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RESEARCH ARTICLE

# Nano-Selenium Supplementation of Semen Extender Improves Post-Thaw Semen Quality of Indonesian Local Madura Bulls: A Preliminary Study

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**ABSTRACT**

Oxidative stress induced by cryopreservation reduces sperm quality, hindering preservation of the superior germ plasm. This study aimed to evaluate the effects of selenium nanoparticles (SeNPs) and sodium selenite ($Na_2SeO_3$) supplementation into semen extender on post-thaw semen quality of Madura bulls. Eight ejaculates were obtained from four bulls over a two weeks period. In the first week, semen was collected twice from two bulls. In the second week, two ejaculates were collected from the remaining two bulls. Following preliminary screening, collected ejaculates were cryopreserved using skim milk-egg yolk-glycerol extender supplemented with SeNPs and $Na_2SeO_3$ at concentrations of 0 (control), 5, 10 and 25ppm and stored in liquid nitrogen ($LN_2$) for 90 days. Post-thaw semen quality was evaluated using CASA for kinematic motility parameters, as well as microscopic assessment of sperm viability, morphological abnormalities, plasma membrane integrity (PMI), acrosomal membrane integrity (AMI), and oxidative parameters, such as reactive oxygen species (ROS), malondialdehyde (MDA) and catalase activity (CAT). Both types of selenium and their concentrations improved sperm motility, viability, PMI, AMI, and oxidative markers ($P \leq 0.05$), but did not affect sperm abnormalities compared to the control. Supplementation of SeNPs (5ppm) provided the best sperm quality in terms of motility, viability, PMI and AMI. The higher $Na_2SeO_3$ dose (25ppm) was associated with increased levels of oxidative markers. However, this condition was not observed in the SeNPs group. In general, all SeNPs doses showed good efficacy by reducing ROS and MDA levels, with increasing CAT activity, indicating superior redox modulation compared to $Na_2SeO_3$. In conclusion, SeNPs at 5ppm represents an optimal, non–pro-oxidant dose for improving post-thaw quality of Madura bull semen, and offers a promising strategy for Indonesian local cattle cryopreservation programs.

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## INTRODUCTION

Cryopreservation of semen is a cornerstone of the successful artificial insemination (AI) programs of livestock and their ex-situ conservation. However, the freeze–thaw process inevitably induces structural and functional damage to the spermatozoa (Khalil *et al.*, 2019; Li *et al.*, 2023). Excessive generation of reactive oxygen species (ROS) during cryopreservation damages plasma and acrosomal membranes, impairs mitochondrial function, accelerates lipid peroxidation and DNA fragmentation, and initiates oxidative stress (OS) in spermatozoa. This leads to reduced sperm motility, viability, and fertilizing capacity due to cryo-induced injury across species (Majzoub and Agarwal, 2018).

Madura is a native cattle breed endemic to Madura Island, which is popular for bull race, and is highly valued

in local cultural and production systems (Azizah *et al.*, 2023). Recent studies have shown that the sperm quality of Madura cattle is generally good in terms of general reproductive capacity, but still not meeting the optimal criteria for frozen sperm production, and requires a cryopreservation protocol to maximize reproductive performance of males of this breed (Rimayanti *et al.*, 2022). In addition, local farmers often prefer to utilize imported European bull semen which is considered to provide high valued offsprings. Thus, the increased threat to the genetic integrity of Madura cattle necessitates the development of an effective semen cryopreservation program to support targeted conservation and breeding programs for Madura cattle (Purwantara *et al.*, 2021; Rimayanti *et al.*, 2022).

Selenium is an essential micro-mineral that acts as an effective antioxidant in the defense mechanism of animal body. It becomes integrated into selenoproteins, such as glutathione peroxidase (GPx) and thioredoxin reductases, which play a key role in maintaining sperm structure, mitochondrial function, and motility (Sitaresmi *et al.*, 2024). Previous studies have shown that addition of sodium selenite ($Na_2SeO_3$) to semen extenders can improve post-thaw sperm motility, viability, membrane integrity, and maximize total antioxidant capacity in post-thaw semen of bulls (Dolník and Mudroňová, 2021) and male goats (Erlindra *et al.*, 2026). However, the window of use of selenium tends to be narrow, where slightly higher doses can induce pro-oxidant and cytotoxic effects that can adversely affect the sperm quality (Dutta *et al.*, 2019; Dolník and Mudroňová, 2021).

Selenium nanoparticles (SeNPs) have a larger surface area, unique redox properties, and lower toxicity than inorganic or conventional forms of selenium (Sitaresmi *et al.*, 2024). Several studies in cattle bulls (Khalil *et al.*, 2019), buffalo bulls (Khalil *et al.*, 2024), rams (Nateq *et al.*, 2020), and poultry (Chen *et al.*, 2025) have shown improved post-thaw sperm motility, plasma membrane integrity (PMI), acrosome membrane integrity (AMI), and antioxidant enzyme activity at low SeNPs concentrations. At the same time, nanoparticles can have dual effects on sperm and seminal fluid, being protective at low doses but potentially disruptive to redox signaling and cell physiology at higher levels. While progress has been made through the inclusion of selenium as an antioxidant in semen extenders, significant knowledge gaps are not removed. Specifically, the application of SeNPs in Indonesian local cattle has not been explored.

