RESEARCH ARTICLE

Evaluation of Centrifuged Egg Yolk Extender with L-Methionine for Improving Cryosurvival of Kankrej Bull Spermatozoa

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Abstract

The present study was undertaken to evaluate the effect of a centrifuged Tris-fructose egg yolk glycerol (TFEYG) extender supplemented with L-methionine on post-dilution and post-thaw semen quality, oxidative stress, and enzymatic activity in Kankrej bull semen. Eighteen ejaculates collected from three healthy, sexually mature Kankrej bulls were processed using three extender formulations: conventional TFEYG (Group I), centrifuged TFEYG (Group II), and centrifuged TFEYG supplemented with 2.5 mM L-methionine (Group III). Semen quality was assessed in terms of individual sperm motility, viability, abnormalities, hypo-osmotic swelling test (HOST)-reacted sperm, malondialdehyde (MDA), superoxide dismutase (SOD), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) at the post-dilution and post-thaw stages. Cryopreservation reduced semen quality in all treatment groups; however, semen extended with centrifuged TFEYG, particularly when supplemented with L-methionine, retained significantly ($p<0.05$) higher sperm motility, viability, and HOST-reacted sperm, along with significantly lower sperm abnormalities than the conventional extender. The most favourable post-thaw semen characteristics were consistently observed in Group III. Although MDA, SOD, and ALT values were not significantly affected by the treatments, post-thaw AST activity was significantly lower in the L-methionine-supplemented group, indicating reduced membrane damage, while the group also showed lower MDA and higher SOD values after thawing. The results suggest that removal of deleterious egg yolk components through centrifugation, together with the antioxidant action of L-methionine, improved the ability of spermatozoa to withstand cryopreservation. Supplementation of a centrifuged TFEYG extender with 2.5 mM L-methionine can therefore be considered a useful approach for improving the post-thaw quality of Kankrej bull semen.

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

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.2026.22.06.26

Introduction

Artificial insemination (AI) has become one of the most successful reproductive technologies for accelerating genetic improvement in cattle through the widespread use of genetically superior sires. The effectiveness of AI programmes depends largely on the availability of high-quality cryopreserved semen that retains its fertilizing ability after thawing. However, the freeze-thaw process exposes spermatozoa to cold shock, osmotic stress, membrane disruption, and oxidative damage, resulting in reduced motility, viability, membrane integrity, and ultimately lower fertility (Said *et al*., 2020; Prasad and Kumar, 2022). Consequently, considerable efforts have been directed toward improving semen extender formulations to minimize cryo-injury and enhance post-thaw sperm survival.

Egg yolk has been used for decades as a key component of semen extenders because of its protective effect against cold shock during cryopreservation. This protection is mainly attributed to its low-density lipoprotein (LDL) fraction, which helps stabilize the sperm plasma membrane and acrosome during freezing and thawing (Moussa *et al*., 2002). Despite these advantages, whole egg yolk also contains several undesirable constituents, including high-density lipoproteins (HDL), calcium ions, steroid hormones, and microorganisms, which may impair sperm function, induce premature capacitation, interfere with semen evaluation, and reduce fertility (Akhter *et al*., 2008).

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How to cite this article: Makawana, G. S., Rathod, B. S., Chaudhary, A. S., Kapadiya, P. S., & Chaudhary, R. K. (2026). Evaluation of Centrifuged Egg Yolk Extender with L-Methionine for Improving Cryosurvival of Kankrej Bull Spermatozoa. Ind J Vet Sci and Biotech, 22(6), 151-155.

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

Conflict of interest: None

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

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To overcome these limitations, centrifugation has been employed to remove the detrimental components while retaining the protective LDL-rich fraction. Improved semen freezability and fertility following the use of clarified or centrifuged egg yolk extenders have been reported in bulls and other domestic species (Fernandez-Santos *et al*., 2006; Nouri *et al*., 2013).

