



Research article

Sodium selenite supplementation diminishes reactive oxygen species and enhances post-thaw spermatozoa membrane integrity of Indonesian indigenous bulls

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Article Info

Article history:

Received 2 February 2026

Revised 17 May 2026

Accepted 28 May 2026

Available online 15 July 2026

Keywords:

Antioxidant,
Indigenous bull,
Post-thaw semen,
Sodium selenite,
Spermatozoa quality

Abstract

Importance of the work: Cryopreservation and thawing induce oxidative stress (OS), which impairs spermatozoa quality. Sodium selenite (Se) supplementation may provide protective and antioxidant effects.

Objectives: To evaluate the effects of Se supplementation on reactive oxygen species (ROS) production and membrane integrity of Aceh and Bali bull semen.

Materials and Methods: In total, 220 semen straws were obtained from superior bulls of both the Aceh and Bali breeds from the Artificial Insemination Center (*Balai Besar Inseminasi Buatan-BBIB*) Singosari, East Java, Indonesia. Samples were allocated to five treatment groups: control (no supplementation) and Se supplementation at 1 part per million (ppm), 5 ppm, 25 ppm and 50 ppm (T1, T2, T3 and T4, respectively). Each solution was stored at 4°C for 60 min. The parameters assessed were: motility, viability, abnormalities, plasma membrane integrity (PMI), acrosome membrane integrity (AMI) and ROS level. Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test and Pearson's correlation analysis.

Results: There were significant ($p < 0.05$) improvements in post-thaw motility, viability, primary abnormality and PMI in all supplemented groups compared to the control groups for both breeds. Secondary abnormalities were observed exclusively in the Aceh bull samples, whereas significant AMI was noted only in the Bali bull samples. Se supplementation significantly reduced ROS levels, with T2 having the greatest efficacy. The Pearson's correlation analysis revealed strong positive correlations ($p < 0.01$) among motility, viability and PMI across both breeds, whereas ROS was negatively correlated with all quality parameters. There were breed-specific differences in the relationship between morphological abnormalities and sperm quality.

Main finding: Se supplementation improved post-thaw spermatozoa quality in both breeds, with 5 ppm being the optimal concentration. These findings highlight the potential of Se as a key additive in semen extenders to support semen quality during cryopreservation.

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<https://doi.org/10.34044/j.anres.2026.60.4.09>

Introduction

Cattle farming in Indonesia is closely linked to cultural practices and community life. The genetic diversity of indigenous breeds is crucial. Indigenous Indonesian cattle support rural livelihoods and sustain sustainable production systems, with smallholder systems providing 90% of the national cattle supply and beef being the second most consumed protein after chicken (Hilmiati et al., 2024). Conservation of genetic resources is crucial for addressing future challenges. Aceh and Bali cattle, developed through hereditary selection, have distinct genetic profiles (Hastarina et al., 2025), including heat and disease resistance and good reproductive performance. Furthermore, elevated levels of propionic acid in Bali cattle enable efficient feed usage and lower methane emissions (Anggraeny et al., 2025).

Despite their valuable traits of Aceh and Bali bulls, their reproductive performance is lower than that of commercial breeds because of the limited adoption of reproductive biotechnology. Their populations are declining due to reproductive inefficiencies in traditional smallholder systems, including extended calving intervals and unregulated mating (Mansur et al., 2024). Genetic degradation and loss of traits may occur in indigenous or local cattle. Preserving genetic diversity requires effective approaches to enhance genetic improvement. Artificial insemination (AI) and *in vitro* fertilization (IVF) can help disseminate genetic material (Rodriguez-Martinez, 2012). However, post-thaw semen has lower motility than fresh semen, which can impair penetration through the cervical mucus (Engdawork et al., 2024). This affects the success of IVF, which requires functional spermatozoa for zona pellucida (ZP) penetration (Leemans et al., 2019). Exposure to reactive oxygen species (ROS) reduces spermatozoa quality through oxidative stress (OS), causing membrane damage and DNA fragmentation (Sengupta et al., 2024). Stimulatory agents and antioxidants can protect spermatozoa and enhance fertility (Ahmed et al., 2020).