These gaps are characterized by three primary limitations: First, no study has systematically compared SeNPs and $Na_2SeO_3$ at various doses in local cattle models, particularly in Madura cattle, which are prioritized for genetic conservation (Kameni *et al*., 2024). Second, previous studies rarely integrated detailed computer-assisted semen analysis (CASA) kinematics with cellular functional characteristics and a panel of redox markers including ROS, malondialdehyde (MDA), and catalase (CAT) after long-term storage, thus limiting the understanding of how antioxidant supplementation alters the redox-functional landscape of frozen-thawed sperm (Khalil *et al.*, 2019; Li *et al.*, 2023). Third, although the concept of the antioxidant paradox, where excessive antioxidant supplementation shifts to pro-oxidant behavior, is increasingly recognized in human andrology, it has not been extensively explored in the context of $Na_2SeO_3$- and SeNPs-based sperm extenders in animals (Dutta *et al*, 2019; Zhang *et al.*, 2023).

This study aimed to investigate dose-dependent effects of SeNPs and $Na_2SeO_3$ supplemented to skim milk-egg yolk-glycerol extender on CASA kinematic parameters, cellular quality traits (viability, abnormalities, PMI, AMI), and oxidative status (ROS, MDA, CAT) of Madura bull spermatozoa after 90 days of cryo-storage. It was hypothesized that SeNPs at an optimal dose would better preserve post-thaw sperm function and redox balance than conventional $Na_2SeO_3$, whereas higher doses of both forms of selenium would exhibit diminished or adverse effects consistent with an antioxidant paradox.

## MATERIALS AND METHODS

**Experimental animals:** Four fertile and proven Madura bulls were used in this study during the period from November 2025 to February 2026. The bulls were aged 3.25±0.50 years with an average body weight of 574.25±44.46kg and body condition score (BCS) of 5.25±0.50 on the scale of 1–9 (Kojima *et al.*, 2022). All bulls were maintained under standard management protocols at the Artificial Insemination Center (Balai Inseminasi Buatan-BIB), Lembang, West Java, Indonesia. These management protocols included standardized feeding regimes, reproductive health assessments, vaccinations, and medical treatments. The daily ration for each bull consisted of hay (Star grass), fresh forage (*Pennisetum purpureum*), and concentrates. The feeding schedule per bull was adopted as follows: 0.2kg of hay at 06:00; 15–20kg of fresh forage and 2–7kg of concentrate at 07:30; 10kg of fresh forage and 1kg of concentrate at 12:00; and a final offering of 15–20kg of fresh forage at 16:00. Feed quantities were adjusted based on the individual requirements of each animal, with water was provided *ad libitum.*

**Characterization of SeNPs and antioxidant activity:** Two antioxidant, $Na_2SeO_3$ (CAS No. 10102-18-8, Merck, Germany) and SeNPs were used in the present study. $Na_2SeO_3$ was diluted using sterile distilled water (Erlindra *et al.*, 2026), while SeNPs were synthesized from $Na_2SeO_3$ using chitosan and sodium tripolyphosphate (STPP) via ionic gelation, a well-established green synthesis approach (Araujo *et al.*, 2021), with particle sizes ranged from 100 to 300nm, with the average diameter was 156.73±10.04nm (Table 1), determined by using Particle Size Analyzer (PSA: Malvern, UK). The antioxidant activity of both selenium forms was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Furthermore, the half-maximal inhibitory concentration ($IC_{50}$) values were calculated to assess antioxidant potency, with activity categorized according to the criteria established by Jumina *et al.* (2019). Antioxidant activity results demonstrated that SeNPS exhibited an $IC_{50}$ of 35.70ppm (very strong), whereas $Na_2SeO_3$ showed an $IC_{50}$ of 138.36ppm (moderate).

**Table 1:** Particle size distribution of SeNPs (Mean±SEM).

| Parameters | SeNPs (n=3) | |
|---|---|---|
| | PSA results | Particle distributions (%) |
| Z-Average (d.nm) | 156.73±10.04 | - |
| PdI | 0.187±0.08 | - |
| Peak (d.nm) | | |
| Peak 1 | 79.30±18.80 | 41.33±29.49 |
| Peak 2 | 62.30±13.49 | 58.66±29.49 |

Z-Average: Zeta average, the mean hydrodynamic diameter of the nanoparticles measured using the dynamic light scattering (DLS) method; PdI: Polydispersity index, representing the degree of uniformity in particle size distribution; Peak 1: The primary population distribution of nanoparticles; Peak 2: A secondary nanoparticle population (indicating a bimodal distribution); and n is the number of replicates of the SeNPs distribution measurement.

**Extender preparation:** A skim milk-egg yolk-glycerol extender was prepared in two fractions (Part A and Part B) according to the standard operating procedure of BIB Lembang (Table 2). The buffer solution was prepared by dissolving skim milk powder in aqua bidestillata, heated to 90°C for 12min, filtered, cooled, and stored at 4°C. Antibiotics (penicillin and streptomycin) were dissolved in aqua bidestillata and mixed with the skim milk buffer at a ratio of 1:100. Part A was used for the first dilution at room temperature, while Part B containing glycerol was used for the second dilution during equilibration at 4–5°C.

**Table 2:** The composition of skim milk-egg yolk-glycerol extender used by BIB Lembang

| Compositions | Extender Solutions | |
|---|---|---|
| | Part A (1,000mL) | Part B (1,000mL) |
| Skim milk buffer solution (mL)* | 940.59 | 762.38 |
| Antibiotic buffer solution (mL)** | 9.41 | 7.62 |
| Egg yolk (mL) | 50.00 | 50.00 |
| Glycerol (mL) | - | 160.00 |
| Glucose (g) | - | 20.00 |

*: Skim milk buffer solution consists of 100.00g skim milk powder and 960.00mL aqua bidestillata; **: Antibiotic buffer solution consists of $3.00\times10^6$ IU penicillin, 3.00g streptomycin, and 30.00mL aqua bidestillata. The mixing ratio of antibiotic buffer solution to skimmed milk buffer solution was 1:100 (v/v).

