RESEARCH ARTICLE

Evaluation of Alternatives to Conventional Egg Yolk Extender for Cryopreservation of Kankrej Bull Semen

Arvind S. Chaudhary[1*], Bharat S. Rathod[2], Gaurav S. Makawana[1], Pratik S. Kapadiya[1], Ravji K. Chaudhary[1]

Abstract

Demand for alternative of egg yolk in freezing extenders have increased in recent years due to variability in egg yolk composition, risk of microbial contamination and presence of steroid hormones. The alternative to egg yolk-based extender can be extender prepared using centrifuged egg yolk (cEY) and liposome based extender (Liposome). Therefore, this study was designed to compare cEY and Liposome with universal EY for evaluation of various physical and biochemical parameters of 18 ejaculates collected from Kankrej bull (n=3). Results revealed that at the post-thawed (PT) stage, individual sperm motility, HOST reactive sperm, and sperm viability were significantly higher ($p<0.05$) in liposome based extender compared to the EY extender. Malondialdehyde (MDA), superoxide dismutase (SOD), aspartate amino transaminase (AST) and alanine amino transaminase (ALT) were estimated from seminal plasma at post-dilution and post-thaw stages of cryopreservation. The Liposome based extender and cEY extender showed a significant ($p<0.05$) decrease in MDA, AST and ALT level, whereas, SOD level non-significant as compared to the EY extender.

Key words: Centrifuged egg yolk extender, Enzymatic parameters, Kankrej bull, Liposome, Oxidative stress, Semen cryopreservation, Sperm quality.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.2026.22.06.25

Introduction

Seminal plasma, which contains a variety of proteins, many of which are secretory products of epididymis and seminal vesicles (Singh *et al*., 2014), affects both the quality and freezability of spermatozoa (Jobim *et al*., 2005). Bovine seminal plasma (BSP) proteins are 'Double edged sword' and have beneficial effect in maintenance of plasma membrane stability, motility (Sanchez-Luengo *et al*., 2004), and capacitation during natural insemination (Therien *et al*., 1998), but harmful to sperm when it comes to sperm storage because during storage cause efflux of phospholipids and cholesterol (Manjunath *et al*., 2007) from the plasma membrane of spermatozoa. Egg-yolk based fractions/milk/plant lipids (liposome) provide phospholipids and lipoprotein. They promote membrane stabilization by forming protective layer on the sperm surface at lower temperature, and limit premature acrosomal membrane activation via replenishing missing phospholipid in the sperm membrane during cryopreservation (Graham and Foote, 1987).

Since the discovery of egg yolk as a protectant for cryopreservation of spermatozoa, it has been widely used to cryopreserve sperm of a variety of species. Due to the wide variation in egg yolk composition (Forouzanfar *et al*., 2010), risk of microbial contamination (Aires *et al*., 2003), presence of high-density lipoproteins, steroid hormones (Lipar *et al*., 1999), interference in microscopic examination due to high viscosity (Vishwanath and Shannon, 2000), fat globules of egg yolk make sperm evaluation challenging (Singh *et al*., 2012), hence demand for replacement of egg yolk in freezing extenders has increased in recent years. In addition to utilizing the entire egg yolk for the semen extender, the yolk can be centrifuged so that just the supernatant is combined with the extender's other essential ingredients (Vidament *et al*., 2000) or the whole yolk can be added to the extender which is then centrifuged (Wall and Foote, 1999). These clarified extenders have been successfully used for bull and stallion semen cryopreservation (Wall and Foote, 1999; Vidament *et al*., 2000). The advantage of removing bigger particles by centrifugation is that fewer particles are interfering with microscopic analyses (Wall and Foote, 1999).

