RESEARCH ARTICLE

Zinc Oxide Nanoparticles-Enriched Extender Enhances Cryosurvival and Antioxidant Defense of Buffalo Spermatozoa

Richakumari M. Patel[1]*, Kirankumar H. Parmar[1], Meet T. Chaudhari[1], Dinesh V. Chaudhari[1], Mohsin M. Pathan[2], Jignesh J. Parmar[3]

Abstract

The present study was conducted at the Central Sperm Station of the College of Veterinary Science in Anand, to evaluate the effect of zinc oxide nanoparticles (ZnONPs) on the post-thaw quality of buffalo bull semen. A total 18 ejaculates from four mature healthy breeding bulls were collected using an artificial vagina and evaluated for semen quality. Semen samples showing >70% initial motility were diluted in Tris fructose egg yolk glycerol (TFYG) extender, divided into four aliquots supplemented with 0 (control), 10, 25, and 50 µg/mL ZnONPs, equilibrated at 4°C, frozen in liquid nitrogen vapour, and stored at -196 °C overnight. Post-thaw semen was evaluated for sperm motility, viability, morphology, acrosome integrity, plasma membrane integrity (HOST), lipid peroxidation (MDA), and total antioxidant capacity (TAC). Statistical analysis revealed that a significant ($p<0.05$) improvement was observed in post-thaw sperm motility, viability, plasma membrane and acrosome integrity, and antioxidant activity in ZnONPs-supplemented groups compared to the control. The 25 µg/mL ZnONPs concentration showed the most significant enhancement ($p<0.05$) in sperm quality parameters, accompanied by a significant reduction ($p<0.05$) in lipid peroxidation levels and an increase in total antioxidant capacity, indicating optimal protection against oxidative damage, followed by 10 µg/mL, while higher concentration (50 µg/mL) showed a detrimental effect.

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

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Introduction

Semen quality plays a critical role in reproductive efficiency since a single bull can inseminate multiple females. Evaluation of semen through tests like the hypo-osmotic swelling (HOS) test and acrosomal integrity assessment provides a reliable estimate of fertility potential (Jarow *et al*., 2002). However, buffalo spermatozoa are highly susceptible to cryo-injury due to their plasma membrane's high polyunsaturated fatty acid (PUFA) content, making them vulnerable to oxidative stress (Hassan *et al*., 2022). Excessive reactive oxygen species (ROS) generation impairs sperm motility, viability, DNA integrity, and fertilizing capacity (Iqbal *et al*., 2022). Addition of antioxidants to semen extenders has been shown to improve post-thaw sperm quality across species by counteracting on oxidative damage (Eidan, 2016). These compounds mitigate lipid peroxidation, maintain sperm membrane integrity, and enhance motility and viability. Among trace minerals, zinc plays a crucial role as a cofactor in numerous metalloenzymes, contributing to spermatogenesis, sperm motility, and chromatin stability (Chia *et al*., 2000; Kumar *et al*., 2006). Zinc deficiency compromises the antioxidant defense system and increases lipid peroxidation, leading to poor semen quality (Agarwal *et al*., 2012).

Recent advances highlight zinc oxide nanoparticles (ZnONPs) as promising antioxidant supplements in semen preservation. Due to their nanoscale size and high surface area, ZnONPs exhibit superior bioavailability and antioxidant efficacy (Malik *et al*., 2023; Ram *et al*., 2025). They reduce ROS generation, stabilize sperm membranes, prevent ice crystal formation, and activate key antioxidant enzymes such as catalase and superoxide dismutase (Ashtari *et al*., 2021). Consequently, the inclusion of ZnONPs in semen extenders has been shown to enhance sperm cryoresistance, motility, and fertility potential, making

[1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat-388001, India.

[2]Department of Physiology and Biochemistry, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat-388001, India.

[3]Veterinary Clinical Complex, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat-388001, India.

Corresponding Author: Dr. Richakumari M. Patel, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat-388001, India. E-mail: richa13022001@gmail.com

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them a valuable tool for improving cryopreservation outcomes of semen of many farm animal species (Farhadi *et al.,* 2022; Hoyzen *et al*., 2023; Ram *et al.,* 2025). Hence, this study was planned to evaluate the effects of zinc oxide nanoparticles inclusion in extender during cryopreservation of buffalo bull semen on its post-thaw sperm quality parameters and antioxidant status.

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 (Gujarat, India) were selected for the present study. Selected animals were maintained under general management practices as followed for bulls on the Sperm Station. The bulls were under twice a week semen collection schedule in the early morning at 7:00 to 8:00 a.m. using the artificial vagina (Danish Model). Total, 18 ejaculates from four bulls obtained at weekly interval were used for this study.