**Semen collection and cryopreservation:** Semen samples were collected from four bulls over a two weeks period using an artificial vagina. In the first week, semen was collected twice from two bulls. In the second week, two ejaculates were collected from each of the remaining two bulls, resulting in a total of eight ejaculates from four bulls. All ejaculates were evaluated and processed individually, with no pooling across bulls. Fresh semen quality was assessed immediately after collection by macroscopic and microscopic examination in terms of ejaculator volume, color, pH, odor, consistency, sperm concentration, motility, viability, morphological abnormalities, PMI and AMI (Supplementary Table 1). Further processing was performed only on ejaculates that exhibited acceptable physical characteristics (abnormalities ≤20%) and sperm motility of ≥70%.

The semen was diluted at 5°C and the dilution rate was calculated to achieve a final sperm concentration of $25\times10^6$ sperm per 0.25mL straw. The diluted semen was chilled at 5°C for 4h. The straws (0.25mL) of seven assorted dosages corresponding to each group, (control/without antioxidant addition), $Na_2SeO_3$ (5, 10, and 25ppm), and SeNPs (5, 10, and 25ppm) were prepared. The antioxidant was mixed with the extender at a 1:150 ratio. After filling and sealing straws, pre-freezing was carried out by reducing the temperature from 5 to -140°C over a period of 9min, followed by the freezing step using a container (Taylor Warten, USA). Subsequently, the semen straws were immersed in liquid nitrogen ($LN_2$) for 24h using programmable freezer and stored in $LN_2$ at -196°C for at least 90 days, simulating long-term cryopreservation conditions.

**Supplementary Table 1:** Fresh semen quality of Madura bulls (n=8 ejaculates)

| Parameters | Mean±SEM |
|---|---|
| Ejaculatory volume (mL) | 6.03±0.15 |
| Color | Milky white |
| pH value | 6.95±0.08 |
| Odor | Distinctive |
| Consistency | Thick |
| Sperm concentration ($10^6$ mL$^{-1}$) | 1,388.71±15.49 |
| Sperm total motility (%) | 86.96±1.63 |
| Sperm progressive motility (%) | 85.70±1.91 |
| Sperm viability (%) | 90.21±0.84 |
| Sperm abnormalities (%) | |
| Primary abnormality | 0.50±0.29 |
| Secondary abnormality | 7.57±1.10 |
| Sperm PMI (%) | 83.79±0.62 |
| Sperm AMI (%) | 95.79±1.08 |

Primary abnormalities: Defects originating during spermatogenesis, like abnormal head shape, double tail, coiled tail at head; Secondary abnormalities: Defects acquired during epididymal transit or handling, such as detached head, bent or coiled tail; PMI: Plasma membrane integrity; AMI: Acrosomal membrane integrity.

**Sample size, replication, and post-thaw evaluation:** A total of 243 straws were produced across all treatments. Post-thaw analyses were performed using three replicates per treatment group for CASA (7×3=21 straws), 16 replicates for sperm morphology parameters (7×16=112 straws), five replicates for ROS measurements across eight groups, including one positive control group (8×5=40 straws), and five replicates for MDA and CAT assays (7×5×2=70 straws, respectively). Each replicate consisted of straws from a given treatment thawed together and assessed under identical conditions, providing an integrated evaluation of the functional and oxidative responses to selenium form and dose. Post-thaw evaluation was carried out after the frozen semen samples were thawed in a water bath at 37°C for 30sec.

**CASA kinematics analysis:** Post-thaw sperm movement characteristics were assessed using CASA system (AndroVision®, Minitube, Germany). The recorded parameters included total sperm motility, progressive motility, and the kinematic parameters such as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), distance curve-line (DCL), distance straight-line (DSL), distance average path (DAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), head abnormality criteria (HAC), wobble (WOB), linearity (LIN), and straightness (STR). To ensure measurement reliability and accuracy, the system was calibrated and standardized at the BIB Lembang laboratory which is accredited by the National Accreditation Committee of Indonesia (KAN). For the analysis, semen samples were placed on preheated glass slides, secured with coverslips, and evaluated using negative phase-contrast microscopy. Observations were

conducted at 400 magnification on a thermal plate maintained at 37°C. A minimum of 500 spermatozoa were evaluated in five observation fields (Raafi *et al.*, 2021; Del Prete *et al.*, 2022).

**Sperm morphology parameters:** Sperm morphological quality traits were evaluated on stained smears and wet preparations using standard laboratory protocols. The following parameters were assessed: sperm viability, morphological abnormalities, PMI, and AMI. Viability and abnormalities of spermatozoa were determined using eosin staining (Leica, Germany), as described earlier by Erlindra *et al.* (2026). Sperm PMI was determined using a hypo-osmotic swelling (HOS) test, as described by Tran *et al.* (2025). Furthermore, sperm AMI was evaluated using Giemsa (Merck, Germany) staining, as described earlier (Li *et al.*, 2023; Erlindra *et al.*, 2026).

