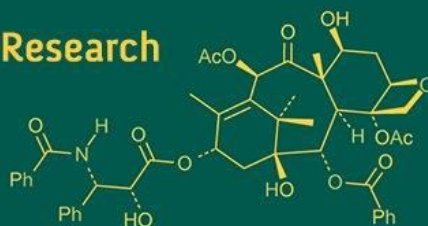


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In vitro study on influence of zinc oxide nanoparticles on seminal attributes of chilled canine semen

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Abstract

The aim of the present study was to evaluate the efficiency of zinc oxide nanoparticles in preserving canine semen in chilled temperature and to evaluate the optimal concentrations of zinc oxide nanoparticles supplemented to an extender for refrigerated canine semen. Canine semen collected manually was evaluated and then diluted in Tris-egg yolk-citrate-fructose (TEYF) extender at 1:4 dilution rate, with different concentrations of zinc oxide nanoparticles (50 mcg/ml, 75 mcg/ml and 100 mcg/ml of extender) and a control (0mcg/ml). Diluted samples were refrigerated at 5 °C and stored for seven days. Extended chilled canine semen was evaluated for progressive motility, viability, morphological abnormality, plasma membrane integrity and DNA integrity on 0, 1, 3, 5 and 7 days. Supplementing zinc oxide nanoparticles to extender showed significantly improved progressive motility at 100mcg/ml zinc oxide nanoparticles across a study period of 7 days, compared to the control, 50 and 75 mcg/ml. Viability, Plasma membrane integrity and DNA integrity were significantly enhanced at 75 and 100mcg/ml zinc oxide nanoparticles. Morphological abnormalities were significantly reduced when the extender was supplemented with 75 and 100 mcg/ml zinc oxide nanoparticles. In conclusion, canine semen extender supplemented with zinc oxide nanoparticles improve seminal attributes preserved at refrigeration temperatures.

Keywords: Canine semen, refrigeration temperature, Tris-egg yolk extender, zinc oxide nanoparticles

Introduction

Assisted reproductive technologies (ARTs) in dogs are increasingly used to enhance reproductive efficiency, preserve genetic diversity and enable safe international exchange of genetic material (Suzuki *et al.*, 2022) ^[1]. Among these technologies, artificial insemination (AI) using chilled or cryopreserved semen is especially valuable (Watson, 1990) ^[2], yet its success is often compromised by oxidative stress during freezing and thawing (Lucia *et al.*, 2016) ^[3]. Canine spermatozoa are highly susceptible to reactive oxygen species (ROS) due to their lipid-rich membranes and limited intrinsic antioxidant defences, leading to membrane disruption, reduced motility and impaired fertilizing ability (Henkel and Schill, 1998; Sanocka-Maciejewska *et al.*, 2005; Schuppe *et al.*, 2008) ^[4, 5, 6]. Although conventional antioxidants have been incorporated into extenders to mitigate this damage, their stability and efficacy can decline under freezing conditions (Khalil *et al.*, 2019) ^[7].

Recent advances in nanotechnology offer a promising alternative. Nanoparticles particularly zinc oxide nanoparticles (ZnO-NPs) exhibit strong and stable antioxidant properties due to their high surface area, enhanced reactivity and improved cellular interaction (Nel *et al.*, 2006) ^[8]. These nanoscale materials can scavenge ROS more efficiently than traditional antioxidants, protect against lipid peroxidation and support the activity of endogenous antioxidant enzymes (Khalil *et al.*, 2019) ^[7]. Their improved stability, bioavailability and lower effective concentrations make them ideal for inclusion in semen preservation media (Dawei *et al.*, 2010) ^[9]. Studies across several species, including humans (Barkhordari *et al.*, 2013; Isaac *et al.*, 2017) ^[10, 11], bulls (Jahanbin *et al.*, 2021) ^[12], rams (Heidari *et al.*, 2019) ^[13], camels (Shahin *et al.*, 2020) ^[14] and goats (Abedin *et al.*, 2023) ^[15] have reported that ZnO-NP supplementation enhances postthaw sperm motility, viability and structural integrity. These findings highlight the potential of nanoparticles to significantly improve canine semen freezing outcomes and support advanced reproductive applications.

The study included six clinically healthy male dogs (2-5 years) presented for routine semen evaluation. Only semen samples with $\geq 2 \times 10^6$ spermatozoa/mL concentration and $\geq 80\%$ motility were used. A Tris-based extender was prepared using Tris, citric acid, fructose, egg yolk, and antibiotics, with final volume adjusted to 100 mL. Semen was collected by digital manipulation following sexual stimulation, and the sperm-rich fraction was divided into four groups. Each group was diluted (1:4) with extender containing different concentrations of ZnO nanoparticles (0, 50, 75, and 100 $\mu\text{g/mL}$) and stored at 4-5 °C for 7 days.