Preparation and Formulation of Semen Extender

Tris fructose egg yolk glycerol (TFYG) extender was used as a base extender in this study. The semen additive, zinc oxide nanoparticles (ZnONPs) used in this study, was obtained from Sigma-Aldrich®. Initially, 50 mg/mL solution of zinc oxide nanoparticles was prepared. From that 2.5, 5.0 and 10 µL solution was added per mL of the TFYG extended semen in three jar to get final concentration of ZnONPs as 10, 25 and 50 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 diluted initially with TFYG extender @ 80 million sperm/mL, and divided into four equal aliquots. Aliquot-1 was then supplemented with 2.5 µL/mL ZnONPs (10 µg/mL ZnONPs group), aliquot-2 was added with 5.0 µL/mL ZnONPs (25 µg/mL ZnONPs group), aliquot-3 was supplemented with 10.0 µL/mL ZnONPs (50 µg/mL ZnONPs 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 Assays

Soon after post-thaw motility evaluation, a portion of thawed semen was centrifuged for 15 min at 2750 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.). The stored seminal plasma samples were thawed to room temperature before subjected to analysis for lipid peroxidation and total antioxidant capacity (TAC) using commercially available assay kits following the procedures recommended by the manufacturer.

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 results illustrating the effect of ZnONPs supplementation in semen extender at varying concentrations (0, 10, 25, and 50 µg/mL) during cryopreservation of buffalo semen on post-thaw sperm motility, viability, morphology, plasma membrane integrity, acrosomal integrity, and oxidative stress parameters are summarized in Tables 1-3.

Sperm Motility, Viability, and Morphology

In the T_{25} (25 µg/mL ZnONPs) group, the mean post-thaw sperm motility and viability were significantly ($p<0.05$) higher with reduced sperm abnormalities than the control and T_{10} groups. Additionally, sperm motility and viability were significantly ($p<0.05$) higher in the groups treated with 10 µg/mL and 50 µg/mL ZnONPs than the control group (Table 1). Sperm viability in 10 µg/mL and 50 µg/mL ZnONPs groups was statistically the same. Similar findings were observed by Hoyzen *et al*. (2023), who reported that 25 µg/mL of ZnONPs significantly ($p<0.05$) increased individual sperm motility of buffalo bull semen. Ram *et al*. (2025) recently noted significantly ($p<0.01$) improved post-thaw sperm motility and viability with reduced sperm abnormalities in Gir bull semen supplemented at 1 µg/mL ZnONPs in AndroMed® extender compared to 1.5 or 0.5 µg/mL levels and the control group, while Jahanbin *et al*. (2021) found 10^{-2} M ZnONPs to be optimal.

A significant ($p<0.05$) increase in sperm motility and viability of cattle and buffalo spermatozoa following the use of various nanoparticles as extender additives at different concentrations has been reported by several researchers, *i.e.,* Li *et al*. (2023) using 0.1 µg/mL cerium oxide NPs, and Al-Janabi *et al*. (2024), Solanki *et al.* (2025) and Chaudhari *et al.* (2026) using 75 µg/mL cerium oxide NPs (CeO_2NPs). A significant ($p<0.05$) decrease in sperm abnormalities with nanoparticle supplementation has also been reported by Khalil *et al*. (2023) using 25 and 37.5 µg/mL thymoquinone nanoparticles (TQNPs) and Abdelnour *et al*. (2020) using 1.5 µg/mL curcumin nanoparticles. Improved sperm motility and viability, along with reduced abnormalities, have further been observed in buck (Khalique *et al*., 2024), and canine semen (Aparnak and Saberivand, 2019) following nanoparticle supplementation.

Since mitochondrial ATP production is crucial for sperm motility, the enhanced motility observed in the present study may be attributed to the ability of ZnONPs to preserve mitochondrial integrity and optimize energy production in post-thawed spermatozoa.

Table 1: Mean (±SE) per cent post-thaw motility, viability and morphology of spermatozoa of buffalo bull semen cryopreserved in TFYG extender with different concentrations of ZnONPs (n=18)

ZnONPs Conc.	Sperm motility (%)	Sperm Viability (%)	Abnormality (%)
Control T_0	50.72^{a}±0.25	57.17^{a}±1.91	15.50^{bc}±0.45
T_{10}	61.08^{c}±0.43	66.11^{b}±1.46	14.50^{ab}±0.50
T_{25}	66.22^{d}±0.52	71.39^{c}±1.09	13.61^{a}±0.52
T_{50}	56.46^{b}±0.67	63.39^{b}±0.54	16.22^{c}±0.43