**Oxidative markers (ROS, MDA and CAT):** ROS levels in post-thawed semen were quantified by flow cytometry using the cell-permeant fluorogenic probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma, Germany) and have been reported as the percentage of ROS-positive spermatozoa, which reflected intracellular oxidative load. In the ROS level assessment, an additional control group (0+) was included, which consisted of the control sample supplemented with $H_2O_2$ solution. This group was used as a positive control to confirm that the assay system was functioning properly and was sufficiently sensitive to detect ROS levels (Ruijter *et al.*, 2024). Semen MDA concentration was determined using an ELISA kit (Catalog No. E-EL-0060, Elabscience, USA) based on a previous study by Shalaby *et al.* (2026). Semen CAT activity was quantified using a commercially available bovine catalase ELISA kit (Enlibio, China) in accordance with manufacturer's protocols and a previous study by Gao *et al.* (2025).

**Statistical analysis:** All results are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA, in which treatment group was included as the fixed factor. When significant differences were observed ($P \leq 0.05$), group means were compared using Duncan's multiple range test. Where appropriate, dose-response trends were further interpreted in terms of inflection-type relationships, potentially indicative of antioxidant paradox phenomena.

## RESULTS

**CASA variables:** All CASA variables differed significantly among treatments ($P \leq 0.05$), indicating that selenium forms and concentrations modulated post-thaw sperm movement patterns. Within the SeNPs group, total and progressive sperm motility values did not differ significantly among 5, 10, and 25ppm, although these values were higher ($P \leq 0.05$) than those of the control group, or in conventional Selenium form at the same dose. Furthermore, the conventional form of selenium treatments ($Na_2SeO_3$) at 10 and 25ppm and to a lesser extent 25ppm SeNPs, exhibited lower progressive motility and higher erratic trajectories, reflected in reduced LIN and STR and less efficient kinematic profiles, similar to observations where excessive SeNPs or selenite impaired motility (Table 3).

Velocity parameters, including VCL, VSL and VAP were highest in the SeNPs 10ppm group and declined progressively at higher $Na_2SeO_3$ concentrations, with the 25ppm $Na_2SeO_3$ group recording values comparable to those of the control group. Similarly, trajectory-related parameters DCL, DSL, and DAP were significantly higher in SeNPs-treated groups compared to the control, with SeNPs 5 and 25ppm yielding the highest values across these measures. For $Na_2SeO_3$, these parameters differed non-significantly among three concentrations. Mean values for both LIN and STR differed non-significantly among all group, except that LIN and STR in 25ppm $Na_2SeO_3$ group were comparable to control group, but LIN was significantly lower in 25ppm $Na_2SeO_3$ than that of 10 and 25ppm SeNPs and 10ppm of $Na_2SeO_3$ groups. Similarly, STR was significantly lower in 25ppm $Na_2SeO_3$ compared to 10ppm SeNPs group ($P \leq 0.05$).

**Table 3:** Effect of different levels of SeNPs and $Na_2SeO_3$ supplementation on sperm motility characteristics in post-thawed semen of Madura bulls (Mean±SEM)

| Parameters | Treatments | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | SeNPs (ppm) | | | $Na_2SeO_3$ (ppm) | | |
| | | 5 | 10 | 25 | 5 | 10 | 25 |
| Total motility (%) | 36.69±2.65$^{a}$ | 78.20±0.19$^{c}$ | 77.39±2.61$^{c}$ | 69.92±5.83$^{c}$ | 69.65±0.49$^{c}$ | 51.32±0.53$^{b}$ | 42.11±4.54$^{ab}$ |
| Progressive motility (%) | 30.61±2.50$^{a}$ | 73.09±2.13$^{c}$ | 74.01±2.46$^{c}$ | 66.35±6.09$^{c}$ | 64.63±0.11$^{c}$ | 46.30±0.92$^{b}$ | 33.03±5.20$^{a}$ |
| VCL (µm/s) | 43.01±2.15$^{a}$ | 86.80±0.88$^{cd}$ | 87.83±3.78$^{d}$ | 74.95±4.23$^{b}$ | 75.93±0.21$^{bc}$ | 53.68±0.62$^{a}$ | 44.82±6.22$^{a}$ |
| VSL (µm/s) | 23.60±2.40$^{a}$ | 47.54±3.02$^{c}$ | 53.12±1.82$^{d}$ | 43.44±3.98$^{c}$ | 41.99±0.09$^{c}$ | 32.45±1.19$^{b}$ | 21.89±3.32$^{a}$ |
| VAP (µm/s) | 26.20±2.17$^{a}$ | 52.00±2.63$^{c}$ | 56.47±2.07$^{d}$ | 46.77±4.13$^{c}$ | 46.08±0.12$^{c}$ | 34.86±1.09$^{b}$ | 24.99±3.63$^{a}$ |
| DCL (µm) | 10.41±0.72$^{a}$ | 34.58±1.89$^{d}$ | 24.17±2.13$^{c}$ | 32.56±2.63$^{d}$ | 20.27±0.32$^{bc}$ | 16.11±2.16$^{ab}$ | 18.681.96$^{bc}$ |
| DSL (µm) | 4.70±0.66$^{a}$ | 18.76±2.17$^{d}$ | 14.05±1.31$^{cd}$ | 18.72±2.26$^{d}$ | 10.50±0.10$^{bc}$ | 9.07±0.84$^{ab}$ | 8.73±1.04$^{ab}$ |
| DAP (µm) | 5.63±0.61$^{a}$ | 20.66±2.05$^{d}$ | 15.18±1.39$^{c}$ | 20.27±2.34$^{d}$ | 11.86±0.22$^{bc}$ | 9.970.96$^{ab}$ | 10.13±1.15$^{ab}$ |
| ALH (µm) | 0.46±0.01$^{a}$ | 0.92±0.02$^{c}$ | 0.84±0.04$^{bc}$ | 0.78±0.05$^{b}$ | 0.77±0.01$^{b}$ | 0.57±0.03$^{a}$ | 0.52±0.05$^{a}$ |
| BCF (Hz) | 7.59±0.44$^{a}$ | 17.79±1.20$^{e}$ | 15.39±0.66$^{de}$ | 17.55±0.55$^{e}$ | 13.31±0.34$^{cd}$ | 10.34±0.08$^{ab}$ | 10.58±1.51$^{bc}$ |
| HAC (rad) | 0.09±0.01$^{a}$ | 0.22±0.01$^{c}$ | 0.20±0.01$^{bc}$ | 0.19±0.02$^{bc}$ | 0.18±0.00$^{b}$ | 0.12±0.00$^{a}$ | 0.12±0.02$^{a}$ |
| WOB (VAP/VCL) | 0.61±0.02$^{ab}$ | 0.60±0.03$^{ab}$ | 0.65±0.01$^{b}$ | 0.62±0.02$^{ab}$ | 0.61±0.01$^{ab}$ | 0.65±0.03$^{b}$ | 0.56±0.01$^{a}$ |
| LIN (VSL/VCL) | 0.55±0.03$^{ab}$ | 0.55±0.03$^{ab}$ | 0.61±0.01$^{b}$ | 0.58±0.02$^{b}$ | 0.55±0.00$^{ab}$ | 0.61±0.03$^{b}$ | 0.49±0.01$^{a}$ |
| STR (VCL/VAP) | 0.90±0.02$^{ab}$ | 0.92±0.02$^{bc}$ | 0.94±0.00$^{c}$ | 0.93±0.00$^{bc}$ | 0.92±0.01$^{bc}$ | 0.94±0.01$^{bc}$ | 0.88±0.01$^{a}$ |

