDL which is an egg yolk component, increases sperm resistance against cold shock, resulting in improved sperm motility. Some of the egg yolk ingredients could impair spermatozoa respiration and motility. Alternatively, it has been shown that centrifuged chicken egg yolk or purified low-density lipoproteins (LDL) from egg yolk can improve the quality of semen (Ward et al. 2003). Egg yolks can be fractionated by centrifugation. Egg yolk plasma consists of 85% LDL whereas its granules consist of 12% LDL. However, egg yolk composition is not uniform and could vary depending on the avian species (Takeo and Nakagata 2010; Yildiz et al. 2013). Many studies have observed that the LDL from egg yolk has cryoprotective properties. The LDL can form an interfacial film during the freezing-thawing process by adhering to the cell membrane, protecting the membrane phospholipid integrity and replacing membrane phospholipids that are lost during the cryopreservation process, or by interacting with or binding harmful proteins present in seminal plasma during cryopreservation (Hu et al. 2011; Yildiz et al. 2013; Varela et al. 2020).

The freezing-thawing process induces thermal, osmotic, and oxidative stresses on the sperm including DNA damage, and reduces their functionality and fertilization rate (Yildiz et al. 2007; Tonieto et al. 2010). In previous studies, 4–12% LDL in semen extenders for different species could achieve better results than traditional egg yolk. It was demonstrated that LDL addition to extenders is a promising alternative to the conventional use of egg yolk (Tonieto et al. 2010; Mahiddine and Kim 2021).

In previous studies, the addition of LDL to cryopreservation extenders was studied in boars (Jiang et al. 2006; Yamauchi et al. 2009; Wang et al. 2014), bulls (Moussa et al. 2002; Amirat et al. 2004; Hu et al. 2011), dogs (Junior et al. 2009; Bencharif et al. 2010), rabbits (Iaffaldano et al. 2014), rams (Therien et al. 1999) and horses (Moreno et al. 2013). To the best of our knowledge, this is the first research report related to low-density lipoprotein on sperm cryopreservation in mice.

The objective of the current study was to evaluate the sperm quality parameters and nuclear DNA integrity after thawing in extenders supplemented with different concentrations of low-density lipoproteins (2.5%, 5.0%, 7.5%, and 10%) in mouse sperm.

MATERIAL AND METHODS

The study was carried out by obtaining permission from Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee dated 21/05/2019 and numbered 20879420-825, and the study was conducted in accordance with the ethics committee rules. All chemicals used in this study were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Animals

The mouse strains of 36 CD-1 breeds used in the study were obtained from the Kobay Laboratories (Ankara, Türkiye). During the experiment time, the mice were housed by the experimental animal unit of Hatay Mustafa Kemal University. Light control (12 h light and 12 h dark) was provided. Feed and water were provided *ad libitum*. All mice used in this study were cared for according to the criteria stipulated in the "Guidelines for the Care and Use of Laboratory Animals" determined by the National Academy of Sciences and published by the National Institutes of Health. Male mice 12-14 weeks old used in the study were sacrificed by the cervical dislocation method under ketamine/xylazine anesthesia, and the cauda epididymis, which was removed from the adipose tissue by surgical method, was dissected.

Extraction of low-density lipoprotein (LDL)

LDL is obtained from egg yolks according to Moussa et al. (2002). The yolk was centrifuged at 10000 g for 45 min at 4 °C after dilution (1:1) with 0.17M NaCl. The supernatant was collected and centrifuged again at 10000 g x 45 mins at 4 °C. After centrifugation, plasma was mixed with %40 (w/v) ammonium sulfate ($(NH_4)2SO_4$) (Sigma 1.01217, Darmstadt, Germany) at pH 8.7 to precipitate subunits at 4 °C. Ammonium sulfate was added to the plasma at a ratio of approximately 1:1 until precipitation was observed. Centrifugation at 10000 g for 45 mins at 4 °C was practiced separating the supernatant from the sediment.

The supernatant was then dialyzed (Sigma Aldrich D6066, Darmstadt, Germany) against distilled water to eliminate ammonium sulfate for 12 h at 4 °C with the distilled water changed every 2 h. After ammonium sulfate elimination, the substance was centrifuged at 10000 g for 45 min, and the LDL-rich supernatant was collected, aliquoted, and stored at -80 °C for further use. The extracted LDL was used as dried.

