RESEARCH ARTICLE

Effect of Cerium Oxide Nanoparticles as an Additive in Cryopreservation of Gir Bull Spermatozoa

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Abstract

The present investigation was carried out on semen of four Gir bulls at Cattle Breeding Farm, Junagadh for a period of 6 months (Feb-July 2024). Semen (n=16 ejaculates) was collected using artificial vagina (Danish model) once weekly from each bull. The semen was evaluated for sperm quality attributes along with oxidative stress parameters at post-thaw stage of cryopreservation using different concentrations of Cerium Oxide Nanoparticles, *viz.,* 25 µg/mL, 50 µg/mL, 75 µg/mL CeO_2NPs and a control AndroMed® extender. AndroMed® extender with 75 µg/mL CeO_2NPs concentration had significantly ($p<0.001$) higher sperm motility, viability, HOST reactive sperm, acrosome integrity with lower sperm abnormality as compared to that of the 25 µg/mL and 50 µg/mL CeO_2NPs and control extender. Mean malondialdehyde (MDA) levels in seminal plasma of post-thaw semen varied significantly between treated and control groups, but the value with 75 µg/mL CeO_2NPs concentration was the lowest and it increased with the decreased level of CeO_2NPs, and was highest in control group, while total antioxidants activity (TAC) increased proportionately with the increase in CeO_2NPs concentration, suggesting its beneficial role as an antioxidant molecule. All the above post-thaw seminal attributes were better in extender containing CeO_2NPs at 75 µg/mL concentration. The findings suggested the cryoprotective and antioxidant effects of CeO_2NPs on bull spermatozoa by lowering free radicals and its associated oxidative stress.

Key words: Cerium oxide nanoparticles, Gir bull, Malondialdehyde, Post-thaw sperm quality, Total antioxidant capacity.

Ind J Vet Sci and Biotech (2025): 10.48165/ijvsbt.21.4.25

Introduction

The Gir cattle breed is a renowned milch cattle breed in India. It originates from the Gir hills and forests of Kathiawar encompassing all the districts of Saurashtra region of Gujarat. Gir cattle are recognized for their stress tolerance and resistance to various tropical diseases (Gaur and Sharma, 2003). While many farmers in the state maintain only 2-3 female cattle at their doorstep, they often face challenges in keeping breeding sires of superior quality. To address this demand sires of superior germplasm are maintained at semen stations to provide high-quality cryopreserved semen for breeding cows through AI, since it is the oldest and most commonly used reproductive biotechnology for accelerating genetic improvement of livestock particularly dairy cattle and buffaloes. However, the success of AI depends on the production of high-quality frozen sperm. Spermatozoa usually suffer damage up to 50% as a result of cryopreservation (Watson, 2000). Semen is exposed to cold shock and ambient oxygen during cryopreservation, which raises the risk of lipid peroxidation by producing more reactive oxygen species (ROS) (Bucak *et al*., 2008). Excess ROS damages a variety of important molecules including lipids, proteins and DNA (Stadtman and Levine, 2000). Lipid peroxidation level is negatively correlated with motility, plasma membrane integrity and fertility of bull semen (Kasimanickam *et al*., 2007; Ram *et al.,* 2025).

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How to cite this article: Solaki, J. Z., Vala, K. B., Ram, D. R., Odedara, A. B., Chaudhary, J. K. & Devmurari, Y. J. (2025). Effect of Cerium Oxide Nanoparticles as an Additive in Cryopreservation of Gir Bull Spermatozoa. Ind J Vet Sci and Biotech, 21(4), 134-137.