Values with different superscripts within the same row indicate significant differences ($P \leq 0.05$); VCL: Curvilinear velocity; VSL: Straight-line velocity; VAP: Average path velocity; DCL: Distance curve-line; DSL: Distance straight-line; DAP: Distance average path; ALH: Amplitude of lateral head displacement; BCF: Beat cross frequency; HAC: Head abnormality criteria; WOB: Wobble; LIN: Linearity; and STR: Straightness.

The WOB ratio showed relatively less variation across treatments, although the 25ppm $Na_2SeO_3$ group recorded the lowest value, which was comparable to control group. ALH and BCF were significantly elevated in SeNPs-supplemented groups relative to the control (P≤0.05), with SeNPs 5ppm showed the highest ALH and SeNPs 5 and 25ppm yielding the highest BCF. HAC values were also significantly higher in selenium-treated groups compared to the control, with SeNPs 5ppm recording the highest value, while values for 10 and 25 ppm $Na_2SeO_3$ groups were comparable to control group. Collectively, these findings indicate that SeNPs at 5–10ppm optimally supported sperm kinematic integrity following cryopreservation. The control group exhibited the lowest total and progressive motility, as well as the least favorable, kinematic parameters, in line with the recognized impact of cryo-induced OS on sperm motility.

**Sperm viability, membrane integrity, and abnormalities:** Antioxidant supplementation both in conventional form or nano-form of selenium at all levels (except $Na_2SeO_3$ at 25ppm for PMI) significantly improved sperm viability, PMI and AMI (P≤0.05) compared to control, but did not affect primary or secondary abnormalities (Table 4). The SeNPs supplemented groups exhibited viability above 70%, particularly at 5ppm (79.50±1.71%), which also showed the highest PMI and AMI values, indicating robust preservation of plasma and acrosomal membranes relative to control and $Na_2SeO_3$ supplemented groups. Both selenium forms improved sperm viability and membrane integrity compared with control. However, conventional form showed a declining pattern in these traits as the dose increased, suggesting a narrow safety margin.

**ROS levels:** The ROS concentrations were significantly different among different treatment groups (P≤0.05). The highest percentage of ROS was recorded in the positive control group of the standard protocol (Table 5). These data showed the effects of high OS in extenders without selenium treatment. The addition of SeNPs at concentrations of 5, 10 and 25ppm significantly reduced (P≤0.05) ROS production in post-thaw Madura semen compared to the control group and the $Na_2SeO_3$ groups (10 and 25ppm). A dose of 5ppm SeNPs was very effective in reducing ROS concentration. Interestingly, while the minimal dose of SeNPs (5ppm) showed significant reduction in ROS production compared to all three concentrations of conventional selenium, the maximal dose of SeNPs still produced almost similar results. These data suggest that the nano form retains antioxidant properties even at minimal doses and provides a wider dose margin at higher concentrations.

**MDA concentration and CAT activity:** The data of the present study showed that selenium supplementation, both in conventional and nano forms, significantly affected MDA levels and CAT activity (P≤0.05). The control group showed the highest semen MDA contents and the lowest CAT activity compared to those for three levels of SeNPs and 5 and 10 ppm of $Na_2SeO_3$. However, MDA contents and CAT activity for 25ppm $Na_2SeO_3$ group were comparable to those of control group (Table 5).

The use of SeNPs, especially at a concentration of 5ppm, showed higher reduction in ROS and MDA levels, with the higher increase in CAT activity, suggesting the establishment of strong protection against lipid peroxidation and an enhanced endogenous antioxidant response. In both forms of selenium, higher doses (10 and 25ppm) were associated with increased MDA and decreased CAT activity compared to 5ppm, highlighting the existence of an optimal concentration window and implying that excessive selenium disrupts redox homeostasis and becomes pro-oxidative. Interestingly, SeNPs showed better results, as the rate of MDA increase with SeNPs was not as high and fast as that with conventional selenium. The MDA pattern closely mirrored the ROS levels in the cellular environment, with a stable response at approximately 5ppm of $Na_2SeO_3$ and a wider range of benefits for SeNPs before a decline at 25ppm.