[1]Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Sciences and Animal Husbandry, Kamdhenu University, Sardarkrushinagar-385506, Gujarat, India

[2]Livestock Research Station, Kamdhenu University, Sardarkrushinagar-385506, Gujarat, India

Corresponding Author: Dr. Arvind S. Chaudhary, M.V.Sc. Scholar, Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Sciences and Animal Husbandry, KU, Sardarkrushinagar-385506, Gujarat, India. e-mail: arvinpatel312@gmail.com

How to cite this article: Chaudhary, A. S., Rathod, B. S., Makawana, G. S., Kapadiya, P. S., & Chaudhary, R. K. (2026). Evaluation of Alternatives to Conventional Egg Yolk Extender for Cryopreservation of Kankrej Bull Semen. Ind J Vet Sci and Biotech, 22(6), 146-150.

Source of support: No specific funding was received for this study.

Conflict of interest: None

Submitted 06/08/2026 **Accepted** 16/09/2026 **Published** 10/11/2026

© The Author(s). 2026 Open Access This work is licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International License.

Liposomes are an alternative to the animal-derived components in semen extenders. The inclusion of liposomes in the extender is a technological advancement in the creation of an appropriate freezing extender. For preparation of the liposomes based extender, the optimal composition and concentration of phospholipids is kept similar to the active component of the egg-yolk that ensure the cell protection during cryopreservation (Bergeron and Manjunath, 2006), and lipids used are non-animal origin and not a vector for infective agent (Manjunath, 2012). The addition of liposomes to extenders is of great relevance because of it being precisely defined in terms of composition and structure and these can be optimized by modulating their phospholipids composition or their size. Therefore, liposomes would be the better option for optimizing the semen extender composition. This study was thus aimed to comparatively evaluate liposome- and centrifuged egg yolk-based extenders as alternatives to conventional egg yolk extender for cryopreservation of Kankrej bull semen.

Materials and Methods

Selection of Bulls and Semen Collection

Total three Kankerj (*Bos indicus*) bulls, aged 5 years maintained at semen collection station, Livestock Research Station, Kamdhenu University, Sardarkrushinagar, were used for study. Eighteen semen samples were collected by homosexual mount using Danish Model of artificial vagina.

Preparation of Centrifuged Egg Yolk Extender

Eggs were manually broken and the albumin was discarded. Yolks were carefully rolled on filter paper to remove albumin and chalazes adhering to the vitelline membrane. These vitelline membranes were then puncture to collect egg yolk in centrifuge tubes and centrifuged at 10,000 x g for 45 min at 4 °C. The supernatant was carefully collected with a glass pipette to avoid any contamination from the pellet (granules) then transferred into a new centrifuge tube for a second centrifugation, under the same conditions. Following the second centrifugation the supernatant was considered as centrifuged egg yolk plasma and used instead of egg yolk in extender preparation.

Semen Evaluation and Processing

Semen ejaculates with more than 75% initial progressive motility were used in the present study. The semen samples were divided into three equal aliquots, and extended at the concentration of 100×10^6 spermatozoa ml^{-1} with 3 different semen extenders. One aliquot was extended with standard Tris Fructose Egg yolk Glycerol (TFEYG) extender as a control. Second aliquot was extended with extender prepared using centrifuged egg yolk (cEY). Third aliquot was extended with commercial liposome based extender Optixcell® (Optixcell®, IMV, France). The extended semen aliquots were filled, sealed and printed in French Mini Straw of 0.25 mL capacity using automatic machine (MRS1 Dual, IMV, France) and then transferred to cold handling cabinet (Frigory SA, France) already maintained at 4 °C and kept for 4 h of equilibration period. The programmable Bio-freezer (MicroDigit Cool, IMV, France) was used for the freezing of semen. The cryopreserved semen was evaluated after 24 h. The seminal plasma was collected from the straws of each aliquot at post-dilution and post thawing by centrifuging at 4000 x g for 10 min.

Physical Attributes, Oxidative Stress and Enzymatic Parameters

The individual sperm motility, viability, total sperm abnormality and hypo-osmotic swelling test (Jeyendran *et al*. 1984) were evaluated from each group at post-dilution and post thawed stages of cryopreservation.