Source of support: Nil

Conflict of interest: None

Submitted 07/05/2025 **Accepted** 10/06/2025 **Published** 10/07/2025

Antioxidants have a protective effect against sperm membrane lipid peroxidation and injuries caused by ROS (Bilodeau *et al*., 2000). The potential uses of nanoparticles in daily life are attracting more and more attention. The Scientific Committee on Emerging and Newly Identified Health Risk (SCENIHR) defines engineered nanoparticles (NPs) as particles with diameters ranging from 1 to 100 nm that possess unique physical, chemical and biological properties. Cerium oxide nanoparticles or CeO_2NPs have drawn a lot of

attention from scientists in recent years because of these unusual characteristics (Walkey *et al*., 2015). In light of their ability to store oxygen ensuing scavenger activity against ROS, similar to that of antioxidant enzymes in biological systems, the application of CeO_2NPs in biomedicine has recently been considered (Pirmohamed *et al*., 2010). Numerous publications in the literature reported that exposure to CeO_2NPs, ZnONPs and other nanoparticles reduced the levels of ROS in a variety of tissues or cells (Farhadi *et al*. 2022; Al-Janabi *et al*., 2024; Ram *et al.,* 2025). NPs also have a positive effect on sperm motility, kinematic parameters or sperm functionality. Because NPs have antioxidative properties they can reduce the harmful effects of ROS that arise from freezing or chilling reconstituted semen, thereby increasing sperm functions and male fertility (Khalil *et al*., 2019; Al-Janabi *et al*., 2024; Khalique *et al.,* 2024; Ram *et al.,* 2025). However, the work on Cerium Oxide Nanoparticles as extender additive is limited on bovine sperm; hence, this study was designed to evaluate the effect of CeO_2NPs as an extender additive in cryopreservation of Gir bull spermatozoa towards improving the post-thaw quality.

Materials and Methods

Four mature Gir bulls from the Cattle Breeding Farm, Kamdhenu University, Junagadh (Gujarat, India) were chosen for this research work. Junagadh is located at 21.52° N longitude and 70.47° E latitude in the foot hill of mountain Girnar in Gujarat on an average 107 meters from the mean sea level. The bulls were maintained on farm under uniform feeding, management and healthcare conditions, and were under weekly twice semen collection schedule using Danish Model of AV. All the glass-wares used in the study were carefully cleaned, dried, and sterilized for 1 h at 160° Celsius in a hot air oven. The artificial vagina was kept overnight at 45°C in an incubator after being autoclaved for 20 min at 5 psi. Rubber articles were autoclaved for 10 min at 5 psi as per MSP guidelines of Government of India.

For the current study, in all 16 ejaculates (4 from each of 4 bulls) obtained at weekly interval were utilized. The tubes containing semen were immediately put in a water bath set at 35°C, and evaluated for quality. Each good quality semen ejaculate was diluted with Andromed© (Mini-tube Germany) extender @ 80 million sperm/mL and sperm motility was evaluated. Samples with more than 70% initial progressively motile spermatozoa were divided into four equal aliquots in 4 jars. Cerium Oxide Nanoparticles (Sigma-Aldrich, USA) dissolved in distilled water @ 50 mg/mL was then added @ 0.5, 1.0 and 1.5 µL per mL of extended semen in the first three jars to get the final concentration of CeO_2NPs as 25 µg/mL, 50 µg/mL and 75 µg/mL, respectively, and the 4th aliquot served as non-added control extender. Each semen aliquot was then thoroughly mixed, filled and sealed in French medium transparent straw (0.5 mL capacity, TBS^{TM}, IMV, France) using an automatic filling and sealing machine and printed using a straw printer. The straws were moved to a cold handling cabinet (Macro Scientific Pvt. Ltd., New Delhi) and arranged in a freezing rack at 4°C for 4 h of equilibration.

After equilibration straws were vapour-freeze using conventional method and then all the straws were submerged in liquid nitrogen at -196°C for storage. Straws were stored cryopreserved for 24 h and then thawed for 30 sec at 37°C in a water bath to assess sperm motility, sperm viability, morphological abnormalities, acrosome integrity and hypo-osmotic swelling test. Post-thawed semen samples were immediately centrifuged for 15 min at 1425 x g to extract seminal plasma, which was stored at -20°C until analyzed for total antioxidant capacity and lipid peroxidation. The stored plasma samples were thawed at room temperature before analyzing the lipid peroxidation (MDA produced) and total antioxidant capacity (TAC) using the standard kits of HiMedia Lab Pvt. Ltd., Mumbai. The data for different parameters were expressed as Mean ±SEs and analyzed using one-way ANOVA and Duncan's *post hoc* test to find out significant differences between the levels of additive CeO_2NPs at $p<0.05$ (Snedecor and Cochran, 1994).