Preparation of sperm extenders

In this study, low-density lipoproteins (LDL) (2.5%, 5.0%, 7.5%, and 10%) were added to the mouse sperm extender, which is a combination of 18% raffinose (Biobasic-RJ392) + 3% skimmed milk powder (Monn-bio) (Moustacas et al. 2011). Spermatozoa in the epididymal tissue were diluted separately with the diluents in the control and study groups and then incubated at 37 °C for 10 min. Epididymal spermatozoa samples were adjusted to a final concentration of 1-3 x 10^7 /ml spermatozoa. The freezing extender (18% raffinose + 3% skimmed milk powder without LDL) was used as a control group. The chemicals

were dissolved in 100 ml of distilled water and cooled and centrifuged at 14000g for 10 min. After centrifugation, the supernatant was collected and passed through filters with 0.22-µm pores. Sperm extenders were prepared individually by adding low-density lipoprotein (LDL) at the determined rates (2.5%, 5.0%, 7.5%, and 10%). After the freezing-thawing process, the sperm samples were transferred into an HTF solution for evaluation. The HTF solution was prepared according to Quinn et al. (1985). The formulation of human tubal fluid (HTF medium) was 101.6 NaCl, 4.69 KCl, 0.20 $MgSO_4 7H_2O$, 0.37 KH_2PO_4, 2.04 $CaCl_2 2H_2O$, 25 $NaHCO_3$, 2.78 glucose, 0.33 Na pyruvate, 21.4 Na lactate, 100 U/ml penicillin, 50 µg/ml streptomycin SO_4, 0.001% (wt/vol).

Sperm collection, freezing, and thawing procedures

Sperm collection from the cauda epididymides of mice was performed according to Takeo and Nakagata (2018). The left and right caudal epididymis were aseptically dissected and placed in the same petri dish (Isolab 60x15mm) containing the extender preheated to 37 °C in an incubator. Under a stereomicroscope, Cauda epididymides were minced using a scalpel and scissors. Spermatozoa were allowed to exit into the extender for 10 min in the incubator. All experimental groups were frozen and thawed using the Nakagata (2000) protocol. Sperm samples were also examined before being processed as fresh sperm. Samples with a motility of less than 50% were not frozen. Briefly, 0.25 ml straws, the ends of which were closed with a press, were frozen in liquid nitrogen (LN_2) vapor for 10 min on the shelves. The level of the straws in the styrofoam was 3.5 cm, and the straws were placed on a shelf approximately 6 cm above the liquid nitrogen level. At the end of 10 min, the straws were taken from the shelf and immersed directly in LN_2. Subsequently, the straws were transferred to nitrogen tanks until use. The frozen samples for thawing were quickly transferred to a 37 °C water bath for 30 sec.

Sperm analysis

Thawed straws were transferred into a microfuge (Isolab) tube containing HTF medium (1:4) and kept in a 37 °C carbon dioxide incubator. Spermatological analyses of thawed sperm samples, which were kept in the incubator for 10 min, were performed.

Assessment of progressive motility

The examinations were performed after taking 10 µl of semen samples**,** diluting the HTF solution with an additional 1/10 ratio, and allowing it to equilibrate for 10 min. Progressive motility determination of sperm samples was performed using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Briefly, 10µl of semen diluted with HTF prepared after incubation was taken on a Makler slide and evaluated at 200x magnification under a phase-contrast microscope (Olympus CX21, Japan). At least 200 spermatozoa were counted from motile spermatozoa that moved forward in the slide area, as strong, smooth linear spermatozoa, and non-motile spermatozoa. In the study groups, progressive motility will be evaluated for 4 hours and recorded as the longevity of progressive motility.

Assessment of the dead spermatozoa

Dead examination of the sperm was performed using the eosin staining method (WHO 1999). Briefly, 5 µL of eosin solution and 5 µL of sperm sample were mixed on a microscope slide, smeared, and quickly (2 seconds) dried in a preheated area at 60 °C. The prepared samples of 300 cells were counted at 400x magnification under a phase-contrast microscope (Olympus CX21, Japan). In the evaluation, while dead spermatozoa were dyed, live spermatozoa did not receive the dye.