**Table 4:** Effect of different doses of SeNPs and $Na_2SeO_3$ supplementation on sperm cellular quality in post-thawed semen of Madura bulls (Mean±SEM)

| Parameters (%) | Treatments | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | SeNPs (ppm) | | | $Na_2SeO_3$ (ppm) | | |
| | | 5 | 10 | 25 | 5 | 10 | 25 |
| Sperm viability | 43.53±0.75$^{a}$ | 79.50±1.71$^{e}$ | 75.87±0.88$^{e}$ | 71.00±2.47$^{d}$ | 66.29±1.43$^{c}$ | 62.36±2.12$^{c}$ | 54.29±1.09$^{b}$ |
| Sperm PMI | 55.29±3.21$^{a}$ | 81.57±2.18$^{d}$ | 78.21±1.27$^{cd}$ | 67.13±1.09$^{b}$ | 73.57±1.62$^{c}$ | 65.86±2.16$^{b}$ | 59.64±1.37$^{a}$ |
| Sperm AMI | 65.83±1.04$^{a}$ | 86.79±1.64$^{e}$ | 83.57±1.70$^{de}$ | 78.57±2.19$^{c}$ | 80.57±1.32$^{cd}$ | 73.27±1.32$^{b}$ | 72.27±1.41$^{b}$ |
| Sperm abnormalities | | | | | | | |
| Primary abnormality | 0.29±0.15 | 0.29±0.15 | 0.29±0.21 | 0.21±0.10 | 0.07±0.07 | 0.00±0.00 | 0.00±0.00 |
| Secondary abnormality | 4.57±0.46 | 4.29±1.26 | 4.93±1.47 | 3.86±1.11 | 2.36±0.72 | 2.64±0.47 | 3.43±0.49 |

Values in the same row with different superscripts differ significantly (P≤0.05); PMI: Plasma membrane integrity; and AMI: Acrosomal membrane integrity.

**Table 5:** ROS, MDA content and CAT activity in post-thawed semen of Madura bulls (Mean±SEM)

| Parameters | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | SeNPs (ppm) | | | $Na_2SeO_3$ (ppm) | | |
| | *0+ | 0 | 5 | 10 | 25 | 5 | 10 | 25 |
| ROS (%) | 74.20±2.42$^{h}$ | 55.90±3.54$^{g}$ | 4.70±0.40$^{a}$ | 9.53±0.09$^{b}$ | 13.50±0.35$^{bc}$ | 17.67±0.64$^{cd}$ | 20.30±0.74$^{de}$ | 25.87±0.24$^{f}$ |
| MDA (µmol/$10^8$ sperm) | - | 2.91±0.04$^{f}$ | 1.33±0.00$^{a}$ | 1.83±0.04$^{b}$ | 2.24±0.04$^{d}$ | 2.03±0.01$^{c}$ | 2.74±0.05$^{e}$ | 2.83±0.03$^{ef}$ |
| CAT (µmol/$10^8$ sperm) | - | 0.34±0.00$^{a}$ | 1.38±0.01$^{d}$ | 1.16±0.12$^{c}$ | 0.95±0.00$^{b}$ | 0.89±0.00$^{b}$ | 0.84±0.00$^{b}$ | 0.46±0.08$^{a}$ |

Values in the same row with different superscripts differ significantly (P≤0.05); ROS: Reactive oxygen species; *0+: The positive control containing $H_2O_2$ solution from the ROS assay kit; MDA: Malondialdehyde; and CAT: Catalase activity.

## DISCUSSION

In the present study, selenium supplementation in semen extender, mainly in the form of SeNPs, has been found to modulate post-thaw sperm CASA motility kinematics, sperm quality, and cellular redox balance in Madura bull semen. These results are consistent with those of a previous study, which indicated that the addition of SeNPs to semen extender improved progressive sperm motility, viability, PMI, and ultrastructure in Holstein bulls, and this was accompanied by improved fertility (Khalil *et al.*, 2019). The application of nanotechnology in the form of selenium antioxidants yields good efficacy, as the nano-sized particles possess a high surface-to-volume ratio (Araujo *et al.*, 2021). This is supported by the findings of this study regarding the size of selenium nanoparticles (Table 1), which indicate that the average diameter of the SeNPs exhibits a relatively narrow overall size distribution. Furthermore, the presence of two distinct peaks (Table 1) further supports a bimodal distribution, which may reflect variations in particle nucleation and growth during synthesis, or partial agglomeration during dispersion (Sitaresmi *et al.*, 2024).

The addition of selenium into semen extender protects seminal plasma and sperm organelles, including the middle section and sperm flagellum, from freezing-induced cellular damage. This protection is particularly important because cold shock and changes in atmospheric oxygen during cryopreservation can cause biochemical, biophysical, ultrastructural, molecular, and functional alterations in sperm (Upadhyay *et al.*, 2021). Selenium plays an important role in the synthesis of GPx enzymes, which protect cell membranes and lipid-containing sperm organelles from post-freezing peroxidative damage (Siena *et al.*, 2026). This protection was reflected in the obtained data, which show better sperm motility kinematics in the selenium treatment groups than in the control group.