Materials and Methods

The present research work was carried out in the small animal outpatient department, Department of Gynecology and Obstetrics, Veterinary College, Hebbal, Bengaluru. All experimental procedures were accepted by the Institutional Animal Ethics Committee VCH/IAEC/2025/38. Zinc oxide nanoparticles were procured from Sigma Aldrich-Merck, Bengaluru, India. Nanoparticle size is <100 nm with an average particle size of <40 nm. All other chemicals used in this study were obtained from HIMEDIA.

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Post-dilution semen evaluation was conducted to assess the effects of ZnO-NPs at different concentrations. Parameters including progressive motility, viability, plasma membrane integrity, morphological abnormalities and DNA integrity were assessed on days 0, 1, 3, 5 and 7. Standard methods were used: Progressive motility by microscopic evaluation (Puja *et al.*, 2018) ^[16], concentration by Neubauer haemocytometer (Johnston *et al.*, 2001) ^[17], viability by eosin-nigrosin staining (Agarwal *et al.*, 2003) ^[18], morphology by Rose Bengal staining (Sousa *et al.*, 2013)

^[19], membrane integrity by HOST (Ramu and Jeyendran, 2013) ^[20] and DNA integrity by acridine orange staining under fluorescence microscopy (Shamsi *et al.*, 2011) ^[21] shown in Fig 1.

Descriptive statistics were calculated as mean $\pm$ standard error for quantitative data and percentages for qualitative data. Differences among treatment groups over time were analyzed using repeated measures ANOVA followed by DUNCAN post hoc test, with analyses performed using online statistical tools.

Results

The sperm-rich fraction of canine semen in all dogs appeared milky to creamy white. Overall, semen quality parameters indicated good seminal characteristics, with high sperm concentration and motility across samples. The mean volume, concentration and motility values are presented in Table 1.

Table 1: Evaluation of fresh canine semen (Mean $\pm$ S.E.)

S. No.	Parameters	Observation
1	Colour	Milky white to creamy white
2	Volume (mL)	2.57 $\pm$ 0.17
3	Concentration ($\times 10^6/\text{mL}$)	471.67 $\pm$ 38.77
4	Motility (%)	89.83 $\pm$ 1.40

Progressive Motility

Progressive motility declined significantly during storage in all groups ($p \leq 0.05$), but higher ZnO-NP concentrations (75 and 100 $\mu\text{g/mL}$) maintained significantly better motility, with the 100 $\mu\text{g/mL}$ group showing the highest values, indicating a dose-dependent protective effect results were shown in Table 2.

Viability, Plasma membrane integrity and DNA integrity

Sperm viability, Plasma membrane integrity and DNA integrity declined significantly during the storage period ($p \leq 0.05$). However, semen treated with higher concentrations of ZnO-NPs (75 and 100 $\mu\text{g/mL}$) consistently maintained significantly better viability, plasma membrane integrity and DNA stability compared to control across all days, indicating a protective effect during chilled storage.

Table 2: Progressive motility (%) with ZnO-NPs at 4-5 °C (Mean $\pm$ S.E.)

ZnO-NPs	Day 0	Day 1	Day 3	Day 5	Day 7
Control	82.17 $\pm$ 1.11 ^{ap}	70.33 $\pm$ 2.14 ^{bp}	53.17 $\pm$ 3.30 ^{cp}	20.83 $\pm$ 2.87 ^{dp}	6.50 $\pm$ 0.85 ^{ep}
50 $\mu\text{g/mL}$	82.33 $\pm$ 1.26 ^{ap}	70.67 $\pm$ 2.23 ^{bp}	55.67 $\pm$ 3.35 ^{cp}	21.67 $\pm$ 2.75 ^{dp}	7.33 $\pm$ 0.71 ^{epq}
75 $\mu\text{g/mL}$	85.50 $\pm$ 0.72 ^{aq}	75.67 $\pm$ 1.74 ^{bq}	58.83 $\pm$ 3.48 ^{cp}	25.83 $\pm$ 2.60 ^{dq}	9.17 $\pm$ 0.48 ^{cq}
100 $\mu\text{g/mL}$	87.50 $\pm$ 0.85 ^{aq}	79.17 $\pm$ 1.74 ^{bq}	63.33 $\pm$ 3.22 ^{cq}	28.33 $\pm$ 2.39 ^{dq}	11.33 $\pm$ 0.67 ^c

Row superscripts (a-e): differences between days ($p \leq 0.05$). Column superscripts (p-r): Differences between groups ($p \leq 0.05$).