Results and Discussion

Effect of CeO_2NPs on Sperm Quality Parameters

The average post-thaw percentage of individual sperm motility, viability and abnormality varied significantly ($p<0.05$) between CeO_2NPs additive groups (Table 1). The mean values of post-thaw sperm motility and viability in the 75 µg/mL CeO_2NPs group were significantly ($p<0.05$) higher with reduced sperm abnormality as compared to the control and other groups. Additionally, post-thaw sperm motility and viability were also significantly ($p<0.05$) higher with reduced sperm abnormality in the groups treated with 50 µg/mL and 25 µg/mL CeO_2NPs than in the control group, and the mean values in the 50 µg/mL and 25 µg/mL CeO_2NPs group also differed significantly, showing the progressive improvement in sperm quality with enhancing the concentration of CeO_2NPs in the extender (Table 1). Similar findings were reported by Al-Janabi *et al*. (2024) and Khalique *et al*. (2024) using CeO_2NPs in HF bull and Beetal buck semen, respectively. Further, significant ($p<0.05$) improvement in sperm motility and viability of Holstein bull semen was also reported by Farhadi *et al*. (2022) with 1.0 µg/mL ZnONPs as compared to control group, while Jahanbin *et al*. (2021) recorded gradual increase in progressive sperm motility and viability in bull semen with addition of Zn Nanoparticles from 10^{-6} M to 10^{-2} M in the extender as compared to control group, and Ram *et al*. (2025) found significantly improved post-thaw motility and viability with reduced sperm abnormality using 1.0 µg/mL ZnONPs compared to higher or lower levels and control extender for cryopreservation of Gir bulls sperm.

The overall post-thaw mean sperm plasma membrane integrity (HOST reacted spermatozoa) and acrosome integrity also varied significantly ($p<0.05$) between 4 treatment groups

(Table 1). The mean percentages of plasma membrane integrity and acrosome integrity in the 75 µg/mL CeO_2NPs group were significantly ($p<0.05$) higher as compared to the control, 25 µg/mL and 50 µg/mL CeO_2NPs groups. However, relative to the control group, the sperm plasma membrane and acrosome integrity percentages in the 25 µg/mL and 50 µg/mL CeO_2NPs groups were shown to be significantly higher, and latter two levels also differed significantly, the values being higher in the 50 µg/mL CeO_2NPs group (Table 1). Similar findings were reported by Hosseinmardi *et al*. (2022) in human sperm and Khalique *et al.* (2024) in buck semen by using CeO_2NPs. Jahanbin *et al.* (2021) and Farhadi *et al*. (2022) reported similar findings of higher sperm plasma membrane and acrosome integrity in Holstein bull semen with ZnONPs supplementation as compared to control group, while Ram *et al.* (2025) noted the best results with 1.0 µg/mL ZnONPs compared to 0.0, 0.5 or 1.5 µg/mL ZnONPs in Andromed extender for cryopreservation of Gir bulls sperm. Acrosome is considered as limiting structure of fertilization, while a biochemically active sperm membrane is required for sperm capacitation, acrosome reaction and sperm binding to the egg surface. Acrosome is highly sensitive to freezing-thawing associated cryo-damage, and hence sperms with defective acrosome fail to bring out acrosomal exocytosis and oocyte-binding required during fertilization. Over production of ROS during freeze-thaw cycle results in rapid decrease in sperm motion behaviour, mitochondrial activity and plasma membrane and acrosome integrity affecting their structural integrity and fertility potential (Bucak *et al*., 2012).