Sodium selenite (Se), an essential component of glutathione peroxidase (GPx), is a trace mineral that protects spermatozoa from OS via its potent antioxidant properties (Muñoz-Benitez et al., 2025). Se contributes to antioxidant defense and reproduction, with insufficient Se linked to abnormal morphology and reduced spermatozoa motility (Siena et al., 2026). Selenoproteins, such as GPx and thioredoxin reductase, contribute to antioxidant defense and are crucial for maintaining sperm structural integrity, mitochondrial function and motility

(Sitaresmi et al., 2024). Supplementing bull semen extender *in vitro* with 1–5 µg/mL Se improved post-thaw motility, viability, membrane integrity and total antioxidant capacity, highlighting the role of sufficient Se in supporting antioxidant protection (Dolník and Mudroňová, 2021). Antioxidants applied pre-freezing or post-thawing reduce ROS levels in the sperm head, improving frozen semen integrity (Erlindra et al., 2026).

Increasingly, semen preservation has positioned Se supplementation as an antioxidant-based strategy to protect sperm function, largely through Se-dependent selenoproteins that support membrane stability, mitochondrial activity and motility under oxidative challenge (Majors et al., 2025). Furthermore, experimental evidence has shown that Se can attenuate OS during *in vitro* sperm storage, although its effects on sperm viability are not uniformly improved and appear to be strongly dependent on the dose and incubation conditions (Dolník and Mudroňová, 2021). Consistently, low-dose Se supplementation in an extender has been reported to reduce ROS production, while improving multiple post-thaw semen quality traits, supporting the ongoing investigation of optimized Se dosing windows for cryopreserved semen (Erlindra et al., 2026).

Although the field has advanced, considerable gaps remain in the supporting evidence. First, Se supplementation in post-thaw spermatozoa has been tested mostly at concentrations up to 5 ppm. Therefore, the effectiveness and safety of higher doses remain unclear and a broader dose-response range has not been defined. Second, the antioxidant paradox of selenium, in which excessive supplementation may shift from protective to harmful by disturbing the redox balance, has not been explored sufficiently in semen preservation studies. Third, integrative analyses examining the relationships between ROS and sperm quality parameters are lacking (Dutta et al., 2022; Erlindra et al., 2026). These gaps are even more pronounced in local cattle models and robust evidence is still limited for Indonesian indigenous breeds, particularly Aceh and Bali bulls, despite their strategic value in genetic conservation and national breeding programs.

Direct antioxidant supplementation at the post-thaw stage may offer a practical advantage because OS and membrane destabilization become more evident after thawing and subsequent handling before AI or IVF. Adding Se-based antioxidants to thawed sperm may help to reduce ROS accumulation, limit lipid peroxidation and stabilize plasma and acrosomal membranes, thereby potentially supporting mitochondrial function and preserving motility and viability, as reported by Hai et al. (2024). This post-thaw approach is

particularly relevant under field conditions, where thawed semen may be held temporarily before use. Furthermore, post-thaw supplementation serves as a controlled *in vitro* model to evaluate the dose-response relationship and potential cytotoxic effects of Se before its direct incorporation into the cryopreservation extender, providing a necessary toxicological baseline for future cryopreservation studies. Therefore, the current study aimed to examine the quality of post-thaw Aceh and Bali bull semen after Se supplementation, with emphasis on its potential to mitigate ROS levels. To the best of our knowledge, this is one of the first investigations to directly apply Se to post-thaw Indonesian indigenous bull semen, thereby offering preliminary insights into the possible protective role of Se in thawed spermatozoa.

Materials and Methods

Ethics statements

This study did not require ethical approval, as it only utilized frozen semen from Aceh and Bali bulls produced by the Artificial Insemination Center (*Balai Besar Inseminasi Buatan-BBIB*) Singosari, East Java, Indonesia. These samples were obtained under ISO 17025:2017 certification and had already undergone quality management systems in accordance with ISO 9001:2015 (Balai Besar Inseminasi Buatan Singosari, 2026).

Experimental animals and study locations

Semen samples were obtained from two Indonesian indigenous breeds, specifically Aceh and Bali bulls. The management of the bulls at the BBIB Singosari followed standardized protocols, which included daily feeding, *ad libitum* access to drinking water, twice-weekly semen collection from each bull and routine health screenings, including vaccinations. The frozen semen used in this study met all quality standards and had undergone prior evaluation based on Indonesian National Standard Agency (2024). Furthermore, all semen straw samples were stored under identical conditions in liquid nitrogen at -196°C . Sperm cellular quality assessments were conducted at the Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Sample size and structure of replication

In total, 220 frozen semen straws were collected from the Aceh and Bali bulls. Semen samples were derived from one superior bull per breed using three distinct cryopreservation batches. Each production batch represented a separate ejaculate to provide a constrained form of biological replication at the ejaculate level. Although this design facilitated the assessment of treatment responses across repeated ejaculations from the same bull, it did not provide independent biological replication across multiple bulls within each breed. For the post-thaw and post-chilled evaluation of sperm cellular quality, each breed comprised five treatment groups with 15 straws per treatment, resulting in 150 straws. For the ROS parameter analysis, each breed was organized into six treatment groups, including a positive control, with five straws per group, totaling 60 straws.