The SeNPs supplemented groups, especially at a concentration of 5ppm, showed relatively better results in terms of CASA kinematic parameters (total motility, progressive motility, VCL, DCL, DSL, DAP, ALH, BCF, and HAC) than the conventional form. In addition, the resulting sperm viability and plasma membrane and acrosomal membrane integrity were better in SeNPs supplemented groups than those in the high-dose conventional selenium group. These data suggest that the use of SeNPs at low dose strengthens the functional robustness of frozen sperm (Li *et al.*, 2023; Esin *et al.*, 2024). Better results were obtained with a dose of 5ppm than higher doses of both selenium forms. However, the administration of selenium in the form of SeNPs at higher doses (up to 25ppm) proved to be more effective and provided better results than the parallel doses of conventional form.

Collectively, starting from 5ppm, conventional selenium showed decreased sperm viability compared to SeNPs, with SeNPs maintaining post-thaw sperm motility above 70% at 5, 10 and 25ppm and a high percentage of PMI and AMI parameters compared to the conventional selenium groups. These findings provide insights and support the view that use of selenium nanomaterials is safer than the use of conventional selenium forms. This is because inorganic selenium compounds like $Na_2SeO_3$ can undergo metabolic conversion to highly toxic hydrogen selenide. According to Li *et al.* (2023), addition of SeNPs has multiple advantages, such as minimal toxicity, efficient catalytic properties, and high adsorption potential, while also helping to lower oxidative stress in sperm through increased GPx activity. The efficiency of nano form is better in scavenging ROS compared to selenite, seleno-methionine and methyl-selenosysteine (Zhu *et al.*, 2025), with much lower toxicity. However, the absence of treatment effects on primary and secondary abnormalities suggests that selenium supplementation primarily modulated membrane stability and survival post-thaw rather than improving pre-existing spermatogenic defects.

The convergence in this study between optimal CASA kinematic parameters and improved redox index at 5ppm SeNPs was consistent with the concept that redox stabilization is a major factor for improved function in cryopreserved bull sperm (Azizah *et al.*, 2023; Li *et al.*, 2023; Ghorbani *et al.*, 2024). The improved antioxidant function at 5ppm SeNPs concentration was accompanied by a better oxidative profile characterized by decreased levels of ROS and MDA, as well as increased CAT activity, suggesting the restoration of cellular redox homeostasis during the freezing and thawing process. Mechanistically, these effects are in accordance with the known role of selenium in seleno-proteins, such as GPx, to detoxify hydrogen, neutralize lipid peroxide activity, and protect the mitochondrial and plasma membranes of sperm (Khalil *et al.*, 2024). According to Esin *et al.* (2024), addition of sodium selenite at 1–2µg/mL to bovine semen extenders increased post-thaw sperm quality and total antioxidant capacity, highlighting the importance of adequate selenium supply for antioxidant defense.

This study demonstrates that the addition of selenium increases the sperm CAT activity in post-thaw semen and provides protection against the OS generated during cryopreservation. Selenium has been shown to enhance CAT production in sperm through a multi-role mechanism involving the enhanced cellular uptake, gene regulation, and metabolic synergy (Sitaresmi *et al.*, 2024). Conventional selenium relies on passive anion transporters (seleno-methionine), whereas SeNPs enter cells more efficiently via endocytosis due to their nanoscale size and high surface area relative to their volume. In sperm, this mechanism is particularly effective because SeNPs can penetrate biological barriers more efficiently to activate antioxidant pathway, resulting in higher post-thaw CAT activity and better protection against cryopreservation-induced damage than conventional forms, as indicated by lower MDA levels (Khalil *et al.*, 2019; Sitaresmi *et al.*, 2024).

Interestingly, contrary to a previous study by Esin *et al.* (2024), higher dosage of selenium and SeNPs (exceeding 5ppm) in this study demonstrated improved sperm quality post-thaw compared to that in the control group. These discrepancies may be attributed to variations in the particle size, SeNPs components, or species utilized. Li *et al.* (2023) demonstrated that biomaterial properties and bioactivity are strongly governed by factors including pore structure, particle characteristics, surface charge profile and mechanical integrity. Selenium

nanoparticles, by virtue of their higher surface area and improved interaction with sperm cells, appear to provide more efficient delivery, as shown in studies where use of SeNPs alone (Abedin *et al.*, 2023), or in combination with glutathione (Li *et al.*, 2023), improved sperm motility, viability, mitochondrial activity, PMI, AMI, and embryo development. The decrease in lipid peroxidation and increase in antioxidant enzyme activity at the optimal SeNPs dose, at 5ppm in this study, supports the earlier findings that SeNPs administration increases the sperm antioxidant system during the freezing and thawing phases by suppressing the concentration of ROS formed (Khalil *et al.*, 2019; Ghorbani *et al.*, 2024).

However, the deterioration of both CASA kinematic parameters and oxidative markers at higher selenium doses underscores the dual nature of selenium and echoes the nonlinear responses reported in other species (Abedin *et al.*, 2023; Esin *et al.*, 2024). At higher concentrations, SeNPs or selenium may promote ROS formation, increase lipid peroxidation, and impair sperm motility and viability, reflecting a transition from beneficial antioxidant activity to harmful pro-oxidant effects once the physiological limit is surpassed (Ghorbani *et al.*, 2024; Paul *et al.*, 2024). A study on the effects of nano-antioxidants and nanomaterial on male fertility indicates that nanoparticles, including SeNPs, can exert oxidative, mitochondrial, and DNA-damaging effects at inappropriate doses or with unfavorable physicochemical properties (Paul *et al.*, 2024). Together with the present study, these findings strengthen the notion of what is known as the antioxidant paradox, in which an excess of conventional or nano-formulated antioxidants can destabilize redox signaling and impair sperm function rather than protect it (Abedin *et al.*, 2023; Li *et al.*, 2023).