Assessment of plasma membrane integrity (Hypo Osmotic Swelling Test/ HOST)

The HOS test method was used to evaluate membrane function integrity (Jeyendran 1984). The HOS test was evaluated by mixing 30 µL of sperm sample with 300 µL of HOST solution with an osmolarity of 100 mOsm (1.351 g fructose + 0.735 g sodium citrate per 100 mL of distilled water) and incubating at 37 °C for 60 min. Viable spermatozoa with active membrane function were identified as swollen and with a curled tail.

Assessment of acrosome integrity

Analysis of acrosome integrity was performed as indicated by Somfai et al (2002). Hancock solution was used for fixation and determination of acrosome damage. It was ensured that 1 ml of Hancock solution and 20 µl of semen were detected in the Eppendorf tube. 50 µl of this mixture was taken on a slide, covered with a coverslip, and examined with a 100x objective with oil immersion. The percentage of spermatozoa with acrosome damage was determined and counted in 200 spermatozoa in total.

Assessment of the fragmented DNA rates

In this study, the TUNEL assay method was used to determine the DNA damage in spermatozoa. For the TUNEL assay, a commercial kit (ApopTag Fluorescein in Situ Detection Kit, Sigma, S7110) was used, and the sperm suspension was prepared and evaluated according to the manufacturer's instructions. However, some parts of the protocol have been modified as follows; to separate the spermatozoa from the extender, it was washed by centrifugation (twice at 500 g, in 1 mL of PBS containing 0.01% polyvinyl-alcohol (PVA). The semen pellet formed after centrifugation was incubated for 40 min in PBS containing 0.1% Triton X-100 to ensure cell permeability. After each treatment to the sperm suspensions, they were washed once in PBS at 500 g for 10 min in centrifugation. TDT enzyme was added as stated in the kit protocol after incubating at room temperature for 10 sec with an equilibration buffer. Incubation was followed for 60 min at 37 °C in a dark chamber. In the next step of the process, STOP solution was added to complete the activation of the TDT enzyme. Subsequently, the sperm suspension was washed twice in PBS. The DNA of the cells was determined by peroxidase-dependent anti-digoxigenin antibody (ADG) after 30 min of incubation in a moist and dark room. Sperm samples were incubated for 1 h with propidium iodide (PI) for counterstaining. Finally, the slides were smeared from the suspension, and all samples were examined under a fluorescent microscope to calculate the percentage of TUNEL-positive sperm (Sharma et al. 2013; Evenson 2016).

Statistical Analysis

All values found at the end of the study were analyzed in SPSS (SPSS 22) using ANOVA. The comparison of LDL groups with the control group was made using Dunnett's test. Data are expressed as mean±SEM. Statistical significance was adjusted at $p<0.05$.

RESULTS

Spermatological values determined in fresh sperm and after freezing-thawing of mouse sperm with different concentrations of low-density lipoproteins (LDL) are given in Tables 1 and 2.

1) Progressive motility: As can be observed in Table 1, fresh sperm progressive motility, 72.4±3.7%, was found to be the highest when compared to the other cryopreserved groups**;** Control, 2.5% LDL, 5.0% LDL, 7.5% LDL, and 10% LDL, as 43.3±2.6%, 56.7±1.3%, 40.7±1.9%, 27.5±1.7%, and 12.5±2.9%, respectively ($p<0.05$). After freezing and thawing, the progressive motility rate in the group containing 2.5% LDL was higher than that in the control and other study groups ($p<0.05$). However, when compared with the progressive motility rate of fresh sperm, the 2.5% LDL group was found to be lower than that of fresh sperm ($p<0.05$). The progressive motility rate of the group with 10% LDL was found to be the lowest among all study groups ($p<0.05$).

2) Longevity of progressive motility: When the progressive motility results up to 4 h in HTF medium after thawing were examined, it was found that the progressive motility rates of fresh sperm at the 1st, 2nd, 3rd, and 4th hours were 69.6±3.4%, 63.2±2.7%, 53.2±3.4%, and 45.4±2.8% respectively, as shown in Table 2, and the results were higher compared with the other research groups ($p<0.05$). Considering the cryopreserved groups, the progressive motility rates of 2.5% LDL, 52.7±2.4%, 47.1±3.6%, 40.9%±1.8%, and 31.3±2.1%, respectively, were found to be higher than the control and other study groups during 4 h. At the same time, the lowest longevity of progressive motility rates was found in the 10% LDL group up to 4 h ($p<0.05$).