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

Acrosome and Plasma Membrane Integrity

The mean post-thaw percent sperm acrosomal integrity and HOST reactive sperm in extender supplemented with T_{10} and T_{25} ZnONPs were significantly ($p<0.01$) higher as compared to those of T_{50} ZnONPs and control groups (Table 2). These findings concurred well with those of Hoyzen *et al*. (2023) and Khalil *et al*. (2023) in buffalo bull semen using zinc oxide NPs and thymoquinone NPs, respectively, Li *et al*. (2023) in Holstein bull semen using GSH+ $SeNP_S$, Khalique *et al*. (2024) in Beetak buck semen using CeO_2NPs, wherein they recorded significant ($p<0.01$) increase in acrosome integrity and HOS reactivity with supplementation of different nanoparticles in the extender. Ram *et al.* (2025) observed significantly ($p<0.01$) higher post-thaw HOST reactivity and acrosomal integrity of Gir bull semen with 1 µg/mL ZnONPs in AndroMed® extender. Further, Shah *et al*. (2017) and Farhadi *et al*. (2022) also showed a positive effect of different nanoparticles on percent acrosome integrity in buffalo bull semen.

The findings of the present study indicate that ZnONPs act as enhancers of sperm motility and membrane stability, significantly improving both sperm motility and structural integrity following the freeze-thaw process.

Table 2: Mean (±SE) post-thaw sperm plasma membrane and acrosome integrity (%) of buffalo bull semen cryopreserved in TFYG extender with different concentrations of ZnONPs (n=18)

ZnONPs conc.	Plasma membrane integrity (%)	Acrosome integrity (%)
Control T_0	50.78^{a}±0.68	63.94^{a}±0.95
T_{10}	59.67^{b}±1.12	62.22^{a}±0.79
T_{25}	64.06^{c}±1.22	66.17^{b}±0.56
T_{50}	64.06^{c}±1.22	62.61^{a}±0.57

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

Lipid Peroxidation and Total Antioxidant Capacity

The mean malondialdehyde (MDA) concentrations in sperm-free post-thaw seminal plasma of T_{25} group with 25 µg/mL ZnONPs supplementation was significantly lower than in all other supplementations including control, which were statistically at par. Thus 25 µg/mL ZnONPs supplementation had a significant protective effect on lipid peroxidation (Table 3). Hozyen *et al.* (2023) reported a significantly lower concentration of MDA in buffalo bull semen extended with 25 µg/mL of ZnONPs. However, the researchers have noted beneficial effects of various other additives on lipid peroxidation of cattle and/or buffalo bull semen (Jahanbin *et al*., 2021; Khalil *et al*., 2023).

Table 3: Mean (±SE) post-thaw seminal plasma levels of malondialdehyde (MDA) and total antioxidant capacity (TAC) of buffalo bull semen cryopreserved in TFYG extender with different concentrations of ZnONPs (n=18)

ZnONPs Conc.	MDA (µmol/L)	TAC (µmol/L)
Control T_0	3.56^{b}±0.35	243.63^{a}±14.33
T_{10}	3.56^{b}±0.35	288.77^{ab}±11.67
T_{25}	2.21^{a}±0.33	343.45^{b}±21.57
T_{50}	3.37^{b}±0.35a	265.66^{a}±13.05

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

The overall mean TAC observed in the control group at post-thaw stage of buffalo bull semen was lowest (243.63±14.33 µmol/L) and at par with and T_{50} group (265.66±13.05 µmol/L), while it was highest in T_{25} group, yet statistical at par with T_{10} group. The mean post-thaw TAC in general was apparently or significantly ($p<0.05$) higher in all ZnONPs supplemented aliquots than in control, suggesting its protective role by ROS scavenging property (Table 3). Similar significant ($p<0.05$) increase in total antioxidant capacity (TAC) was observed by Shah *et al*. (2017) and Khalil *et al*. (2023) in buffalo bull semen and Aparnak and Saberivand (2019) in canine semen by adding different nanoparticles. Solanki *et al.* (2025) and

Chaudhari *et al.* (2026) observed significantly ($p<0.001$) lower MDA and higher TAC in bull semen cryopreserved in extender with 75 µg/mL CeO_2NPs concentration as compared to 25 and 50 µg/mL CeO_2NPs and control extender. 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. This protective action of nanoparticles may be attributed to their antioxidant and membrane-stabilizing properties, which preserve the acrosomal and plasma membranes during freezing.

Conclusions

The present experimental findings revealed that supplementation of the TFYG extender with 25 µg/mL zinc oxide nanoparticles (ZnONPs) significantly improved post-thaw semen quality by enhancing sperm motility, viability, plasma membrane and acrosome integrity, and reducing abnormalities. It also reduced MDA production by inhibiting lipid peroxidation, and increased TAC, indicating strong antioxidative protection. Concentrations of 10-25 µg/mL ZnONPs were found optimal for cryopreservation, however further *in vivo* studies are needed to validate their effectiveness in enhancing fertility outcomes.

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