

Zinc Oxide Nanoparticles (ZnONPs) Supplementation in Semen Extender Improves Post-Thaw Quality and Antioxidant Capacity of Gir Bull Spermatozoa

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ABSTRACT

A study was conducted on the semen of four Gir bulls over a six-month period to evaluate the impact of different concentrations of Zinc Oxide Nanoparticles incorporated into the Andromed extender on sperm quality and oxidative stress parameters of cryopreserved semen. A total of 16 semen ejaculates (4 ejaculates per bull) with initial motility more than 70% were used. Semen was extended in Andromed extender @ 80 million sperm per mL, divided into four equal aliquots, and then 1 mg/mL stock solution of ZnONPs was appropriately added to achieve final concentrations of 0.0, 0.5, 1.0, and 1.5 µg/mL. The diluted semen was filled into 0.5 mL French Medium straws, sealed, equilibrated at 4°C for 4 h, and frozen in liquid nitrogen vapour using a conventional wide-mouth freezer. Thawing was done next day in a water bath at 37°C for 30 sec, and various sperm quality parameters and oxidative markers were assessed. Results showed that post-thaw sperm motility, viability, HOST reactive sperm, and acrosomal integrity were significantly ($p < 0.05$) higher at the 1.0 µg/mL ZnONPs concentration ($61.31 \pm 0.35\%$, $74.00 \pm 0.39\%$, $64.68 \pm 0.29\%$, and $80.68 \pm 0.31\%$, respectively), while sperm abnormality ($14.19 \pm 0.19\%$) was significantly ($p < 0.05$) lower compared to the control, 1.5 µg/mL, and 0.5 µg/mL ZnONPs groups. Additionally, the mean malondialdehyde (MDA) level in post-thaw seminal plasma was lower (1.70 ± 0.10 µM/L, $p < 0.05$) and total antioxidant activity was higher (321.19 ± 9.15 µmol/L, $p < 0.05$) at the 1.0 µg/mL ZnONPs concentration compared to the control, 0.5 µg/mL, and 1.5 µg/mL ZnONPs groups. The study suggests that the addition of 1.0 µg/mL ZnONPs to the semen extender enhances the post-thaw sperm quality and antioxidant capacity of cryopreserved Gir bull semen.

Keywords: Cryopreservation, Gir bull semen, Oxidative marker, Sperm quality parameters, Zinc Oxide Nanoparticles (ZnONPs),

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INTRODUCTION

The production of high-quality frozen sperm is essential for artificial insemination (AI) in dairy bovine. However, the cryopreservation process itself is inherently damaging, often compromising sperm viability and function. Antioxidative protection system is mainly cytoplasmic in origin. Spermatozoa lose a large proportion of cytoplasm during the last phase of differentiation which make them highly sensitive to damages caused by reactive oxygen species (ROS) during the freezing-thawing process. Lower concentration of free radicals have regulatory role in specific sperm cell function especially capacitation and acrosome reaction (Seifi-Jamadi *et al.*, 2016). Balance between ROS and protective antioxidants have crucial role to prevent spermatozoa from damages which can occur before and during the cryopreservation processes (Mousavi *et al.*, 2019). The mitochondrial system is the main source of intracellular ROS with the respiratory chain acting in an auto-oxidation process (Moretti *et al.*, 2012). Phospholipids are essential for the structure, function and integrity of the plasma membrane and are vulnerable to attack by ROS, initiating a lipid peroxidation cascade, which seriously compromise the functional integrity of the membranes. 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

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fertility of bull semen (Kasimanickam *et al.*, 2007). The thiol group of the enzyme tyrosine phosphatase, which plays a crucial role in sperm capacitation, is sensitive to ROS attack. Other abnormalities that can result from oxidation events include DNA damage, a decreased capacity for sperm-oocyte fusion capacity (Ahmad *et al.*, 2015), poor fertilization rates and alterations during embryogenesis (Simoes *et al.*, 2013).

Cryopreservation reduces the fertilizing ability of spermatozoa by damaging the acrosomal membrane and mitochondria. However, the addition of Zinc Oxide Nanoparticles (ZnONPs) is the most common form of Zn used in animals because of its high Zn content and high absorption rates. Thus, due to its anti-oxidative properties, addition of ZnONPs to the semen extender can prevent adverse effects of ROS and can effectively reduce DNA damage and lipid peroxidation during semen freezing (Zhandi *et al.*, 2020). There are some studies which demonstrated the positive effects of Zinc Oxide Nanoparticles on reproductive cells and functions in rat (Afifi *et al.*, 2015), mouse (Talebi *et al.*, 2013), human (Barkhordari *et al.*, 2013) and bull (Farhadi *et al.*, 2022). Hence, the present study was planned to assess the effects of different concentrations of ZnONPs to the semen extender on sperm quality and oxidative parameters of Gir bull spermatozoa.