Se solution preparation

A stock solution was prepared by dissolving 50 mg of Se (Merck; Germany) in 10 mL of sterile water (OneMed; Indonesia) according to the method described by Erlindra et al. (2026). Subsequently, the stock solution was diluted to obtain final working concentrations of 1 part per million (ppm), 5 ppm, 25 ppm and 50 ppm (T1, T2, T3 and T4, respectively). The required dilutions were calculated using the following Equation 1:

$$V_1 \times C_1 = V_2 \times C_2 \quad (1)$$

where V_1 is the initial volume, C_1 is the initial concentration, V_2 is the final volume and C_2 is the final concentration.

Semen thawing, Se supplementation and chilling

The frozen semen straws were thawed for 30 s in a water bath at 37°C (Galeati et al., 2020). For each breed, semen samples were handled individually (not pooled) and divided into five equal aliquots corresponding to the control (T0) and the Se treatment groups. Each thawed straw was transferred to a microtube and Se was added to achieve the designated concentration. Then, the semen and Se solution were mixed at a 1:1 (volume per volume) ratio, a proportion selected to evaluate the optimal mixing ratio. The resulting mixtures were chilled at 4°C for 60 min and then evaluated.

Evaluation of spermatozoa quality

The semen samples mixed with Se were evaluated based on multiple microscopic evaluations using a light microscope (Yazumi 107 BN; Japan) coupled with an advanced observer system (Optilab; USA). For motility evaluation, a 10 μ L semen sample was dripped onto an object glass and covered with a cover glass. Motility was rated on a 0–100% scale in 5% intervals (Fitriana et al., 2025). The evaluation of other sperm quality parameters, including viability, morphological abnormalities, plasma membrane integrity (PMI) and acrosome membrane integrity (AMI), were performed by observing 200 spermatozoa at 400 $\times$ magnification, with the results expressed as percentages.

Spermatozoa viability and abnormalities were evaluated using eosin staining on the same smear preparation (Leica; Germany). Specifically, 10 μ L semen sample was mixed with 5 μ L of eosin and then the smear was heat-fixed over a Bunsen burner for approximately 5 s until the liquid film dried (El-Zeftawy et al., 2020), to rapidly fix the preparation and minimize further dye uptake. Nonviable spermatozoa absorbed the eosin and appeared stained (pink or red), whereas viable spermatozoa remained unstained with white heads (Fitriana et al., 2025). Sperm abnormalities, including large or swollen, small, or misshapen heads, as well as double tails and double heads, were categorized as primary abnormalities (PAs). The secondary abnormalities (SAs) included bent tails, loose heads, coiled tails and loose tails. The PAs originate during spermatogenesis, whereas the SAs occur after ejaculation (Ngcobo et al., 2023).

The PMI was assessed via the hypo-osmotic swelling (HOS) test by mixing 30 μ L of HOS solution with 10 μ L of semen and incubating it at 37°C for 60 min. Following incubation, a drop of the solution was placed on a glass object, overlaid with a cover glass and allowed to stand until movement of the spermatozoa ceased. Spermatozoa with coiled tails showed a positive response to the HOS solution, indicating an intact plasma membrane, whereas uncoiled tails showed a negative response due to membrane damage (Bintara et al., 2024).

The AMI was assessed using modified Giemsa staining (Merck; Germany) adapted from the method described by Ruthrakumar et al. (2024). First, semen smears were fixed in methanol (Merck; Germany) for 10 min and then stained using a Giemsa staining solution containing 2 mL phosphate-buffered saline (PBS; Sigma, Germany), 3 mL absolute Giemsa and 35 mL distilled water. The preparation was incubated in

a 37°C water bath in Giemsa solution for 2 hr. The slides were rinsed with running water and air-dried after staining. The partially stained heads indicated an intact acrosome, whereas the unstained or completely stained heads indicated acrosomal damage (Erlindra et al., 2026).

Reactive oxygen species measurement

Intracellular ROS levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining (Sigma, Germany). To prepare the working reagent, DCFH-DA was diluted in dimethyl sulfoxide to a final concentration of 20 μ M. Frozen semen straws were thawed and resuspended in PBS to obtain a final concentration of 10 μ M. The cell suspension was centrifuged at 1,000 $\times$ g for 10 min, after which the supernatant was removed. The resulting pellet was resuspended in the DCFH-DA solution. One group for each breed was supplemented with hydrogen peroxide (H_2O_2) to serve as a positive control (T0+), ensuring the assay's sensitivity and functionality in detecting ROS levels. Then, the cells were incubated in the dark at 37°C for 60 min. Following incubation, the suspension was centrifuged at 1,000 $\times$ g for 10 min to remove residual extracellular probes, 20 μ L of PBS were added and flow cytometry was used to analyze the samples (Benedetti et al., 2022).