3) Dead spermatozoa rate: Considering the rates of dead spermatozoa, the fresh sperm group had the lowest rate of dead sperm compared with the other cryopreserved study groups, which are Control, 2.5% LDL, 5.0% LDL, 7.5% LDL, and 10% LDL, 18.6±1.7%, 11.6±2.9%, 24.2±1.4%, 19.6±1.5%, 56.4±1.3%, respectively ($p<0.05$). When comparing values between cryopreserved LDL and control groups after freezing-thawing, 2.5% LDL had the lowest dead spermatozoa rate whereas 10% LDL had the highest percentage of dead sperm ($p<0.05$).

4) Plasma membrane integrity (HOST): Contemplating the values of the HOS Test showing membrane integrity, curled-tailed sperm rates of fresh sperm were the highest (78.1±3.9%) compared with other studied groups**;** 48.9±3.1%, 63.0±2.7%, 41.1±1.9%, 33.7±2.6%, 14.3±1.8%, respectively ($p<0.05$). After freezing-thawing, the integrated membrane rate was found to be higher in the group containing 2.5% LDL than in the other study groups ($p<0.05$).

5) Abnormal acrosome: Considering the acrosome integrity rates, there was no statistical difference between fresh sperm and all other frozen and thawed study groups in terms of acrosome damage, as shown in Table 1 ($p>0.05$). While the lowest sperm acrosome damage rate was found in fresh sperm (0.8±1.3%), the highest acrosome damage was found at 6.4±1.3% in the 7.5% LDL group after freezing and thawing.

6) DNA fragmentation rate: Fragmented DNA values of mouse sperm detected by the TUNEL assay after freezing-thawing with different concentrations of low-density lipoproteins are given in Figure 1. The cryopreservation process in our studies also significantly increased the rate of abnormal sperm DNA in all cryopreserved groups compared with the rate in fresh control in mouse sperm ($p<0.05$). There were no statistical differences in the rate of nuclear DNA integrity in the comparison with the control and by adding different concentrations of LDL in 2.5%, 5.0%, 7.5%, and 10% groups, respectively ($p>0.05$).

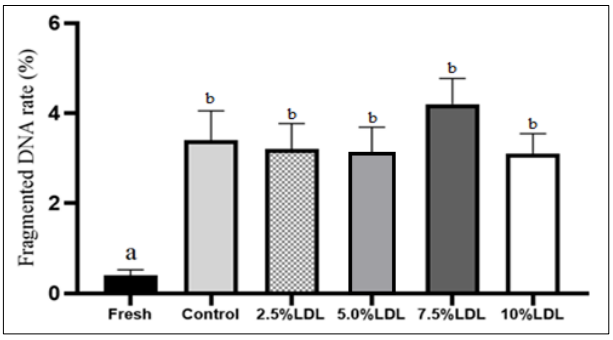

Figure 1: Fragmented DNA rates detected after freezing-thawing of mouse sperm with different concentrations of low-density lipoproteins (n:6). $p<0.05$: Different letters on bars are statistically significant.

Table 1: Spermatological values determined after freezing-thawing of mouse sperm with different concentrations of LDL (n:6).

Study Groups	Progressive motility (%)	Dead sperm (%)	Plasma membrane integrity (%)	Abnormal acrosome (%)
Fresh Sperm	72.4±3.7^{a}	1.6±1.9^{a}	78.1±3.9^{a}	0.8±1.3^{a}
Control (RSM)	43.3±2.6^{b}	18.6±1.7^{c}	48.9±3.1^{c}	5.4±1.9^{b}
RSM + 2.5% LDL	56.7±1.3^{c}	11.6±2.9^{b}	63.0±2.7^{b}	4.7±1.8^{b}
RSM + 5.0% LDL	40.7±1.9^{b}	24.2±1.4^{c}	41.1±1.9^{cd}	5.2±2.4^{b}
RSM + 7.5% LDL	27.5±1.7^{d}	19.6±1.5^{c}	33.7±2.6^{d}	6.4±1.3^{b}
RSM + 10% LDL	12.5±2.9^{e}	56.4±1.3^{d}	14.3±1.8^{e}	4.9±3.2^{b}

^{a} $p<0.05$: Different letters on the same column are statistically significant.

