RESEARCH ARTICLE

Effect of Egg Yolk-Derived Low-Density Lipoprotein and Melatonin Supplementation on Post-Thaw Semen Quality of Salem Black Bucks

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Abstract

The aim of this study was to evaluate egg yolk-derived low-density lipoprotein (LDL) as a semen extender and melatonin as an antioxidant in goat semen cryopreservation by assessing post-thaw semen quality parameters. Semen ejaculates (n=24) were collected twice weekly from Salem Black bucks (n=2) using an artificial vagina. After preliminary evaluation, screened pooled samples were extended using six different extenders: Tris–egg yolk–glycerol extender (control), Tris–6% LDL–glycerol extender (Group 2), Tris–8% LDL–glycerol extender (Group 3), control with 0.5 mM melatonin (Group 4), control with 1 mM melatonin (Group 5), and Tris–8% LDL–glycerol extender with 0.5 mM melatonin (Group 6). Semen samples were diluted according to the appropriate dilution rate, filled into straws, and cryopreserved at -196 °C following standard protocols. Physiological characteristics of fresh, freshly diluted (22 °C), pre-freeze (5 °C), and post-thaw semen were evaluated on days 0, 7, and 30 of cryostorage. Post-thaw evaluation on day 30 of cryostorage revealed significantly higher ($p < 0.01$) progressive motility, sperm livability, plasma membrane integrity, and acrosomal integrity, with values of 54.50%, 57.33%, 54.50%, and 51.33%, respectively, in the treatment group 6 followed by group 4 than others. The results indicate that the extender replacing egg yolk with 8% LDL and supplemented with melatonin 0.5 mM is effective for semen preservation in Salem Black bucks. Melatonin improved post-thaw semen quality by reducing oxidative damage during cryopreservation.

Key words: Buck semen, Cryopreservation, LDL, Melatonin, Semen quality.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.22.2.26

Introduction

Cryopreservation of semen is well established in dairy cattle and buffalo breeding; however, it is not widely adopted in goat breeding programs. The availability of cryopreserved buck semen would facilitate large-scale breeding and genetic improvement, thereby promoting goat production. Egg yolk is commonly used in mammalian semen extenders to protect spermatozoa from cryoinjuries during the freeze–thaw process (Salmani *et al.,* 2014). However, a major limiting factor in goat semen preservation is the presence of bulbo-urethral gland protein fractions, such as egg yolk–coagulating enzyme (phospholipase A) or SBUIII (BUSgp60; 55-60 kDa glycoprotein lipase), which adversely affect sperm survival when semen is diluted in egg yolk- or milk-based extenders (Pellicer-Rubio and Combarnous, 1998).

The choice of a suitable semen extender is therefore a critical determinant of success in artificial insemination programs in goats. Furthermore, variability in egg yolk composition among chicken breeds, the presence of unidentified inhibitory factors and the high-density lipoprotein (HDL) content of egg yolk necessitate the replacement of whole egg yolk with its low-density lipoprotein (LDL) fraction. Previous studies have demonstrated that supplementation of semen extender with LDL (6-9%) improves sperm motility, reduces DNA damage and stabilizes sperm plasma membrane and acrosomal integrity. The extraction of LDL from egg yolk (Moussa *et al.,* 2002) has been employed to eliminate the detrimental components of whole egg yolk during semen cryopreservation. Ali-Al-Ahmad *et al.* (2008) and Perumal (2018) reported improved semen freezability using 8% LDL and observed higher zona-binding percentages in buck and mithun semen, respectively.

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How to cite this article: Sreeranjani, R., Selvaraju, M., Sathyabama, T., & Jayachandran, S. (2026). Effect of Egg Yolk-Derived Low-Density Lipoprotein and Melatonin Supplementation on Post-thaw Semen Quality of Salem Black Bucks. Ind J Vet Sci and Biotech, 22(2), 143-147.