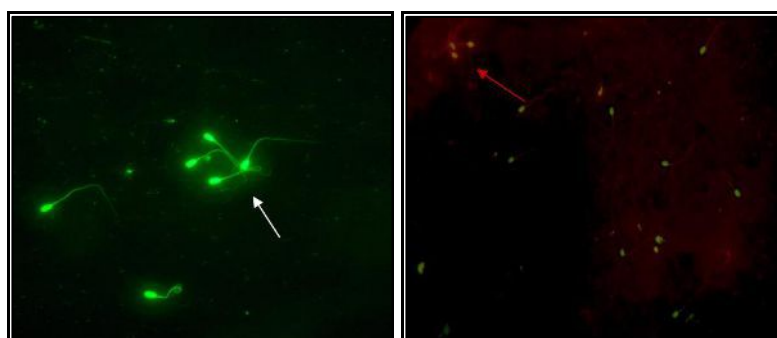


Fig 1: Sperm DNA integrity evaluation by Acridine Orange stain under fluorescent microscope (white arrow-Spermatozoa with intact DNA, Red arrow-Spermatozoa with denatured DNA) (400X magnification)

Discussion and Conclusion

ZnO-NPs at higher concentrations (75 and 100 µg/mL) effectively improved the preservation of chilled canine semen by reducing storage-induced declines in motility, viability, morphology, membrane integrity, and DNA integrity over seven days at 4-5 °C.

Fresh Semen Characteristics: Semen was milky to creamy white, reflecting high sperm concentration. The sperm-rich fraction volume, concentration and motility were within or above established norms, with variations attributable to breed, age, collection methods and evaluator factors.

Progressive Motility: ZnO-NPs at 75 and 100 µg/mL significantly improved motility, particularly at higher doses, likely via enhanced mitochondrial ATP production, membrane function and signaling pathways supporting sperm hyperactivation. These trends echo Fayez *et al.* (2023)^[22] in canine semen (100-200 µg/mL), Lyngdoh *et al.* (2024)^[23] in boars (10 µM), and studies in rams, camels, and goats (Omidi *et al.*, 2024; Shahin *et al.*, 2021; Abedin *et al.*, 2023)^[24, 25, 15]. Zinc from ZnO-NPs boosts mitochondrial ATP, tail membrane function, and GPR39/CatSper signaling for hyperactivation via cAMP/PKA pathways (Allouche-Fitoussi and Breitbart, 2020; Michailov *et al.*, 2014)^[26, 27].

Viability: Sperm viability declined over storage, with losses exceeding those of motility. ZnO-NPs at 75 and 100 µg/mL significantly preserved viability throughout, whereas 50 µg/mL showed no appreciable effect compared to controls. Results corroborate Fayez *et al.* (2023)^[22], Lyngdoh *et al.* (2024)^[23] and multi-species data (Omidi *et al.*, 2024; Heidari *et al.*, 2019; Abedin *et al.*, 2023)^[24, 13, 15]. Zinc supports ATP production, mitochondrial function, and ROS scavenging, stabilizing membranes (Afifi *et al.*, 2015; Maleki *et al.*, 2025)^[28, 29].

Sperm Morphology: ZnO-NPs at 75-100 µg/mL consistently reduced defects, while 50 µg/mL had a moderate effect on early days. Supporting data from rams, goats, and camels show similar reductions (Omidi *et al.*, 2024; Abedin *et al.*, 2023; Shahin *et al.*, 2021)^[24, 15, 25]. Zinc elevates antioxidant enzymes (superoxide dismutase, catalase), competes with pro-oxidants and stabilizes acrosomal membranes (Khalique *et al.*, 2023; Cruz *et al.*, 2015; Hu *et al.*, 2025)^[30, 31, 32].

Plasma membrane integrity: Membrane integrity declined significantly ($p \leq 0.05$) over storage. While 50 µg/mL offered no advantage ($p > 0.05$), 75-100 µg/mL significantly outperformed controls daily ($p \leq 0.05$), aligning with Fayez *et al.* (2023)^[22], Lyngdoh *et al.* (2024)^[23] and others (Omidi *et al.*, 2024; Farhadi *et al.*, 2022; Abedin *et al.*, 2023)^[24, 33, 15].

DNA integrity: DNA integrity dropped non-significantly day 1 ($p > 0.05$), then significantly later ($p \leq 0.05$), except minimally in 50 µg/mL. Higher doses (75-100 µg/mL) significantly protected versus controls across days. Findings match Fayez *et al.* (2023)^[22], Omidi *et al.* (2024)^[24], Abedin *et al.* (2023)^[15] and human and bull studies (Isaac *et al.*, 2017; Farhadi *et al.*, 2022)^[13, 33]. Zinc forms protective S-S bonds, protamine interactions and nanoparticle coatings against peroxidation (Balhorn, 2007; Isaac *et al.*, 2017)^[34, 13].

ZnO-NPs effectively preserved sperm progressive motility, viability, normal morphology, plasma membrane integrity for up to five days and DNA integrity for seven days. Among the tested concentrations, 100 µg/mL consistently improved progressive motility, while both 75 µg/mL and 100 µg/mL were identified as optimal for maintaining overall sperm quality during storage.

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