MATERIALS AND METHODS

The current study was undertaken at the Cattle Breeding Farm, Kamdhenu University, Junagadh, Gujarat, India during the period from February to July, 2024 on four Gir bulls kept under identical management and nutritional practices. All the bulls were in good health, under proper veterinary care and maintained in uniform sanitary conditions. Total 16 semen ejaculates were collected in artificial vagina from four Gir bulls under twice a week collection schedule and were evaluated. The sperm concentration and dilution rate were determined automatically by the photometer (Accucel, IMV, France). The semen was diluted with AndroMed extender (Minitube, Germany) at rate of 80 million sperm per mL and was evaluated for motility. Semen ejaculates with more than 70 % progressive motile spermatozoa on dilution were divided into four equal aliquots. Aliquot-1 was kept without additive and served as control. While the aliquots 2, 3 and 4 were added with Zinc Oxide Nanoparticles (ZnONPs; powder; Sigma-Aldrich; USA; 1 mg/mL stock solution) in such a way to get 0.5 µg/mL, 1.0 µg/mL and 1.5 µg/mL final concentrations in the extended semen. The diluted aliquots were filled and sealed in French Medium straws (0.5 mL capacity, TBS™, IMV, France) by automatic filling sealing machine (MRS1 Dual, IMV, France). Straws were then transferred to cold handling cabinet maintained at 4 °C and were equilibrated for 4 h prior to freezing. After equilibration, straws were vapour-frozen using conventional method in a wide mouth freezer and then submerged in liquid nitrogen at -196°C for storage. Straws were cryopreserved for 24 h and then thawed for 30 sec at 37°C in a water bath to assess post-thaw individual sperm motility, sperm viability, sperm abnormality, plasma membrane integrity (HOST) and acrosome integrity.

The contents of frozen-thawed semen straws of each aliquot were centrifuged for 15 min at 1425 x g to separate the seminal plasma for biochemical and enzymatic estimation, which was stored in sterile vials at -20 °C until analysed. The

stored 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., The data were expressed as Mean ± SE and analyzed by ANOVA test followed by the Duncan's post hoc test to find significant differences at $p < 0.05$ between levels of additive (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Effect on Sperm Motility, Viability and Morphology

In the present study, the mean post-thaw percent sperm motility and viability were found significantly ($p < 0.05$) higher in extender supplemented with 1.0 µg/mL as compared to 0.5 µg/mL and 1.5 µg/mL Zinc Oxide Nanoparticles (ZnONPs) and control extender (Table 1). Similar significantly ($p < 0.05$) improved sperm motility and viability of Holstein bull semen were observed by Farhadi *et al.* (2022) with 1.0 µg/mL ZnONPs as compared to control group. Jahanbin *et al.* (2021) also recorded gradual increase in progressive sperm motility and viability in bull semen with addition of Zn Nanoparticles @ 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} M in the extender as compared to control group. Li *et al.* (2023) and Chaudhary (2024) also reported that addition of Glutathione + Selenium Nanoparticles (GSH+SeNPs) and Selenium Nanoparticles (0.5 µg/mL SeNPs) groups significantly ($p < 0.001$ and $p < 0.05$) improved post-thaw mean sperm motility and viability as compared to control group in Gir and HF bull semen, respectively. Khalil *et al.* (2023) observed significantly ($p < 0.05$) improved post-thawed progressive motility in buffalo bull sperm, with values of 48.60 ± 1.43 % in 50 µg/mL Thymoquinone nanoparticles (TQNP) compared to 41.40 ± 0.92 % in the control group.

The percent sperm abnormality was significantly ($p < 0.05$) lower in extender supplemented with 1.0 µg/mL Zinc Oxide Nanoparticles as compared to all other groups (Table 1). Similarly, Chaudhary (2024) found that SeNPs treatment reduced the sperm abnormality (8.33 ± 0.58 %) in post-thawed Gir bull semen as compared to the control group (8.75 ± 0.80 %), however, the difference was non-significant. Contrary to the present study, Khalil *et al.* (2023) observed significantly lower sperm abnormalities with 25 and 37.5 µg/mL Thymoquinone Nanoparticles (TQNP) (7.9 ± 0.34 and 8.1 ± 0.46 %, respectively) as compared to control group (9.7 ± 0.57 %) in post-thawed semen of buffalo bull.