Source of support: Nil

Conflict of interest: None

Submitted 19/01/2026 **Accepted** 13/02/2026 **Published** 10/03/2026

Inclusion of melatonin during semen cryopreservation has been shown to reduce oxidative damage to spermatozoa. Owing to its amphiphilic nature, melatonin derived from pineal (Sugden, 1989) and extra-pineal sources (Reiter *et al.,* 2013), can readily cross physiological barriers and stabilize the mitochondrial electron transport chain (Appiah *et al.,* 2019). Several studies have reported that melatonin preserves mammalian spermatozoa and improves their morphological characteristics (Zamiri, 2020). It has also been shown to counteract the adverse effects of the freeze–thaw process at concentrations of 2.0-3.0 mM in bull semen (Ashrafi *et al.,* 2013) and 1.0 mM in ram semen (Succu *et al.,* 2011). Based on these considerations, the present study was undertaken to evaluate the effects of egg yolk-derived LDL 8% in place of whole egg yolk in semen extender and 0.5 mM melatonin as an antioxidant supplementation on the quality of cryopreserved semen in Salem Black bucks.

Materials and Methods

Experimental Animals

Two Salem Black bucks, 4-5 years old, maintained at the Frozen Semen Bank, Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Namakkal, Tamil Nadu, were selected for the study.

Collection and Screening of Semen

Semen ejaculates were collected from the bucks twice weekly using an artificial vagina. Two ejaculates from each buck were utilized per collection. Immediately after collection, the semen samples were maintained at 34 °C in a water bath and transported to the semen processing laboratory for further evaluation. A total of 40 ejaculates were used for the entire study. Following a pilot study conducted in May 2023 using 16 ejaculates to optimize LDL replacement of egg yolk and melatonin inclusion levels, 24 ejaculates (four ejaculates per day) were collected in six batches during the subsequent experiment on six combinations of basic Tris–egg yolk–glycerol extender. All collected samples were subjected to macro- and microscopic evaluation to assess semen quality and were pooled before further processing.

Macroscopic and Microscopic Evaluation

Immediately after collection, routine macro- and microscopic evaluation of semen was done. Sperm concentration in the pooled semen sample was determined using an Accucell® photometer (IMV Technologies, France). Semen samples with a sperm concentration of ≥ 2500 million spermatozoa per mL were selected for further study (Manivannan, 2017).

Progressive individual sperm motility was assessed in diluted semen with Tris buffer under a phase contrast microscope (20X magnification), while sperm viability was assessed using the eosin (5 %) and nigrosin (10 %) staining technique under a bright field microscope, and expressed as percentage motile or live sperm. Similarly, the percentage of sperm abnormality was assessed using same eosin-nigrosin stained smears. For assessment of plasma membrane integrity, the hypo-osmotic swelling test was carried out using 75 mOsm media of citrate-fructose, incubated in a 37°C water bath for 30 min. and then examined under a phase-contrast microscope by observing the tail curling. The acrosome integrity was assessed using Giemsa staining.

Only semen samples exhibiting mass activity of +++ or higher and initial progressive motility exceeding 80% were considered. Additionally, samples containing more than 80% viable spermatozoa, less than 20% abnormal spermatozoa, more than 80% HOST-reacted spermatozoa and intact acrosomes were used for subsequent experiments.

Extender Formulations and Dilution of Semen

Six combinations of extender used under split-sample technique included Tris–egg yolk–glycerol as standard Control (Group 1), Tris + glycerol + 6% LDL (Group 2), Tris + glycerol + 8% LDL (Group 3), Control + melatonin 0.5 mM (Group 4), Control + melatonin 1.0 mM (Group 5), and Tris + glycerol + 8% LDL + 0.5 mM melatonin (Group 6). EY was completely replaced with extracted LDL from EY in Group 2, 3 & 6.

In a preliminary trial, supplementation of melatonin in Tris–egg yolk–glycerol extender (Control) at 0.5 mM, 1.0 mM, 1.5 mM and 3.0 mM was tested, wherein 1.5 mM and 3.0 mM did not yield satisfactory (exceed 50%) sperm motility. Therefore, lower concentrations of melatonin (0.5 mM and 1.0 mM) were subsequently selected and evaluated. Similarly replacing egg yolk with 8% LDL rather than 6% gave better results. Since 8% LDL and 0.5 mM melatonin individually produced the best results among the LDL and melatonin treatment groups, their combination was employed in Group 6.

The corresponding extenders were initially added to the pooled semen samples under split-sample technique at a 1:1 ratio and equilibrated at 22 °C for 10 min under laminar airflow conditions. Subsequently, the semen samples were further diluted with their respective extenders according to the predetermined dilution rate.

