RESEARCH ARTICLE

Nano-Cerium: A Promising Additive for Enhancing Cryosurvival and Antioxidant Defence in Buffalo Bull Semen

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Abstract

The present study was aimed to evaluate the effect of cerium oxide nanoparticles (CeO_2NPs) supplementation in Tris-fructose-yolk-glycerol (TFYG) extender on post-thaw semen quality of buffalo bulls. Semen ejaculates (n=18) collected from four healthy, sexually mature bulls using an artificial vagina were pooled and divided into four treatment groups containing 0 (control), 25, 50, and 75 µg/mL CeO_2NPs in TFYG extender. The samples were processed, equilibrated, cryopreserved, and evaluated post-thaw for sperm motility, viability, morphology, plasma membrane integrity, acrosome integrity, malondialdehyde (MDA), and total antioxidant capacity (TAC). Post-thaw sperm motility was significantly higher ($p<0.05$) in extender supplemented with 50 and 75 µg/mL CeO_2NPs compared to 25 µg/mL and control groups, while 75 µg/mL CeO_2NPs showed a marked improvement in sperm viability and reduced sperm abnormalities compared to other treatments. The extender with 75 µg/mL CeO_2NPs also maintained significantly better sperm plasma membrane and acrosome integrity than the lower concentrations and control. Moreover, the MDA level was significantly reduced ($p<0.05$) and TAC was increased in semen supplemented with 75 µg/mL CeO_2NPs, indicating enhanced antioxidant protection and reduced oxidative stress during cryopreservation. It is concluded that supplementation of TFYG extender with 75 µg/mL cerium oxide nanoparticles is optimal for maintaining superior post-thaw sperm quality by improving structural and functional attributes as well as antioxidant status in buffalo bull semen, though further *in vivo* fertility trials are required to confirm these findings.

Key words: Antioxidant status, Buffalo bulls, Cerium oxide nanoparticles, Cryopreservation, TFYG extender.

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Introduction

Animal husbandry plays a vital role in supporting rural livelihoods, contributing significantly to its economy. Globally, the buffalo population is estimated at 208 million, with over 98% in Asia and India contributing about 57% (FAO, 2024). Buffaloes are valued for their high milk yield, quality meat, and draught power, as well as their ability to utilize low-quality feed and support soil fertility (Pasha and Hayat, 2012). Despite their economic importance, buffaloes exhibit poor reproductive efficiency due to late maturity, silent estrus, and prolonged calving intervals (Srirattana *et al*., 2022). Artificial insemination (AI) is an effective tool for genetic improvement, yet conception rates with frozen semen remain low (42.5-51.1%) compared to cattle (Kumaresan *et al*., 2006; Bhagat *et al*., 2020). This limitation largely arises from cryoinjury during semen freezing and thawing, which causes loss of motility, membrane integrity, and fertility (Bucak *et al*., 2008).

Cryopreservation imposes osmotic, thermal, and oxidative stress on spermatozoa, reducing viability by nearly half (Kumar *et al*., 2015). Excessive generation of reactive oxygen species (ROS) during the freeze–thaw process leads to lipid peroxidation, DNA damage, and impaired fertilization capacity (Tiwari *et al*., 2021). Although semen possesses antioxidant defenses, their depletion during cryopreservation makes buffalo sperm highly susceptible to oxidative injury (Bucak *et al*., 2008). Cerium oxide nanoparticles (CeO_2NPs) have recently gained attention for their regenerative antioxidant activity, attributed to reversible redox cycling between Ce^{3+} and Ce^{4+}states (Karakoti *et al*., 2010). Acting as mimetics of superoxide dismutase and catalase, CeO_2NPs can effectively scavenge ROS and protect

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cells from oxidative stress (Das *et al*., 2013). Given the critical role of oxidative stress in cryo-induced sperm damage, CeO_2NPs may serve as a promising additive to improve bull (Al-Janabi *et al*., 2024; Solanki *et al.,* 2025), buck (Khalique *et al*. (2024) and human (Hosseinmardi *et al.,* 2022) semen cryopreservation. Therefore, the present study was planned to evaluate the effects of cerium oxide nanoparticles during semen cryopreservation on post-thaw sperm quality and antioxidant status of buffalo bulls.