The importance of sperm morphology has been understood for a long time. Cryopreservation has been associated to morphological alterations in sperm, including damage to mitochondria, the acrosome and the sperm tail, when ROS exceeds the defence mechanisms of sperm (Khalil *et al.*, 2019). This damage can reduce post-thawed fertility of spermatozoa causing zygotes or embryos to often fail to reach full-term pregnancy (Khan *et al.*, 2021). The acrosome and associated dense fibers of the middle piece in sperm



are covered by mitochondria that generate energy from intracellular stores of ATP and these are responsible for motility of spermatozoa. The supplement might display cryo-

protective effect on the functional integrity of mitochondria by generating energy from ATP and may be responsible for improved post-thaw sperm motility (Reddy *et al.*, 2010).

Table 1: Mean (\pm SE) post-thaw sperm quality parameters of Gir bull semen cryopreserved in AndroMed extender with different concentration of ZnONPs.

Groups (ZnONPs, $\mu\text{g/mL}$)	Post-Thaw Stage				
	Individual sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Plasma Membrane Integrity (%)	Acrosome Integrity (%)
C	53.18 \pm 0.26 ^a	64.12 \pm 0.39 ^a	18.31 \pm 0.26 ^d	55.75 \pm 0.35 ^a	73.37 \pm 0.30 ^a
T _{0.5}	54.81 \pm 0.29 ^b	66.37 \pm 0.38 ^b	17.56 \pm 0.22 ^c	57.62 \pm 0.31 ^b	74.75 \pm 0.34 ^b
T _{1.0}	61.31 \pm 0.35 ^d	74.00 \pm 0.39 ^d	14.25 \pm 0.19 ^a	64.68 \pm 0.29 ^d	80.68 \pm 0.31 ^d
T _{1.5}	57.43 \pm 0.24 ^c	69.37 \pm 0.22 ^c	16.68 \pm 0.23 ^b	60.62 \pm 0.20 ^c	77.25 \pm 0.21 ^c
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.

T 0.5 = ZnONPs 0.5 $\mu\text{g/mL}$, T1.0 = ZnONPs 1.0 $\mu\text{g/mL}$, T1.5 = ZnONPs 1.5 $\mu\text{g/mL}$ extender.

Findings of the present study suggested that ZnONPs substantially improved post-thaw progressive sperm motility and viability in Gir bull semen at the concentration of 1.0 $\mu\text{g/mL}$. In other studies, ZnONPs offered improved progressive motility at lower or higher concentrations compared to the present one, which may be due to species and breed difference, semen collection technique, extender composition, additives used, preservation protocol, source and concentration of ZnONPs used, thawing time and time taken by the observer etc.

Sperm Plasma Membrane and Acrosome Integrity

A biochemically active sperm membrane is required for sperm capacitation, acrosome reaction and spermatozoa binding to the egg surface, all of which entail changes in membrane characteristics. Acrosome is considered as limiting structure of fertilization. Intact acrosome is required for bringing out sperm-oocyte fusion and it is highly sensitive to freezing-thawing associated cryo-damage. Spermatozoa with defective acrosome fail to bring out acrosomal exocytosis 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 (Bucak *et al.*, 2012) affecting their structural integrity and fertility potential. In the present study, the mean post-thaw sperm plasma membrane integrity (HOS reacted spermatozoa) and acrosome integrity were significantly ($p < 0.05$) higher in all ZnONPs supplemented groups as compared to control. Furthermore, the values of both the parameters were also significantly ($p < 0.05$) higher in 1.0 $\mu\text{g/mL}$ ZnONPs treated group as compared to other groups (Table 1).

Similar findings of higher sperm plasma membrane and acrosome integrity were also observed in Holstein bull semen by Farhadi *et al.* (2022) with 0.1, 1.0 and 10 $\mu\text{g/mL}$ ZnONPs supplementation as compared to control group, although the results were better with lower concentration of ZnONPs. Chaudhary (2024) found significantly higher

($p < 0.05$) acrosome intact spermatozoa with concentration of 0.5 $\mu\text{g/mL}$ SeNPs in Gir bulls' semen as compared to control group, while Jahanbin *et al.* (2021) reported significantly increased ($p < 0.05$) percentage of post-thawed HOST reacted and acrosome intact spermatozoa with addition of 10^{-6} and 10^{-2} M of Zn Nanoparticles in bull semen as compared to the control group or 10^{-5} , 10^{-4} , and 10^{-3} M supplements. Various scientists (Khalil *et al.*, 2023; Li *et al.*, 2023; Khalique *et al.*, 2024) have also reported higher sperm plasma membrane and acrosome integrity by addition of different concentrations of various nanoparticles (TQONPs, a combination of GSH and SeNPs, CeO₂NPs) in different animals (buffalo bull, Holstein bull, Beetal buck, respectively). Variable effect of the ZnONPs observed in literatures might be due to species variation, freezing extender composition, preservation protocol and concentration of ZnONPs used.