Preparation of LDL and Melatonin Extenders

The LDL extraction was made as described by Moussa *et al.* (2002) with slight modification in ammonium sulphate as 50 %. Extracted LDL was incorporated into the base diluent at concentrations of 6% and 8% (w/v), as per the experimental design in Groups 2 and 3 extenders, and in Group 6 @ 8% along with melatonin at 0.5 mM

Melatonin (≥ 99% purity; HiMedia) was supplemented into the control extender at concentrations of 0.5 mM (0.116 mg/mL) and 1.0 mM (0.232 mg/mL) in groups 4 and 5, respectively and also in Group 6 @ 0.5 mM along with 8% LDL.

Cryopreservation and Post-Thaw Evaluation of Semen

The semen was diluted to the concentration of 100 million sperms per straw (0.25 mL). Using automated filling and

sealing machine (IMV Technologies, France), the extended semen samples were filled into French mini straws at 22°C. The details of the semen samples were printed on the straws and were kept in the cold handling cabinet at 5°C for 3 h equilibration. After equilibration, the straws arranged in freezing rack were transferred to a biofreezer (IMV Technologies, France) in which straws were freezed using standard preset programme. The temperature was reduced in a fast and controlled manner from 5°C to -140°C in 7 min. Then the straws were stored in liquid nitrogen container at -196°C for storage until further evaluation.

Frozen semen straws were thawed at 37 °C for 30 seconds in water bath on days 0, 7 and 30 of cryostorage in LN2, and evaluated for post-thaw semen quality parameters, including progressive individual sperm motility, livability, abnormalities, acrosomal integrity and plasma membrane integrity, as explained above for fresh/freshly diluted semen.

Statistical Analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA) with IBM® SPSS® Statistics version 23.0 for Windows®. Duncan's multiple range test was applied for *post hoc* comparison of mean values among different treatment groups for various semen quality parameters (Snedecor and Cochran, 1994).

Results and Discussion

The post-thaw sperm quality parameters of buck semen cryopreserved in different extenders with LDL and melatonin supplementation observed after 0, 7 and 30 days of cryostorage are presented in Table 1.

Progressive Individual Motility

On day 0, Group 6 (8% LDL + 0.5 mM melatonin) showed significantly higher ($p < 0.01$) motility compared with all other groups. Groups 2 (6% LDL) and 5 (1 mM melatonin) recorded significantly lower ($p < 0.05$) values than the remaining groups. A similar trend was observed on day 7, with Group 6 exhibiting the highest motility (57.17%), followed by Groups 4, 3 and control, while Groups 2 and 5 remained significantly lower ($p < 0.01$). On day 30, Group 6 maintained significantly higher ($p < 0.01$) progressive motility (>10% increase) compared with Group 2, followed by Groups 3 and 4. Groups 2 and 5 did not differ significantly from each other and showed the lowest motility. The beneficial effect of melatonin supplementation on post-thaw motility observed in the present study agreed with reports in ram (Succu *et al.,* 2011), bull (Ashrafi *et al.,* 2013) and horse (Izadpanah *et al.,* 2015), but contradicted with the findings of Moazzami *et al.* (2014) in rams.

Sperm Livability

On day 0, sperm livability was highest in Group 6 (64.17%), followed by Groups 4 and 3, and lowest in Groups 2 and 5 ($p < 0.01$). On day 7, Groups 6, 4 and 3 showed significantly higher ($p < 0.01$) livability than control, whereas Groups 2 and 5 remained significantly lower. On day 30, Group 6 recorded the highest livability (57.33%), significantly exceeding all other groups ($p < 0.01$). The lowest values were observed in Group 5 followed by Group 2. Improved sperm viability with LDL supplementation has been reported in bovines (Hu *et al.,* 2010), ovines (Hong *et al.,* 2008; Tonieto *et al.,* 2010), mithun (Perumal *et al.,* 2017), porcines (Hu *et al.,* 2006) and buffaloes (Akhter *et al.,* 2011). Species differences may arise due to variation in membrane lipid composition and susceptibility to cryoinjury (Perumal *et al.,* 2017). The present findings at 8% LDL agree with these reports. The beneficial effect of 0.5 mM melatonin is consistent with earlier observations in ram, bull and horse (Succu *et al.,* 2011; Ashrafi *et al.,* 2013; Izadpanah *et al.,* 2015), but differs from Moazzami *et al.* (2014), who reported no benefit at higher concentrations.