Another important factor affecting semen quality is bacterial contamination introduced during semen collection, processing, and storage. Microorganisms stimulate excessive production of reactive oxygen species (ROS), leading to lipid peroxidation, membrane damage, and deterioration of sperm motility and viability (Gloria *et al*., 2014). Since mature spermatozoa possess only limited endogenous antioxidant defence, supplementation of semen extenders with antioxidants has become an effective strategy to reduce oxidative damage during cryopreservation (Bilodeau *et al*., 2002) L-methionine, a sulfur-containing amino acid and precursor of glutathione, is recognized for its antioxidant and membrane-protective properties. It readily reacts with ROS, thereby limiting oxidative injury and improving sperm survival during freezing. Previous studies have shown that methionine supplementation enhances post-thaw sperm motility while reducing dead and acrosome-damaged spermatozoa (Bucak *et al*., 2010; Kim *et al*., 2014; El-Battawy and El-Nattat, 2018). Although the individual benefits of clarified egg yolk extenders and antioxidant supplementation have been well documented, information regarding their combined application in Kankrej bull semen cryopreservation remains limited. Therefore, the present study was undertaken to evaluate the protective role of a centrifuged Tris-fructose egg yolk glycerol (TFEYG) extender supplemented with 2.5 mM L-methionine on post-dilution and post-thaw semen quality, oxidative stress, enzymatic activity, microbial load, and field fertility of cryopreserved Kankrej bull semen.

Materials and Method

Experimental Design and Semen Processing

The experiment was conducted using semen collected from three clinically healthy, sexually mature Kankrej (*Bos indicus*) bulls maintained at the Livestock Research Station, Kamdhenu University, Sardarkrushinagar, India. A total of 18 ejaculates (six ejaculates from each bull) were collected at weekly intervals using a Danish model artificial vagina with the homosexual mount technique. Only ejaculates showing an initial progressive motility of ≥70% were included in the study.

The Tris-fructose-egg yolk-glycerol (TFEYG) extender was prepared according to the Minimum Standard Protocol for bovine semen production (Government of India, 2024). Clarified egg yolk was prepared as described by Wall and Foote (1999). Briefly, TFEYG extender centrifuged twice at 10,000 g for 45 min at 4°C. The supernatant obtained after the centrifugation was used as dilutor. Each ejaculate was divided into three equal aliquots and diluted with one of the following extenders: conventional TFEYG (Group I; control), centrifuged TFEYG extender (Group II), and centrifuged TFEYG extender supplemented with 2.5 mM L-methionine (Group III). Semen was diluted to a final concentration of 80×10^6 spermatozoa/mL using an automated photometer (Accucel, IMV Technologies, France). The extended semen was packaged in 0.25-mL French mini straws, equilibrated at 4°C for 4 h, cryopreserved using a programmable freezer, and stored in liquid nitrogen (−196°C). After 24 h of storage, the straws were thawed at 37°C for 30 sec. and evaluated at the post-thaw stage.

Evaluation of Post-Dilution and Post-Thaw Semen Quality

Semen quality was assessed immediately after dilution and following thawing. Progressive sperm motility was evaluated by light microscopy, while sperm viability was determined using the eosin-nigrosin staining technique. Morphological abnormalities were assessed after examining 200 spermatozoa under oil immersion in the eosin-nigrosin stained smears, and expressed as the percentage. Functional integrity of the sperm plasma membrane was evaluated using the hypo-osmotic swelling test (HOST) following the procedure of Jeyendran *et al*. (1984).

Determination of Oxidative Stress and Enzymatic Activity

Seminal plasma was separated from post-dilution and post-thaw semen samples by centrifugation at 4000 g for 10 min. Malondialdehyde (MDA), an indicator of lipid peroxidation, and superoxide dismutase (SOD) activity were quantified using commercially available bovine ELISA kits (Puregene, Genetix Biotech Asia Pvt. Ltd., New Delhi, India) according to the manufacturer's recommendations.

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), used as indicators of sperm membrane damage, were determined in seminal plasma using commercially available colorimetric diagnostic kits (LiquiCHEK, Agappe Diagnostics Ltd., Kerala, India). Enzyme activities were measured using an automated biochemical analyser and expressed as U/L.

Statistical Analysis

Data were analysed using IBM SPSS Statistics version 20. Normality of the data was assessed using the Shapiro-Wilk test, and percentage data were subjected to arcsine transformation before statistical analysis. Differences

among treatment groups at the post-dilution and post-thaw stages were analysed using one-way analysis of variance. Results are presented as mean ± standard error (SE), and differences were considered statistically significant at $p < 0.05$.

Results and Discussion

The findings on post-dilution and post-thaw stage individual subjective motility, sperm viability, HOST-reactive sperm and sperm abnormality are presented in Table 1. Individual subjective motility (%), sperm viability (%), HOST-reactive sperm (%) and sperm abnormality (%) differed significantly ($p<0.05$) among the treatment groups at both stages of semen processing. At the post-dilution stage, Group III exhibited significantly higher individual subjective motility (78.05±4.24%), sperm viability (82.27±1.02%) and HOST-reactive sperm (76.16±1.10%), along with significantly lower sperm abnormality (5.61±0.18%) than Group I. Group II also showed significantly improved values for these parameters compared to the control group, whereas the differences between Group II and Group III for individual motility and HOST response were non-significant. At the post-thaw stage, individual subjective motility, sperm viability and HOST-reactive sperm remained significantly ($p<0.05$) higher in Group III followed by Group II than in Group I. Likewise, sperm abnormality at the post-thaw stage was significantly lower in Group III followed by Group II compared with Group I (Table 1).