Materials and Methods

Four sexually mature healthy breeding buffalo bulls, which were in regular semen collection at Central Sperm Station of Department of Veterinary Gynaecology and Obstetrics of the College in Anand (India) were selected for the present study. Selected bulls were maintained under general management practices as followed for bulls at Central Sperm Station, Anand. Semen collections were obtained in the early morning at 7:00 to 8:30 a.m., twice per week, from each bull using the artificial vagina (Danish Model). Total, 18 ejaculates from four bulls were used for this study.

Preparation and Formulation of Semen Extender

In this study, a Tris fructose egg yolk glycerol (TFYG) extender was used. The semen additive, cerium oxide nanoparticles, used in this study was obtained from Sigma-Aldrich®. Initially, 50 mg/mL solution of cerium oxide nanoparticles was prepared. From that 0.5, 1.0 and 1.5 µL solution was added per mL of the TFYG extended semen in three jar to get final concentration of cerium oxide nanoparticles (CeO_2NPs) as 25, 50 and 75 µg/mL, respectively. These were then used in the study employing one aliquot as non-added control extender.

Experimental Design and Groups

Immediately after collection, semen was evaluated for routine physical characteristics. Ejaculates containing more than 70 % initial progressive motile spermatozoa were divided into four equal aliquots. Aliquot-1 was diluted with 0.5 µL/mL CeO_2NPs (25 µg/mL CeO_2NPS group), aliquot-2 was diluted with 1 µL/mL CeO_2NPs (50 µg/mL CeO_2NPs group), aliquot-3 was diluted with 1.5 µL/mL CeO_2NPS (75 µg/ mL CeO_2NPs group), and aliquot-4 served as a control and was kept without additive.

After final dilution, semen samples were filled and sealed in 0.5 mL French medium straws at room temperature (22-25°C) using an automatic filling and sealing machine (IS4, IMV, France). The filled straws were placed in freezing racks and equilibrated at 4°C for at least 4 h to ensure gradual cooling. Subsequently, freezing was carried out in liquid nitrogen vapour using a thermocol box, following the standard bovine semen freezing protocol, for 10 min. The frozen straws were then stored in liquid nitrogen (-196 °C) overnight. After 24 h of storage, the straws were thawed in a water bath at 37 °C for 30 s and immediately evaluated for sperm quality and oxidative parameters.

Sampling Protocol for Biochemical Assay

Soon after post-thaw motility evaluation, a portion of thawed semen was centrifuged for 15 min at 750 xg in order to separate out the seminal plasma. A drop of seminal plasma was viewed at a 40X magnification to confirm absence of spermatozoa. Seminal plasma was then preserved at -20°C in sterile vials until used for estimation of oxidative stress markers. Both the total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in seminal plasma were estimated using standard assay kits supplied by Puregene (Genetix Biotech Asia Pvt. Ltd.).

Statistical Analysis

Data for various parameters were analysed using one-way ANOVA, followed by Duncan's multiple range test in SPSS (Statistical Package for the Social Sciences), Version 20.0 to determine significant differences among groups. Results were expressed as Mean ± SE.