The activities of lipid peroxidation (LPO) and superoxide dismutase (SOD) in seminal plasma at post-diluted and post-thawed stages were estimated using the Bovine Malondialchehyde ELISA Kit, and Bovine Superoxide Dismutase ELISA Kit (Puregene, Genetix Biotech Asia Pvt. Ltd., New Delhi), respectively. The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured from the seminal plasma to know the damage to the sperm membrane by calorimetric kits (liqui CHEK, Agappe Diagnostic LTD, Kerala).

Statistical Analysis

Data were first checked for normality using Shapiro-Wilk test. The percentage data were transformed into the arcsine before the analysis. The mean differences among the groups for different variables were seen using one-way ANOVA. The data were presented as Mean ±SE. Significance was set at 95%. Data analysis was done with SPSS software (IBM® SPSS® statistics, version 20.0).

Results and Discussion

The findings of semen samples examined for individual sperm motility, viability, plasma membrane integrity and sperm abnormality at post-dilution and post-thawed stages are presented in Table 1.

The individual sperm motility (%), live sperm (%), HOST reacted sperm (%), and sperm abnormality (%) differed non-significantly at post-dilution stage between control, centrifuged egg yolk (cEY) and liposome group. In accordance to present study, Singh *et al*. (2020) also found non-significant difference in individual motility, live sperm, HOST reacted sperm and sperm abnormality between egg yolk (EY) and egg yolk plasma (EYP) extender in Beetal

Table 1: Efficacy of different extender for qualitative parameters (Mean ±SE) in Kankrej bull semen at different stages of cryopreservation

Parameters	Treatment group	Stage		Group
		Post-dilution	Post-thaw	
Individual sperm motility (%)	TFEYG	73.67±0.01	51.77±0.003[b]	p=0.0085
	cEY	74.81±0.01	53.84±0.008[b]	
	Liposome	75.33±0.008	55.61±0.009[a]	
Total viability (%)	TFEYG	78.77±0.02	56.66±0.01[b]	p=0.001
	cEY	79.99±0.02	59.03±0.01[b]	
	Liposome	80.31±0.01	61.43±0.01[a]	
HOS reacted sperm (%)	TFEYG	72.43±0.01	55.07±0.01[b]	p=0.0407
	cEY	73.40±0.02	56.18±0.01[b]	
	Liposome	73.93±0.02	57.60±0.01[a]	
Sperm abnormality (%)	TFEYG	6.29±0.01	10.00±0.01	p=0.5293
	cEY	5.99±0.01	10.08±0.01	
	Liposome	5.77±0.01	10.12±0.01	

Means bearing different subscripts (a,b,c) differ significantly within column (p<0.05).

buck semen. Chaudhari *et al*. (2016) found non-significant difference in individual motility, live sperm, HOST reacted sperm and sperm abnormality between Tris-citrate-fructose-egg yolk-glycerol and liposome based extender in Gir bull semen.

At post-thaw stage, individual subjective motility, HOST reactive sperm and sperm viability were significantly (p<0.05) higher in liposome and non-significantly higher in cEY groups than control group (Table 1). Like present findings, García *et al*. (2018) in ram semen, Singh *et al*. (2020) and Lukusa *et al*. (2021) in buck semen, and Kamusasa *et al*. (2023) in bull semen also noticed non-significant differences in these traits between the egg yolk and egg yolk plasma extender at post-thawed stage. Similar to present findings, Kumar *et al*. (2015) in Murrah buffalo bull, Chaudhari *et al*. (2016) in Gir bull, Singh *et al*. (2020) in Sahiwal bulls reported significantly (p<0.05) higher post-thaw sperm progressive motility, HOST reacted sperm and sperm viability in liposome extender compared to egg yolk extender.