Table 2: Longevity of progressive motility values determined after freezing-thawing of mouse sperm with different concentrations of LDL in HTF (n:6)

Study Groups	Longevity of progressive motility (%) (hours)			
	1	2	3	4
Fresh Sperm	69.6±3.4[a]	63.2±2.7[a]	53.2±3.4[a]	45.4±2.8[a]
Control (RSM)	41.4±1.9[b]	23.0±2.7[b]	20.1±2.5[b]	11.6±3.2[b]
RSM + 2.5% LDL	52.7±2.4[c]	47.1±3.6[c]	40.9±1.8[c]	31.3±2.1[c]
RSM + 5.0% LDL	38.8±3.2[b]	26.2±1.7[b]	23.6±2.8[b]	10.3±3.3[b]
RSM + 7.5% LDL	24.6±1.8[d]	20.4±1.9[b]	18.8±3.2[b]	9.2±1.3[b]
RSM + 10% LDL	10.7±2.6[e]	8.9±1.6[d]	7.3±2.1[d]	2.6±3.3[d]

p<0.05: Different letters on the same column are statistically significant.

DISCUSSION AND CONCLUSION

Most mammalian species require cryoprotectants during the freezing and thawing processes, and egg yolk is one of the most commonly used substances (Scott and Baynes 1980). A resistance component that serves to protect sperm from the effects of cold shock and storage factors that aid in retaining sperm motility and viability are responsible for egg yolk's favorable effect on sperm cryopreservation (Jamieson and Leung 1991). Despite the advantages of egg yolk in extenders, the rising interest in international semen transit legislation has raised questions about egg yolk use among authorities involved in biosecurity issues. Inconsistent composition, egg yolk granules that impair sperm motility, cryoprotectant antagonists, and other negative aspects of egg yolk have also been described, according to certain sources (Ansari et al. 2010). Furthermore, high-density lipoproteins in egg yolks decrease the quality of semen by causing an efflux of cholesterol from the sperm plasma membrane and resulting in a change in fluidity that increases the sensitivity to cold shock (Amirat et al. 2005; Yildiz et al. 2013). However, low-density lipoproteins (LDL) may adhere to the sperm cell membrane and form an interfacial film during the freezing process (Anton et al. 2003; Bergeron et al. 2004). Phospholipids released from LDL can form a protective film at the surface of the sperm membranes, which prevents the efflux of phospholipids and cholesterol (Hu et al. 2006). In addition, LDL forms a complex with seminal plasma proteins (BSP-A1/A2, BSP-A3, and BSP-30-kDa), which interacts with the sperm membranes, resulting in a reduced efflux of phospholipids and cholesterol and increased sperm protection against cold shock-induced damage (Bergeron et al. 2004; Takeo and Nakagata 2018). Despite the stated positive and negative effects of egg yolk, in this study, after freezing and thawing, the progressive motility, live sperm rate, membrane integrity, and longevity of progressive motility rates in the group containing 2.5% LDL were found to be better than the other tested groups and control ($p<0.05$). In other words, when we looked at the sperm viability markers together (table 1), 2.5% LDL was well protected and improved post-thawed spermatologic parameters compared with the other studied groups (5.0%, 7.5%, and 10%) and control during the cryopreservation process ($p<0.05$). The ideal amount of supplemented LDLin the extender is critical for obtaining better cryo survival. In previous studies, optimal rates of LDL addition to cryopreservation extenders against cold shock were 4-6% (Yamauchi et al. 2009), 9.0% (Jiang et al. 2006), and 0.09 g/ml (Wang et al. 2014) in boars, 8.0% in bulls (Moussa et al. 2002; Amirat et al. 2004; Hu et al. 2011); 8.0% in Mithun bulls (Patil et al. 2020); 12% (Dalal et al. 2020) and 10% in buffaloes (Akhter et al. 2011), 6% and 6.0%, 8.0%, or 10% in dogs (Junior et al. 2009; Bencharif et al. 2010), 20% in peccaries (Souza et al. 2015); 10% in rabbits (Iaffaldano et al. 2014); 4.0% in roosters (Shahverdi et al. 2015), 8.0% in rams (Tonieto et al. 2010), and 2.0% in horses (Moreno et al. 2013). We found that 2.5% LDL had greater cryosurvival results compared with control group in the mice. In addition, according to Bencharif et al. (2010) differences in the optimal LDL concentrations used for the cryopreservation of semen from various animal species, including the collared peccaries, could be due to variations in the biochemical composition of the sperm plasma membrane. Therefore, it can be assumed that the success rates of cryosurvival are LDL dose and spacious-dependent. In addition, for useful effects of LDL, has also been reported that LDL supplementation to extender can increase the amount of cellular total antioxidants against reactive oxygen radicals. Perumal et al. (2016) noted that the use of 8.0% LDL for the freezing of semen from Mithun (Bos frontalis) produced a post-thawing increase in total antioxidants, as well as a reduction in the lipid peroxidation of semen, as we found affirmative effects of 2.5% LDL on general viability parameters (Table 1).