Compared with conventional selenium, SeNPs exhibited lower pro-oxidant activity and were associated with less sperm damage at equivalent doses, although sperm quality still declined at higher concentrations. At the cellular level, SeNPs is known to exhibit high bioavailability due to their neutral oxidation pattern and unique nanoscale properties, which facilitate more controlled cell interactions than the performance pattern of conventional selenium (Ding *et al.*, 2026). In contrast to conventional selenium, which rapidly generates excessive ROS when used above the optimal limits and depletes cellular thiols, such as glutathione, causing DNA damage and apoptosis, which renders the use of SeNPs as safe. The SeNPs are integrated directly into the enzyme defense system of the body with low toxicity. They specifically increase the activity of protective selenium proteins, such as GPx and superoxide dismutase (SOD), and act as effective free radical scavengers. The balanced cellular uptake of this nano form avoids the aggressive pro-oxidant pathways associated with traditional selenium, thus protecting cells from OS and selenosis, even at higher doses (Chen *et al.*, 2025).

As observed in the present study, improved post-thaw sperm motility, viability, and membrane integrity following supplementation of SeNPs can increase the possibility of meeting the dose of quality sperm that can be used for AI (Silvestre *et al.*, 2021; Esin *et al.*, 2024; Erlindra *et al.*, 2026). This is expected to improve fertility and increase the preservation of local livestock, as shown by the higher pregnancy rates in cows inseminated with SeNPs-treated bull semen (Khalil *et al.*, 2019; Li *et al.*, 2023). For local livestock, such as Madura cattle, which play a key role in local adaptation and food security but face genetic erosion, optimizing extender formulations with SeNPs could strengthen cryopreservation seed banks and long-term national conservation strategies (Abedin *et al.*, 2023; Esin *et al.*, 2024). Regarding the broader application of nanobiotechnology, the current dose-response data complement the views advocating nano-antioxidants as promising additives to manage infertility in bulls due to oxidative stress. However, the toxicity patterns and endocrine disruption of nano-specific applications should be rigorously evaluated (Jurado-Campos *et al.*, 2023; Paul *et al.*, 2024). Integrating optimized SeNPs dosage with advanced CASA kinematics, protein biomarkers or metabolites associated with fertility, and other nano-based sperm selection approaches may help identify bulls with superior resistance to cryopreservation-induced stress (Vigolo *et al.*, 2022; Shalaby *et al.*, 2026).

The relatively small sample size in this study, comprising four bulls and eight ejaculates, limits the generalizability of the findings. Therefore, the results should be regarded as preliminary. This study only focused on *in vitro* parameters and did not measure re-failure rate or birth outcome (Khalil *et al.*, 2019; Li *et al.*, 2023). Oxidative assessment was limited to ROS, MDA, and CAT. However, sperm motility and kinematic parameters were assessed using a standardized CASA system in an accredited laboratory, ensuring high measurement reliability. Conventional evaluation complemented these data by addressing variables not covered by CASA, specifically sperm viability, morphology, PMI, and AMI. Furthermore, ROS levels were evaluated via flow cytometry, while MDA and CAT were determined spectrophotometrically, providing a comprehensive assessment of post-thaw semen quality.

Nevertheless, the study proved that SeNPs had a positive effect on post-thaw semen quality. Investigations on additional markers, such as SOD and GPx activity, mitochondrial membrane potential, DNA fragmentation, and protein oxidation, that would provide deeper mechanistic insights, are needed (Khalil *et al.*, 2019; Dolník and Mudroňová, 2021; Shalaby *et al.*, 2026). In addition, the physicochemical properties of SeNPs have not been fully characterized, despite their significant impact on the behavior of nanoparticles in biological systems and their ability to modulate both effectiveness and toxicity (Silvestre *et al.*, 2021; Yuan *et al.*, 2024).

**Conclusions:** Supplementation of Madura bull semen extender with 5ppm SeNPs optimally stabilized redox homeostasis and preserved post-thaw sperm quality, as reflected by improved CASA kinematics, sperm viability, and membranes integrity. In contrast, $Na_2SeO_3$ conferred comparatively limited protection, and both selenium forms demonstrated a dose-dependent shift towards pro-oxidant activity and deleterious effects at higher concentrations. These findings support the strategic inclusion of carefully titrated SeNPs in semen cryopreservation protocols for local cattle species. Importantly, these findings underscore that

nanotechnology-based antioxidant interventions must be applied within well-defined therapeutic windows, guided by functional sperm parameters and oxidative balance, to maximize benefits while minimizing potential risks.

**Ethical approval:** Madura bull management, sample collection and experimental procedures were carried out in accordance with BIB Lembang's established standards and protocols. In addition, the BIB Lembang testing laboratory is nationally recognized and internationally accredited under SNI and ISO/IEC 17025:2017.

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**Authors contribution:** DTW and MP conceptualized the study. Data curation was performed by FGP, DFFD and PIS. Analysis was conducted by FGP, PIS and DFFD. DTW, MP and AK developed the methodology, while FGP, DFFD and PIS managed the software. The original draft was written by DTW, MP, PIS, FGP and DFFD. Finally, DTW, DFFD, FGP, PIS, AK and MP contributed to the writing, review and editing of the manuscript.

**Data availability:** All data supporting the findings of this study are included in this manuscript. Further information may be obtained from the corresponding author upon reasonable request.

**Declaration of competing interest:** No potential conflict of interest is related to this manuscript.

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