During cryopreservation of semen, reduction in progressive motility of spermatozoa occurs due to the damage of mitochondrial apparatus and axoneme (Jones and Stewart, 1979). Because of the reduced size, liposomes can fuse with plasma membrane more effectively than egg yolk and protect the internal structures of spermatozoa during cryopreservation. In this way the cryo-damage of mitochondrial apparatus and axoneme might be lesser in spermatozoa cryopreserved in liposome based extender and thus, comparatively better progressive motility and viability could be maintained after thawing. It is assumed that reduced motility and viability of spermatozoa in egg yolk-based extenders might be attributed to delayed energy production mechanism at cellular level due to the presence of high-density lipoproteins (HDL) and other granular substances of egg yolk (Amirat *et al*., 2005). Liposomes, because of their small size might have integrated with the spermatozoon plasma membrane more competitively than egg yolk and maintained significantly better functional membrane integrity.

The percentage of total sperm abnormality at the post-thawed stage differed non-significantly between the control, centrifuged egg yolk (cEY) and liposome group. Chaudhari *et al*. (2016) and Kamusasa *et al*. (2023) reported a non-significantly lower percentage of sperm abnormality in post-thawed bull semen treated with cEY and liposome based extender, respectively, compared to control group.

The semen samples were assessed for MDA, SOD, AST and ALT at post-dilution and post-thawed stages (Table 2). A significantly (p<0.05) decreased MDA levels during post-dilution and post-thawed stage were recorded in semen aliquot containing liposome group compared to control group. Whereas, MDA levels non-significantly differed at post-dilution and significantly decreased at post-thawed stage between cEY and control group, respectively.

Bull spermatozoon plasma membrane is very sensitive to oxidative stress generated during freeze-thaw process, as it contained high level of unsaturated fatty acids and low level of cholesterol. Excessive production of reactive oxygen species (ROS) results in disruption of disulfide bonds between proteins, modification of sperm glycocalyx and lipid peroxidation. With higher the level of LPO (lipid peroxidation), more fatty acid chains are broken into aldehyde compound so that more MDA is formed (Wahjuningsih *et al*., 2021). Dilution in the antioxidant systems of seminal plasma on extension and the release of L-aromatic amino oxidase from dead spermatozoa are mainly contributing to the increased production of ROS during cryopreservation of spermatozoa. L-aromatic amino acid, particularly L-phenyl alanine, which is abundantly present in egg yolk, is the target of L-aromatic amino oxidase.

Table 2: Efficacy of different extender for oxidative stress parameters and enzymatic activity (Mean ±SE) in Kankrej bull semen at different stages of cryopreservation

Parameters	Treatment group	Stage		Group
		Post-dilution	Post-thaw	
MDA (nmol/mL)	TFEYG	6.63±0.18[a]	13.93±0.32[a]	p=0.001
	cEY	6.65±0.15[a]	10.77±0.34[b]	
	Liposome	5.36±0.13[b]	10.27±0.51[b]	
SOD (ng/mL)	TFEYG	46.64±0.67	36.22±0.77	p=0.6061
	cEY	46.17±0.94	36.43±1.08	
	Liposome	47.24±0.69	35.21±0.89	
ALT (U/L)	TFEYG	5.32±0.37[a]	10.36±0.98[a]	p=0.001
	cEY	5.38±0.33[a]	7.75±0.32[b]	
	Liposome	4.09±0.49[b]	7.08±0.49[b]	
AST (U/L)	TFEYG	25.11±0.38[a]	31.93±0.93[a]	p=0.009
	cEY	25.00±0.35[a]	30.63±0.99[a]	
	Liposome	15.95±0.35[b]	20.29±0.83[b]	

Means bearing different superscripts (a, b, c) differ significantly within column ($p<0.05$)

Liposome based extender is free of this demerit. Centrifuged egg yolk gets rid of the L-phenyl alanine substances to some extent so the MDA concentration is lower. The SOD concentration differed non-significantly during post-dilution and post-thawed stage between control, centrifuged egg yolk (cEY) and liposome group.