Effect on Oxidative Stress Parameters

The mean malondialdehyde (MDA) concentration in the seminal plasma of 75 µg/mL CeO_2NPs group was significantly ($p<0.05$) lower and total antioxidant capacity (TAC) was higher ($p<0.05$) than in the control, 25 µg/mL and 50 µg/mL CeO_2NPs groups. Furthermore, relative to the control group, the mean malondialdehyde (MDA) values in the 25 µg/mL and 50 µg/mL CeO_2NPs groups were found to be significantly lower with higher total antioxidant capacity (TAC). Among the latter two levels, the 50 µg/mL CeO_2NPs group also showed significantly superior result with lower mean MDA and high TAC values than the 25 µg/mL CeO_2NPs group (Table 2). These findings on oxidative stress response of bull sperm following use of CeO_2NPs in semen extender compared well with Khalique *et al.* (2024) in buck semen. Further, Jahanbin *et al*. (2021) and Farhadi *et al.* (2022) found reduced post-thawed MDA level compared to control group by treating Holstein bull semen with different levels of ZnONPs. Khalil *et al.* (2023) and Ram *et al.* (2025) also reported lower MDA and higher TAC levels in post-thawed semen of buffalo bull and Gir bull semen by adding different concentrations of thymoquinone NPs, and ZnONPs, respectively,

Table 2: Mean (± SE) post-thaw sperm Lipid peroxidation (µM) and Total antioxidant capacity (µM) in Gir bull semen cryopreserved in AndroMed® extender with different concentration of CeO_2NPs

Groups (CeO_2NPs levels)	Post-thaw stage	
	Lipid peroxidation (MDA, µM)	Total antioxidant capacity (TAC, µM)
Control	4.26 ± 0.16^{c}	238.51 ± 9.91^{a}
T_{25}	3.71 ± 0.16^{bc}	263.40 ± 3.79^{ab}
T_{50}	3.31 ± 0.15^{b}	284.74 ± 6.19^{b}
T_{75}	2.64 ± 0.11^{a}	320.94 ± 9.21^{c}
p value	<0.001	<0.001

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

The natural seminal antioxidant content is inadequate for the prevention of lipid peroxidation during freezing and thawing process, which further decreases the antioxidant defence capacity of mammalian semen. Nanoparticles exhibit protective effects against cold shock and oxidative damage by inhibiting lipid peroxidation, as a result of powerful free radical scavenging activity (Bucak *et al.,* 2012; Ram *et al.,* 2025), and hence improve the post-thaw sperm quality. The addition of CeO_2NPs in extender under current study also maintained the sperm membrane integrity accompanied by decreasing MDA production and thus improved the post-thaw quality of bull spermatozoa. However, the ultimate goal, *in vivo* fertility evaluation of such treated semen needs to undertaken to validate the net results.

Table 1: Mean (± SE) post-thaw sperm quality parameters of Gir bull semen cryopreserved in AndroMed® extender with different concentration of CeO_2NPs (µg/mL)

Groups (CeO_2NPs levels)	Post-thaw stage				
	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
Control	52.06 ± 0.21^{a}	63.00 ± 0.35^{a}	21.00 ± 0.26^{a}	49.63 ± 0.33^{a}	62.25 ± 0.21^{a}
T_{25}	53.63 ± 0.26^{b}	65.19 ± 0.31^{b}	20.00 ± 0.22^{b}	51.44 ± 0.27^{b}	63.63 ± 0.20^{b}
T_{50}	56.31 ± 0.22^{c}	68.31 ± 0.25^{c}	18.81 ± 0.21^{c}	54.50 ± 0.18^{c}	66.13 ± 0.13^{c}
T_{75}	60.06 ± 0.29^{d}	72.81 ± 0.36^{d}	16.94 ± 0.14^{d}	58.44 ± 0.20^{d}	69.44 ± 0.13^{d}
p value	<0.001	<0.001	<0.001	<0.001	<0.001

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

Conclusion

Use of 75 µg/mL Cerium oxide nanoparticles (CeO_2NPs) as an additive in AndroMed® extender significantly ($p<0.05$) improves the post-thawed sperm motility, viability, HOS reactive sperm and acrosome integrity, reduces the percentage of sperm abnormalities, increases total antioxidant capacity and reduces MDA level followed by 50 µg/mL CeO_2NPs concentration compared to 25 µg/mL CeO_2NPs concentration and the control group. The findings suggested the cryoprotective and antioxidant effects of CeO_2NPs on bull spermatozoa by lowering free radicals and its associated oxidative stress. However *in vivo* fertility trials are warranted before advocating this level in route bovine semen cryopreservation to semen stations.

Acknowledgement

We are grateful to the authorities of the Kamdhenu University and Cattle Breeding Farm, KU, Junagadh, for the facilities and cooperation extended in this research work.

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