Results and Discussion

The mean post-thaw motility (%) differed significantly ($p<0.05$) among treatment groups, with the highest value recorded in T75 (58.19 ± 0.93%), followed by T50 (55.19 ± 0.99%), T25 (52.34 ± 0.96%), and the control (50.92 ± 0.84%). Motility in the T75 group was significantly higher ($p<0.05$) than in all other groups, while the T50 group also differed significantly ($p<0.05$) from the control and T25. No significant ($p>0.05$) difference was observed between the control and T25 groups (Table 1). Similar trends were reported by Al-Janabi *et al*. (2024), who observed higher post-cooled motility at 75 µg/mL CeO_2NPs (40.00 ± 0.00%) compared to the control (36.67 ± 1.67%), while higher concentrations reduced motility. Solanki *et al*. (2025) also recorded significantly ($p<0.001$) higher post-thaw sperm motility and viability of Gir bull semen in AndroMed® extender with 75 µg/mL CeO_2NPs concentration as compared 25 µg/mL and 50 µg/mL CeO_2NPs and control extender. Hosseinmardi *et al*. (2022) found that 0.1 µg/mL CeO_2NPs significantly enhanced post-thaw progressive motility in human spermatozoa. Likewise, Khalique *et al*. (2024) reported improved total and progressive motility ($p<0.001$; $p=0.003$) at 25-50 µg/mL CeO_2NPs in Beetal bucks. The improvement in motility at lower CeO_2NPs concentrations may be attributed to their cryoprotective and antioxidant effects, which preserve mitochondrial function and ATP synthesis, thereby sustaining sperm motility post-thaw (Reddy *et al*., 2010). Recently, 1 µg/mL ZnONPs in AndroMed® extender compared to higher or lower levels was also found to improve significantly ($p<0.01$) the post-thaw sperm motility of Gir bull semen (Ram *et al.,* 2025).

Sperm viability in the control group was 54.11 ± 0.80%, while treatments T25, T50, and T75 recorded 56.61 ± 0.89%, 58.39 ± 0.89%, and 60.28 ± 0.93%, respectively (Table 1). The

T75 group showed significantly ($p<0.05$) higher post-thaw viability than the control, T25, and T50 groups, whereas T25 and T50 also differed significantly ($p<0.05$) from the control, but not from each other ($p>0.05$). Similar trends were reported by Al-Janabi *et al*. (2024) and Hosseinmardi *et al*. (2022), who observed improved sperm viability at lower CeO_2NP concentrations, while higher doses had suppressive effects. Khalique *et al*. (2024) also found enhanced viability in Beetal bucks at 25-50 µg/mL CeO_2NPs, while Solanki *et al*. (2025) found significantly ($p<0.001$) higher sperm viability in Gir bull semen with 75 µg/mL CeO_2NPs. The improvement at lower concentrations may be attributed to the antioxidant properties of CeO_2NPs, which scavenge reactive oxygen species, maintain mitochondrial function, and preserve plasma membrane integrity during cryopreservation. Further Ram *et al*. (2025) observed significantly ($p<0.01$) improved post-thaw sperm viability with 1 µg/mL ZnONPs in AndroMed® extender for Gir bull semen compared to higher or lower levels.

The mean sperm abnormality was 17.83 ± 0.57% in the control group, while treatments T25, T50, and T75 recorded 15.89 ± 0.49%, 14.44 ± 0.46%, and 12.89 ± 0.57% values, respectively. The T75 group showed the lowest sperm abnormality, which was significantly ($p<0.05$) lower than all other groups. Both T25 and T50 also exhibited significantly ($p<0.05$) reduced sperm abnormalities compared with the control, while no significant ($p>0.05$) difference was observed between T25 and T50 (Table 1). Solanki *et al*. (2025) also found similar significantly ($p<0.001$) reduced sperm abnormalities with higher sperm viability of Gir bull semen cryopreserved in AndroMed® extender with 75 µg/mL CeO_2NPs concentration compared lower levels of CeO_2NPs and control extender. In contrast, Hosseinmardi *et al*. (2022) did not find significant effect of CeO_2NPs on morphology of human sperm. However, findings by Khalil *et al*. (2023) and El-Kholy *et al*. (2023) demonstrated reduced post-thaw sperm abnormalities following supplementation with thymoquinone nanoparticles and selenium nanoparticles, respectively, in buffalo semen. These observations suggest that nanoparticles supplementation can mitigate cryo-induced structural damage, improving sperm morphology and post-thaw semen quality. Variations among studies may be attributed to species differences, extender composition, and differences in cryopreservation protocols or CeO_2NPs concentrations.