Table 1: Effect of different extender on semen qualitative parameters in Kankrej bull semen at post dilution and post thawed stage (Mean ± SE, n=18)

Parameters	Treatment group	Stage	
		Post-dilution	Post-thaw
Individual subjective motility (%)	Group I	72.22±1.29^{a}	49.72±0.63^{a}
	Group II	76.94±1.22^{b}	56.11±0.76^{b}
	Group III	78.05±4.24^{b}	58.61±0.88^{c}
	P value	**0.002**	**0.001**
Total viability (%)	Group I	76.61±1.34	53.33±0.51^{a}
	Group II	80.77±1.26	61.00±0.93^{b}
	Group III	82.27±1.02	63.16±1.04^{b}
	P value	**0.005**	**0.001**
Sperm abnormality (%)	Group I	8.05±0.25^{a}	11.83±0.24^{a}
	Group II	6.22±0.19^{b}	8.77±0.30^{b}
	Group III	5.61±0.18^{c}	8.00±0.26^{c}
	P value	**0.001**	**0.001**
HOST reacted sperm (%)	Group I	70.16±1.55^{a}	50.61±0.54^{a}
	Group II	75.55±1.38^{b}	56.39±1.04^{b}
	Group III	76.16±1.10^{b}	57.94±1.16^{b}
	P value	**0.005**	**0.001**

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

The present findings closely agreed with earlier reports demonstrating the beneficial effects of centrifuged egg yolk extender and methionine supplementation on sperm quality during cryopreservation. Çoyan *et al*. (2010), Bucak *et al*. (2010), Dheerib *et al*. (2020), Verma *et al*. (2023), and Alam *et al*. (2025) reported improved post-thaw sperm motility, viability and membrane integrity following methionine supplementation. Similarly, Singh *et al*. (2020) and Chaudhari *et al*. (2017) observed superior post-thaw semen quality in semen extended with centrifuged egg yolk or modified egg yolk extenders compared with conventional egg yolk extenders. Reduced sperm abnormalities following antioxidant supplementation have also been reported by Bucak *et al*. (2010), Sariozkan *et al*. (2014) and El-Hawary *et al*. (2017), whereas improved HOST response after antioxidant supplementation has been documented by Nair *et al*. (2006) and Wafa *et al*. (2021).

During cryopreservation, spermatozoa are exposed to cold shock, osmotic stress and excessive production of reactive oxygen species, resulting in disruption of plasma membrane integrity, mitochondrial dysfunction and lipid peroxidation. These changes ultimately reduce sperm motility, viability and membrane functionality while increasing morphological abnormalities. The comparatively better post-thaw semen quality observed in Groups II and III may be attributed to the removal of detrimental egg yolk components, such as high-density lipoproteins, yolk granules and other particulate substances, through centrifugation, thereby enriching the low-density lipoprotein fraction responsible for membrane stabilization. Supplementation of centrifuged TFEYG extender with 2.5 mM L-methionine appears to provide additional protection by scavenging reactive oxygen species, reducing oxidative damage and preserving mitochondrial activity as well as plasma membrane integrity during freezing and thawing. Consequently, spermatozoa cryopreserved in centrifuged TFEYG supplemented with methionine retained higher motility, viability and functional membrane integrity, together with lower structural abnormalities, than semen cryopreserved in the conventional extender.

The semen samples were further assessed for malondialdehyde (MDA), superoxide dismutase (SOD), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at the post-dilution and post-thaw stages, and the values are presented in Table 2. The MDA and SOD concentrations did not differ significantly among the treatment groups at either the post-dilution or post-thaw stage. Although the differences were statistically non-significant, Group III recorded better value of MDA concentrations at both the post-dilution (7.80±1.28 nmol/mL) and post-thaw (9.16±1.41 nmol/mL) stages

compared with the control group (8.45±1.47 and 9.82±1.51 nmol/mL, respectively). Likewise, SOD concentration remained statistically comparable among all treatment groups at both stages. AST activity did not differ significantly at the post-dilution stage; however, at the post-thaw stage, it was significantly ($p<0.05$) lower in Group III followed by Group II than in Group I. ALT activity showed non-significant differences among the treatment groups at both post-dilution and post-thaw stages, although numerically lower ALT values were recorded in Groups II and III than in the control group after thawing (Table 2).