Sperm Abnormality

On day 0, no significant differences were observed among groups (12.00-13.00%). On day 7, significantly lower ($p < 0.01$) abnormal sperm percentages were recorded in Groups 6, 3 and 4 compared with Groups 2 and 5. On day 30, Group 6 showed the lowest abnormality (15.83%), followed by Group 4, while Groups 2 and 5 showed the highest values ($p < 0.01$). These results agreed with Perumal *et al.* (2017), who reported lower sperm abnormalities in semen extended with 8% LDL compared to egg yolk extenders.

Plasma Membrane Integrity

On day 0, plasma membrane integrity was highest in Group 6 (57.83%), followed by Group 4 ($p < 0.01$). Groups 2 and 5 showed significantly lower values. On day 7, Groups 6, 4, 3 and control showed comparable integrity, while Groups 2 and 5 remained significantly lower. On day 30, Group 6 again recorded the highest integrity (54.50%), followed by Group 4. These findings corroborated with reports in bulls where LDL extenders preserved membrane integrity better than egg yolk extenders (Amirat *et al.,* 2004; Hong *et al.,* 2008; Hu *et al.,* 2010), possibly due to sequestration of BSP proteins by LDL. The beneficial effect of melatonin concurred with Succu *et al.* (2011), Ashrafi *et al.* (2013) and Izadpanah *et al.* (2015), but not with Moazzami *et al.* (2014).

Acrosomal Integrity

On day 0, acrosomal integrity was highest in Group 6 (59.17%), followed by Groups 4 and 3. On day 7, Group 6 showed significantly higher ($p < 0.01$) acrosomal integrity, whereas Groups 2 and 5 recorded the lowest values. On day 30, Group 6 (51.33%) and Group 4 (48.83%) were superior to Groups 3 and control, with Groups 2 and 5 being the poorest. These results agree with findings in bulls using 8% LDL extenders (Amirat *et al.,* 2004; Hu *et al.,* 2010) and melatonin supplementation studies in ram, bull and horse (Succu *et al.,* 2011; Ashrafi *et al.,* 2013; Izadpanah *et al.,* 2015).

Table 1: Mean (± SE) values of post-thaw sperm quality parameters of Salem Black bucks semen cryopreserved in various extenders containing LDL and Melatonin after 0, 7 and 30 days of cryostorage (n=18)