Oxidative Stress Markers (LPO, TAC)

The mean lipid peroxidation (MDA, $\mu\text{M/L}$) was found to be highest in control group as compared to ZnONPs treated groups, and the lowest in 1.0 $\mu\text{g/mL}$ ZnONPs group. The mean total antioxidant capacity ($\mu\text{M/L}$) was significantly ($p < 0.05$) higher in 1.0 $\mu\text{g/mL}$ ZnONPs group as compared to control and other treated groups (Table 2). MDA production was also significantly lower with higher total antioxidant capacity in post-thawed semen with ZnONPs levels 0.5 and 1.5 $\mu\text{g/mL}$ as compared to control group.

In comparison to the present study, Farhadi *et al.* (2022) found that treating post-thawed Holstein bull semen with 1.0 $\mu\text{g/mL}$ ZnONPs significantly reduced MDA levels to 0.6 ± 0.1 nmol/mL, compared to 1.1 ± 0.1 nmol/mL in the control group. Jahanbin *et al.* (2021) reported a non-significantly decreased MDA level with all levels of Zn Nanoparticles (10^{-6} and 10^{-2} M) supplement in cryopreserved bulls' semen as compared to control group. Hozyen *et al.* (2023) also reported that supplementation of semen extender with 25 $\mu\text{g/mL}$ ZnONPs on bull semen significantly ($p < 0.05$) decreased the concentration of MDA compared to the control group. Various

scientists (Khalil *et al.*, 2023; Chaudhary, 2024; Khaliq *et al.*, 2024) have also reported lower MDA and higher TAC levels by addition of different concentrations of various nanoparticles (TQONPs, SeNPs, CeO₂NPs) in different animals (buffalo bull, Gir bull, Beetal buck, respectively).

Table 2: Mean (\pm SE) post-thaw lipid peroxidation and total antioxidant capacity in the seminal plasma of cryopreserved Gir bull semen in AndroMed extender with different concentrations of ZnONPs.

Groups (ZnONPs, $\mu\text{g/mL}$)	Post-Thaw Stage	
	Lipid Peroxidation ($\mu\text{M/L}$)	Total Antioxidant Capacity ($\mu\text{M/L}$)
C	3.36 \pm 0.21 ^c	238.58 \pm 9.94 ^a
T _{0.5}	3.26 \pm 0.21 ^{bc}	263.49 \pm 3.81 ^{ab}
T ₁	1.70 \pm 0.10 ^a	321.19 \pm 9.15 ^c
T _{1.5}	2.56 \pm 0.21 ^b	288.79 \pm 5.89 ^b
p value	<0.001	<0.001

Means with different superscripts within column differ significantly at $p < 0.01$ level.

The susceptibility of mammalian spermatozoa to oxidative stress is because of their higher concentrations of unsaturated fatty acids and of limited repair mechanisms. Semen processing and cryopreservation decrease the antioxidant defence capacity of semen. Moreover, seminal antioxidant content is inadequate for the prevention of lipid peroxidation during freezing and thawing process (Storey, 1997). During cryopreservation, the semen is exposed to cold shock at atmospheric oxygen which in turn increases the susceptibility to lipid peroxidation because of higher production of reactive oxygen species (Perumal *et al.*, 2009). ZnONPs exhibits 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). The addition of ZnONPs maintains the sperm membrane integrity accompanied by decreasing MDA. As such, it may prevent peroxidative change to the sperm membrane structure, thus may maintain motility and decrease structural and functional alteration to the gamete. At post-thaw stage, we found lower lipid peroxidation level with higher TAC at 1 and 1.5 $\mu\text{g/mL}$ ZnONPs concentration than control group. This highlights that addition of ZnONPs sustained the seminal total antioxidant capacity during cryopreservation.

CONCLUSION

The present findings revealed that ZnONPs added as a semen additive in the semen extender, at a concentration of 1.0 $\mu\text{g/mL}$ ZnONPs, significantly ($p < 0.05$) improves post-thaw sperm motility, sperm viability, HOST reactive sperm and acrosomal integrity with lower sperm abnormality as compared to control and other treated groups. It significantly ($p < 0.05$) reduced MDA level and increased total antioxidant activity in post-thawed semen of Gir bulls in comparison to control, 0.5 $\mu\text{g/mL}$ and 1.5 $\mu\text{g/mL}$ ZnONPs concentrations, suggesting that ZnONPs controlled oxidative stress.

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