In the present study, the mean ALT enzymatic value differed non-significantly between control and cEY groups during post-dilution and post-thawed stages, whereas mean ALT activity was significantly lower during post-dilution and post-thawed stage in liposome group compared to control group. Similarly, liposome group significantly ($p<0.05$) minimized the leakage of AST enzyme from spermatozoa during both post-dilution and post-thawed stage, while cEY group significantly ($p<0.05$) minimized the leakage of AST enzyme only during post thawed stage.

The existing studies revealed ALT and AST activity of frozen semen to be significantly advantageous in groups which are extended with centrifuged egg yolk or liposome extender than control group. It is widely accepted that the protective agent in the egg yolk is LDL fraction of egg yolk plasma (Anton *et al*., 2006). Thus, there are several mechanisms by which LDL of EY plasma decreases damage to the sperm during cooling and cryopreservation. One possibility is that the LDLs, particularly the phospholipids, associated with the sperm membrane and thereby provide membrane stabilization (Ricker *et al*., 2006). In egg yolk, some unknown factors are reported to destabilize the sperm plasma membrane and cause premature capacitation (Watson and Martin, 1975) which can be removed by high speed centrifugation.

Conclusion

The liposome extender demonstrated a modest safeguarding effect on the sperm plasma membrane, evident through a reduction in lipid peroxidation (LPO) and enzymatic activity (ALT and AST) especially during the post-thawed stage when compared to the control group, while the use of a centrifuged egg yolk extender devoid of large particulate matter significantly improves the quality of cryopreserved semen, which may be attributed to reduced enzymatic activity and improved microscopic evaluation of spermatozoa.

Acknowledgments

The authors acknowledge the Kamdhenu University, Sardarkrushinagar, for their support, and extend their gratitude to the Livestock Research station, KU, for providing facilities and technical assistance.

References

Aires, V. A., Hinsch, K. D., Mueller-Schloesser, F., Bogner, K., Mueller-Schloesser, S., & Hinsch, E. (2003). In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology, 60*(2), 269–279.

Amirat, L., Anton, M., Tainturier, D., Chatagnon, G. R., Battut, I., & Courtens, J. L. (2005). Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction, 129*, 535–543.

Anton, M., Nau, F., & Nys, Y. (2006). Bioactive egg components and their potential uses. *World's Poultry Science Journal, 62*(3), 429–438.

Bergeron, A., & Manjunath, P. (2006). New insights towards understanding the mechanisms of sperm protection by egg

yolk and milk. *Molecular Reproduction and Development, 73*(10), 1338–1344.

Chaudhari, D. V., Dhami, A. J., Hadiya, K. K., & Patel, J. A. (2016). Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (−196°C) of buffalo semen. *Veterinary World, 8*(2), 239.

Forouzanfar, M., Sharafi, M., Hosseini, S. M., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H. R., & Nasr-Esfahani, M. H. (2010). In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology, 73*(4), 480–487.

García, W., Tabarez, A., & Palomo, M. J. (2018). Effect of the type of egg yolk, removal of seminal plasma and donor age on ram sperm cryopreservation. *Animal Reproduction, 14*(4), 1124–1132.

Graham, J. K., & Foote, R. H. (1987). Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology, 24*(1), 42–52.

Jeyendran, R. S., Van der Ven, H. H., Perez-Pelaez, M., Crabo, B. G., & Zaneveld, L. J. D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Reproduction, 70*(1), 219–228.

Jobim, M. I. M., Oberst, E. R., Salbego, C. G., Wald, V. B., Horn, A. P., & Mattos, R. C. (2005). BSP A1/A2-like proteins in ram seminal plasma. *Theriogenology, 63*(7), 2053–2062.

Jones, R. C., & Stewart, D. L. (1979). The effects of cooling to 5°C and freezing and thawing on the ultrastructure of bull spermatozoa. *Reproduction, 56*(1), 233–238.

Kamusasa, P. T., Chatiza, F. P., Machabango, R., & Gororo, E. (2023). Effect of whole and clarified egg yolk based extenders on post-thaw quality of bull semen. *Research Square, 23*(7), 1–12.