Days of evaluation	Parameters (%)	Group 1 (TEYC)	Group 2 (6% LDL)	Group 3 (8% LDL)	Group 4 (0.5 mM melatonin)	Group 5 (1 mM melatonin)	Group 6 (8%LDL + 0.5mM melatonin)	F Value
0th day	Progressive individual motility	$55.17^{b}\pm 0.792$	$50.33^{a}\pm 0.558$	$55.00^{b}\pm 0.730$	$56.83^{b}\pm 0.946$	$49.00^{a}\pm 0.632$	$59.00^{c}\pm 0.730$	26.741**
	Sperm livability	$61.17^{c}\pm 0.654$	$55.00^{a}\pm 0.447$	$59.00^{b}\pm 0.683$	$61.50^{c}\pm 0.719$	$54.00^{a}\pm 0.447$	$64.17^{d}\pm 0.833$	37.608**
	Sperm abnormality	12.67 ± 0.715	13.00 ± 0.365	12.33 ± 0.715	12.17 ± 0.872	12.83 ± 0.601	12.00 ± 0.57	0.357^{NS}
	Plasma membrane integrity	$55.17^{b} \pm 0.307$	$52.00^{a} \pm 0.577$	$55.50^{bc} \pm 0.428$	$56.67^{cd} \pm 0.494$	$51.83^{a} \pm 0.307$	$57.83^{d} \pm 0.307$	34.404**
	Acrosomal integrity	$56.00^{bc} \pm 0.632$	$54.67^{ab} \pm 0.882$	$56.17^{bc} \pm 0.703$	$58.17^{cd} \pm 0.703$	$53.00^{a} \pm 0.730$	$59.17^{d} \pm 0.703$	9.504**
7th day	Progressive individual motility	$53.17^{b} \pm 0.980$	$47.83^{a} \pm 0.749$	$53.33^{b} \pm 0.760$	$54.67^{b} \pm 0.882$	$47.17^{a} \pm 0.749$	$57.17^{c} \pm 0.792$	22.831**
	Sperm livability	$56.33^{b} \pm 0.919$	$53.17^{a} \pm 0.307$	$57.00^{bc} \pm 0.683$	$59.00^{c} \pm 0.816$	$51.83^{a} \pm 0.401$	$62.33^{d} \pm 0.843$	29.902**
	Sperm abnormality	$14.83^{abc} \pm 0.872$	$15.00^{bc} \pm 0.365$	$14.17^{abc} \pm 0.792$	$14.00^{ab} \pm 0.577$	$16.00^{c} \pm 0.516$	$13.00^{a} \pm 0.577$	3.474**
	Plasma membrane integrity	$53.17^{b} \pm 0.307$	$49.83^{a} \pm 0.654$	$53.00^{b} \pm 0.577$	$54.67^{b} \pm 0.494$	$48.67^{a} \pm 0.803$	$56.83^{c} \pm 0.307$	29.852**
	Acrosomal integrity	$54.17^{b} \pm 0.749$	$50.17^{a} \pm 0.946$	$53.83^{b} \pm 0.543$	$55.83^{bc} \pm 0.792$	$50.33^{a} \pm 0.882$	$57.83^{c} \pm 1.138$	12.321**
30th day	Progressive individual motility	$51.33^{b} \pm 0.955$	$43.17^{a} \pm 1.327$	$51.50^{bc} \pm 0.671$	$52.83^{bc} \pm 1.014$	$43.83^{a} \pm 0.910$	$54.50^{c} \pm 1.057$	22.779**
	Sperm livability	$51.33^{c} \pm 0.715$	$49.00^{b} \pm 0.516$	$52.67^{cd} \pm 0.843$	$54.67^{d} \pm 0.803$	$45.67^{a} \pm 0.843$	$57.33^{e} \pm 0.882$	28.245**
	Sperm abnormality	$18.50^{b} \pm 0.619$	$21.00^{c} \pm 0.730$	$18.00^{b} \pm 0.683$	$16.83^{ab} \pm 0.749$	$20.67^{c} \pm 0.494$	$15.83^{a} \pm 0.749$	9.198**
	Plasma membrane integrity	$50.33^{b} \pm 0.333$	$46.17^{a} \pm 1.108$	$51.00^{b} \pm 0.577$	$52.50^{bc} \pm 0.619$	$45.83^{a} \pm 0.946$	$54.50^{c} \pm 0.500$	22.368**
	Acrosomal integrity	$45.00^{b} \pm 1.125$	$40.83^{a} \pm 1.014$	$44.33^{b} \pm 0.989$	$48.83^{c} \pm 1.249$	$39.50^{a} \pm 0.764$	$51.33^{c} \pm 0.667$	21.059**

Means bearing same superscript within the row do not differ significantly. **Significant at 1% level, ^{NS}Non-significant ($p>0.05$). ($p<0.01$)

Conclusion

The post-thawed sperm quality parameters of buck semen on days 0, 7 and 30 after cryostorage were significantly higher ($p < 0.01$) in semen diluted with 8% LDL–glycerol extender supplemented with 0.5 mM melatonin compared to the control and other treatments. Replacement of egg yolk with 8% egg yolk-derived LDL significantly improves post-thaw semen quality in Salem Black bucks. Similarly, supplementation of 0.5 mM melatonin further enhances sperm motility, viability and membrane integrity by protecting spermatozoa from oxidative damage during cryopreservation. These findings indicate that LDL is a superior alternative to egg yolk as a cryoprotective agent in buck semen extenders and that low-dose melatonin effectively enhances post-thaw semen quality by mitigating oxidative damage. The combination of 8% LDL and 0.5 mM melatonin can therefore be recommended for preparation of buck semen extenders for large-scale cryopreservation and artificial insemination programmes. Field fertility trials are suggested to validate the fertilizing potential of semen processed using this extender formulation.

Acknowledgement

The authors sincerely thank the Frozen Semen Bank, Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Namakkal, TANUVAS, for providing laboratory facilities and manpower to conduct the research work.

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