Supplementation of 7.5% LDL and 10% LDL to extender caused the lowest sperm quality parameters after thawing (Table 1). Excess of the optimal LDL concentration decreased sperm quality after cryopreservation in both groups. Similar studies have reported that a high level of LDL leads to LDL aggregation, resulting in the depression of LDL functions (Moussa et al. 2002). The excessive concentration of LDL causes high fluidity of the plasma membrane and reduces osmotic pressure when LDL concentration increases. It could also be that LDL inactivation of the effect of aggregated LDL and sperm cells more vulnerable to membrane damage, these detrimental effects lead to a decrease in sperm quality after cryopreservation (Moussa et al. 2002; Hu et al. 2011; Perumal et al. 2016).

The EY-based extender might be responsible for capacitation-like changes during cryopreservation because of its calcium ingredients, progesterone, and HDL components. To overcome this problem, the LDL extracted from EY showed a better post-thaw semen quality than the LDL cryopreserved in the EY-based extender (Moussa et al. 2002; Witte et al. 2009). LDL has a protective effect against

sperm cryopreservation. Thereafter, many studies have reported that the LDL-based extender improved the post-thaw sperm motility and fertility in different species (Amirat et al. 2004; Li et al. 2006; Moustacas et al. 2011; Patil et al. 2020). Dalal et al. (2020) indicated that a 12% LDL concentration increases sperm quality for the cryopreservation of buffalo sperm. Dong et al. (2011) LDL did not offer any additional benefits in terms of post-thaw motility compared with 20% egg yolk in monkeys. This finding does not agree with our study. An increase in LDL concentration in the extender above 10% leads to a decrease in sperm quality after cryopreservation (Moussa et al. 2002). This result is in agreement with our study.

Considering the acrosome integrity rates, there were no statistical differences among the study groups in terms of acrosome damage, as shown in Table 1 ($p>0.05$). However, there were statistical differences between fresh sperm and other tested LDL groups and the control ($p<0.05$). The protective properties of egg yolk, low-density lipoprotein (LDL), and skim milk against the effects of cold shock on plasma and acrosomal membranes during cryopreservation have been previously documented (Therien et al. 1999; Bencharif et al. 2008; Hu et al. 2011) and also stated that extenders based on LDL gave higher proportions of acrosome and plasma-intact sperm, repairing acrosomal membrane phospholipids, after post-thawed.

The paternal genetic contribution to healthy offspring depends critically on the integrity of mammalian sperm DNA. The accurate transmission of paternal genetic information depends on the integrity of the sperm DNA.

Prerequisites for fertilization include the normal stabilization and decondensation of sperm chromatin in the nucleus after sperm penetration into the cytoplasm of the oocyte (Flaherty et al. 1995). The effects of cryopreservation on the integrity of the sperm nucleus are not well understood. However, it has been reported that the freezing and thawing steps of the cryopreservation process can lead to an increase in inappropriate chromatin condensation in the sperm of boars (Fraser and Strzezek 2004), horses (Li et al. 2006), rams (Peris et al. 2004), and humans (Royere et al. 1988; Donnelly et al. 2001; Hammadeh et al. 2001) and Chohan et al. (2004) recently reported that after the freeze-thaw process in human sperm, normal chromatin packaging is drastically reduced. Likewise, the cryopreservation process in our studies also significantly increased the rate of abnormal sperm DNA in all control and LDL cryopreserved groups compared with the rate of fresh sperm in mouse sperm ($p<0.05$) (Figure 1).

The results indicate that an extender supplemented with 2.5% LDL provides greater beneficial effects on progressive motility, cell viability, and membrane integrity during cryopreservation in the mouse sperm. A freezing extender supplemented with 2.5% LDL is suitable for mouse sperm cryopreservation.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: CY, İE
Supervision / Consultancy: CY
Data Collection and / or Processing: İE
Analysis and / or Interpretation: CY
Writing the Article: CY, İE
Critical Review: CY

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