Table 2: Effect of centrifuged extender and methionine on oxidative stress markers and biochemical parameters in Kankrej bull semen at post dilution and post-thawed stage (Mean ± SE, n=18)

Parameters	Treatment group	Stage	
		Post-dilution	Post-thaw
MDA (nmol/mL)	Group I	8.45 ±1.47	9.82±1.51
	Group II	7.80±1.28	10.22±1.58
	Group III	7.80±1.28	9.16±1.41
	P value	**0.919**	**0.880**
SOD (ng/mL)	Group I	40.14±1.61	37.83±1.63
	Group II	36.91±1.83	34.53±1.86
	Group III	37.22±2.07	34.67±2.05
	P value	**0.402**	**0.372**
AST (U/L)	Group I	26.01 ±1.54^{a}	33.87±1.77^{a}
	Group II	22.57± 1.86^{a}	28.44±2.16^{ab}
	Group III	20.76±2.31^{a}	26.35±2.34^{b}
	P value	**0.161**	**0.041**
ALT (U/L)	Group I	12.82±2.23^{a}	17.82±2.42^{a}
	Group II	10.75±2.22^{a}	14.84±2.34^{a}
	Group III	11.33±2.53 ^{a}	15.67±3.16^{a}
	P value	**0.806**	**0.719**

Means bearing different superscripts (a, b) differ significantly within row ($p<0.005$)

The present observations agreed with Çoyan *et al*. (2010), Bucak *et al*. (2010) and Akalin *et al*. (2015), who reported non-significant changes in MDA concentration following methionine supplementation during semen cryopreservation. Similar non-significant variation in SOD activity after antioxidant supplementation has been reported by Singh *et al*. (2020), whereas Verma *et al*. (2023) and Alam *et al*. (2025) observed reduced AST and ALT leakage in methionine-treated semen, indicating better preservation of sperm membrane integrity. Comparable reductions in enzyme leakage following improvement of extender composition have also been reported in bull semen by Kapadiya *et al*. (2018) and other researchers (Chaudhary *et al.,* 2026).

Cryopreservation induces oxidative stress through excessive generation of reactive oxygen species, leading to lipid peroxidation, membrane destabilization and leakage of intracellular enzymes from spermatozoa. Although MDA and SOD values remained statistically comparable among the treatment groups in the present study, the consistently lower numerical MDA concentration observed in Group III suggests a favourable trend towards reduced lipid peroxidation. Likewise, the significantly lower AST activity and numerically reduced ALT activity observed in Groups II and III indicate better preservation of sperm plasma membrane integrity during freezing and thawing. The beneficial effects of centrifuged TFEYG extender may be attributed to the removal of harmful egg yolk constituents while retaining the protective LDL fraction, which stabilizes the sperm membrane. The additional improvement observed with methionine supplementation is likely due to its antioxidant properties, which minimize oxidative damage, preserve membrane phospholipids and reduce leakage of intracellular enzymes during cryopreservation. These findings further support the superior cryoprotective efficiency of centrifuged TFEYG extender supplemented with 2.5 mM L-methionine for maintaining post-thaw semen quality in Kankrej bulls.

Conclusion

The present findings indicate that the use of centrifuged TFEYG extender, particularly when supplemented with 2.5 mM L-methionine, resulted in better preservation of post-thaw semen quality in Kankrej bulls. The treatment improved individual sperm motility, viability and plasma membrane integrity, while reducing sperm abnormalities and AST leakage compared with the conventional TFEYG extender. Although the differences in MDA, SOD and ALT were not statistically significant, better values observed in the methionine-supplemented group suggest improved protection against cryo-induced oxidative stress. The membrane-stabilizing effect of the centrifuged egg yolk extender together with the antioxidant property of L-methionine appears to have contributed to improved preservation of sperm functional characteristics during cryopreservation. Therefore, supplementation of centrifuged TFEYG extender with 2.5 mM L-methionine may be considered an effective approach for improving cryosurvival and post-thaw semen quality in Kankrej bull semen.

Acknowledgement

The authors acknowledge the Kamdhenu University, Sardarkrushinagar, for their support, and extend their gratitude to the Banas Dairy, Palanpur, Gujarat, for providing facilities and technical assistance.

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