Mean sperm plasma membrane integrity (HOST-reactive sperm) was 45.56 ± 0.88%, 47.33 ± 0.81%, 49.28 ± 0.92%, and 51.44 ± 0.91% in the control, T25, T50, and T75 groups, respectively (Table 1). The T75 group recorded significantly ($p<0.05$) higher membrane integrity than the control and T25, while T50 also differed significantly from the control, but not from T25. No significant difference was detected between T25 and the control. Comparable results were reported by Hosseinmardi *et al*. (2022), who observed improved post-thaw membrane functionality in human spermatozoa supplemented with 0.1 µg/mL CeO_2NPs (66.1 ± 1.85% vs. 55.4 ± 1.85%; $p<0.05$). Solanki *et al*. (2025) found significantly ($p<0.001$) higher sperm plasma membrane and acrosome integrity of Gir bull semen cryopreserved in AndroMed® extender with 75 µg/mL CeO_2NPs concentration compared 25 and 50 µg/mL CeO_2NPs and control extender. Likewise, Khalique *et al*. (2024) noted higher membrane integrity ($p = 0.003$) in Beetal buck semen at 25-50 µg/mL CeO_2NPs. Other nanoparticles, including ZnO-NPs (Hozyen *et al*., 2023; Ram *et al*., 2025), Se-NPs (El-Kholy *et al*., 2023), and thymoquinone nanoparticles (Khalil *et al*., 2023), have also been shown to enhance membrane stability in cryopreserved bovine semen. Such improvements are primarily attributed to the antioxidant and membrane-protective effects of nanoparticles, although variations among studies may arise from species differences, extender composition, and protocol-specific factors.

Supplementation of semen extenders with CeO_2NPs positively influenced acrosomal integrity, yielding post-thaw mean values of 56.17 ± 0.81%, 57.94 ± 0.84%, 60.06 ± 0.90%, and 62.39 ± 0.95% in the control, T25, T50, and T75 groups, respectively (Table 1). The T75 group exhibited significantly ($p<0.05$) higher acrosome integrity than the control and T25 groups but comparable to T50. The T50 group also differed significantly ($p<0.05$) from the control, while T25 showed no significant ($p>0.05$) difference. Comparable enhancement of acrosomal integrity was reported by Khalique *et al*. (2024) in Beetal bucks semen supplemented with 25-50 µg/mL CeO_2NPs, and by El-Kholy *et al*. (2023) and Hozyen *et al*. (2023) following supplementation with Se-NPs and ZnO-NPs, respectively. Ram *et al*. (2025) observed significantly ($p<0.01$) higher post-thaw HOST and acrosomal integrity with 1 µg/mL ZnONPs in AndroMed® extender for Gir bull semen. The protective action of nanoparticles may be attributed to their antioxidant and membrane-stabilizing properties,

Table 1: Effect of different concentration of cerium oxide nanoparticles (CeO_2NPs) on post-thaw sperm quality parameters of buffalo bulls semen (Mean ±SE, n=18).

Groups (CeO_2NPs levels)	Individual sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	HOST reactivity (%)	Acrosome integrity (%)
Control	50.92±0.84^{a}	54.11±0.80^{a}	17.83±0.57^{c}	45.56±0.88^{a}	56.17±0.81^{a}
T25	52.34±0.96^{a}	56.61±0.89^{b}	15.89±0.49^{b}	47.33±0.81^{ab}	57.94±0.84^{ab}
T50	55.19±0.99^{b}	58.39±0.89^{bc}	14.44±0.46^{b}	49.28±0.92^{bc}	60.06±0.90^{bc}
T75	58.19±0.93^{c}	60.28±0.93^{c}	12.89±0.57^{a}	51.44±0.91^{c}	62.39±0.95^{c}

Means with different superscripts within the column differ significantly ($p<0.05$)

which preserve the acrosomal and plasma membranes during freezing. As the acrosome is highly sensitive to cold-induced structural disruption (Church and Graves, 1979), the stabilization of membrane systems by CeO_2NPs likely minimizes enzyme leakage and maintains functional integrity essential for fertilization.