Kumar, P., Saini, M., Kumar, D., Balhara, A. K., Yadav, S. P., Singh, P., & Yadav, P. S. (2015). Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Animal Reproduction Science, 159*, 38–45.

Lipar, J. L., Ketterson, E. D., Nolan, V., Jr., & Casto, J. M. (1999). Egg yolk layers vary in the concentration of steroid hormones in two avian species. *General and Comparative Endocrinology, 115*(2), 220–227.

Lukusa, K., Hassen, A., & Lehloenya, K. C. (2021). Dietary selenium supplementation, clarified egg yolk extender and slow cooling improve cryopreserved sperm characteristics of Saanen buck. *Asian Pacific Journal of Reproduction, 10*(1), 43–48.

Manjunath, P. (2012). New insights into the understanding of the mechanism of sperm protection by extender components. *Animal Reproduction, 9*(4), 809–815.

Manjunath, P., Bergeron, A., Lefebvre, J., & Fan, J. (2007). Seminal plasma proteins: Functions and interaction with protective agents during semen preservation. *Society of Reproduction and Fertility Supplement, 65*, 217.

Ricker, J. V., Linfor, J. J., Delfino, W. J., Kysar, P., Scholtz, E. L., Tablin, F., Crowe, J. H., & Meyers, S. A. (2006). Equine sperm membrane phase behavior: The effects of lipid-based cryoprotectants. *Biology of Reproduction, 74*(2), 359–365.

Sanchez-Luengo, S., Aumüller, G., Albrecht, M., Sen, P. C., Röhm, K., & Wilhelm, B. (2004). Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine sperm membrane Ca2+-ATPase. *Journal of Andrology, 25*(2), 234–244.

Singh, A. K., Singh, V. K., Narwade, B. M., Mohanty, T. K., & Atreja, S. K. (2012). Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5°C) in egg yolk- and soya milk-based extenders. *Reproduction in Domestic Animals, 47*(4), 596–600.

Singh, B., Kaur, D., Singla, M., Cheema, R. S., Sethi, A. P. S., Singhal, S., & Malik, D. S. (2020). Effects of egg yolk and egg yolk plasma Tris-base extenders on Beetal buck semen preservation. *International Journal of Current Microbiology & Applied Sciences, 9*(11), 1853–1864.

Singh, M., Ghosh, S. K., Prasad, J. K., Kumar, A., Tripathi, R. P., Bhure, S. K., & Srivastava, N. (2014). Seminal PDC-109 protein vis-à-vis cholesterol content and freezability of buffalo spermatozoa. *Animal Reproduction Science, 144*(1–2), 22–29.

Therien, I., Moreau, R., & Manjunath, P. (1998). Major proteins of bovine seminal plasma and high-density lipoprotein induce cholesterol efflux from epididymal sperm. *Biology of Reproduction, 59*(4), 768–776.

Vidament, M., Ecot, P., Noue, P., Bourgeois, C., Magistrini, M., & Palmer, E. (2000). Centrifugation and addition of glycerol at 22°C instead of 4°C improve post-thaw motility and fertility of stallion spermatozoa. *Theriogenology, 54*(6), 907–919.

Vishwanath, R., & Shannon, P. (2000). Storage of bovine semen in liquid and frozen state. *Animal Reproduction Science, 62*(1–3), 23–53.

Wahjuningsih, S., Ihsan, M., Siswoyo, D. A., Fatmila, D. T., & Firmawati, A. (2021). Extract of cincau (*Mesona palustris* B.) supplementation in semen extender improves Boer goat sperm cryopreservation. *Journal of Advanced Veterinary Research, 11*(4), 247–253.

Wall, R. J., & Foote, R. H. (1999). Fertility of bull sperm frozen and stored in clarified egg yolk-Tris-glycerol extender. *Journal of Dairy Science, 82*(4), 817–821.

Watson, P. F., & Martin, I. C. A. (1975). The influence of some fractions of egg yolk on the survival of ram spermatozoa at 5°C. *Australian Journal of Biological Sciences, 28*(2), 145–152.