Post-thawed seminal plasma of buffalo bull semen cryopreserved in TFYG extender supplemented with varying concentrations of CeO_2NPs (Table 2) revealed that T75 group had significantly ($p<0.05$) lower MDA concentrations than the control and T25 groups, while T50 also differed significantly ($p<0.05$) from the control. No significant ($p>0.05$) variation was observed between T50 and T75, or between T25 and control. These findings agreed with Hosseinmardi *et al*. (2022) and Khalique *et al*. (2024), who observed marked reductions in lipid peroxidation following CeO_2NPs supplementation in human and buck semen, respectively. Solanki *et al.* (2025) observed significantly ($p<0.001$) lower MDA and higher TAC in Gir bull semen cryopreserved in AndroMed® extender with 75 µg/mL CeO_2NPs concentration as compared to 25 and 50 µg/mL CeO_2NPs and control extender. Comparable antioxidative effects were reported by Hozyen *et al*. (2023) and Ram *et al.* (2025) with ZnO-NPs, Khalil *et al*. (2023) with thymoquinone nanoparticles, and Eliraqy *et al*. (2024) with lecithin nanoparticles. The reduction in MDA concentration indicates that CeO_2NPs effectively attenuate lipid peroxidation and protect sperm membrane lipids during cryopreservation through their potent free radical scavenging activity.

Table 2: Effect of different concentration of cerium oxide nanoparticles (CeO_2NPs) on biochemical attributes (Mean ± SE) in buffalo bulls (n=18 ejaculates/group)

Groups (CeO2NPs levels)	Post-thaw	
	Lipid peroxidation (µmol/L)	Total antioxidant capacity (µmol/L)
Control	4.12 ± 0.1^{8}c	239.55 ± 13.6^{7}a
T25	3.70 ± 0.2^{4b}c	263.09 ± 12.1^{0}a
T50	3.15 ± 0.3^{3a}b	273.30 ± 11.0^{9}a
T75	2.66 ± 0.2^{4}a	312.05 ± 16.0^{7}b

Means with different superscripts within column differ significantly at $p<0.05$ level.

The mean total antioxidant capacity (TAC) in the post-thawed seminal plasma of buffalo bull semen cryopreserved in TFYG extender supplemented with CeO_2NPs was significantly ($p<0.05$) higher in T75 group (312.05 ± 16.07 µmol/L) compared to all other groups including control 239.55 ± 13.67 µmol/L), whereas differences among T25 and T50 were non-significant (Table 2). Similar enhancement in antioxidant capacity following nanoparticle supplementation was reported by Khalil *et al*. (2023) and Eliraqy *et al*. (2024) in buffalo semen using thymoquinone and lecithin nanoparticles, respectively. The higher TAC observed at 75 µg/mL CeO_2NPs concurred with the findings of Solanki *et al*. (2025), and indicated improved oxidative balance and protection against cryo-induced lipid peroxidation during preservation of bull semen. Ram *et al.* (2025) noted significantly ($p<0.01$) higher post-thaw seminal plasma TAC and reduced MDA levels with 1 µg/mL ZnONPs in AndroMed® extender for Gir bull semen compared to 1.5 or 0.5 µg/mL and control extender.

Conclusions

The current research findings showed that the supplementation of TFYG extender with cerium oxide nanoparticles (CeO_2NPs), particularly 75 µg/mL, significantly improved post-thaw sperm motility, viability, plasma membrane and acrosome integrity, while reducing sperm abnormalities compared to lower concentrations and the control. The observed decrease in malondialdehyde (MDA) levels alongside an increase in total antioxidant capacity (TAC) at this concentration indicates enhanced antioxidant defence and reduced oxidative damage during cryopreservation. Overall, supplementation with 75 µg/mL CeO_2NPs can be considered the optimal for maintaining post-thaw sperm quality in buffalo bull semen. Nevertheless, further *in vivo* fertility studies are warranted to validate these results and establish practical recommendations for field application.

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