Statistical analysis

Data for each breed were analyzed separately using one-way analysis of variance (ANOVA) to evaluate the dose-dependent effects of Se concentration. The experimental design involving a single independent variable with multiple levels justified the use of the one-way model. Breeds were independently analyzed to eliminate confounding genetic and physiological variables, ensuring biological homogeneity within each dataset. The analysis consisted of five treatment levels for sperm quality and seven for ROS (including fresh-thawed and positive controls). Prior to the analysis, the normality and homogeneity of variance were verified to confirm the suitability of the ANOVA model. Significant ($p < 0.05$) differences were further analyzed using Duncan's multiple range test. Pearson's correlation coefficient was used to assess the relationships between parameters. Analyses were performed using the SPSS software version 26.0 (IBM Corp.; Chicago, IL, USA), with data presented as mean $\pm$ SD values.

Results and Discussion

Effect of Se supplementation on quality of post-thaw spermatozoa

Fresh-thawed semen quality was generally high in both breeds (Table 1). The effects of Se supplementation on post-thaw semen quality parameters in the Aceh and Bali bulls are presented in Tables 2 and 3. The T0 groups in both breeds exhibited a pronounced functional decline after 60 min at 4°C (Tables 2 and 3), as evidenced by reduced motility, viability and PMI. In contrast, in the samples supplemented with Se at various concentrations, spermatozoa quality decreased but was still significantly maintained. Based on the fresh-thawed data (Table 1), Aceh semen showed slightly higher motility than Bali semen, indicating marginally better post-thaw motion in the Aceh bull than in the Bali bull.

After chilled storage, motility decreased markedly in the T0 groups in both breeds (40%), indicating that short-term post-thaw holding accelerated the functional decline. Se supplementation affected motility and the T2 groups consistently improved motility in both breeds compared with the T0 groups. The fresh-thawed viability was high and comparable between the Aceh and Bali bulls (>80%). However, after 60 min, it declined in the T0 group. Se significantly improved viability, with the most consistent increase observed at T2, reflecting that an optimal Se dose helped to maintain sperm survival during post-thaw storage. These results were consistent with the literature stating that post-thaw semen should have a minimum motility and viability of 40 and 50%, respectively, for AI programs (Indonesian National Standard Agency, 2024; Fitriana et al., 2025).

Se supplementation showed potential for maintaining post-thaw sperm motility and viability through its antioxidant properties and role in optimizing mitochondrial activity. Motility and viability reflect the ability of spermatozoa to survive and move after thawing, which is essential for ZP penetration and fertilization. However, free radicals damage cells by inducing lipid peroxidation, disrupting motility and damaging the nucleus (Tamargo et al., 2024). In the current study, Se supplementation improved the motility of post-thaw semen in both bull breeds (Tables 2 and 3) through enhanced mitochondrial oxidative phosphorylation. This improvement is related to catalytic sites in antioxidant enzymes, particularly selenocysteine-containing enzymes such as GPx (Hariharan and Dharmaraj, 2020). Selenium, as GPx4, functions as an energy

Table 1 Mean $\pm$ SD of post-thaw semen quality parameters in Aceh and Bali bulls (%).

Parameter	Fresh-thaw semen	
	Aceh bull	Bali bull
Motility	57.67 $\pm$ 1.45	54.00 $\pm$ 1.48
Viability	84.17 $\pm$ 1.91	83.33 $\pm$ 0.74
PA	0.37 $\pm$ 0.14	0.17 $\pm$ 0.06
SA	5.57 $\pm$ 0.65	11.67 $\pm$ 0.38
PMI	82.48 $\pm$ 0.53	81.57 $\pm$ 2.53
AMI	99.20 $\pm$ 0.25	99.23 $\pm$ 0.22

PA = primary abnormality; SA = secondary abnormality; PMI = plasma membrane integrity; AMI = acrosome membrane integrity

source for flagellar movements enhancing motility through GPx activity, thereby supporting mitochondrial function and energy transfer (Salimi et al., 2024). Selenoproteins protect against ROS, which reduces adenosine triphosphate (ATP) levels and increases membrane permeability, compromising cell viability (Yuan et al., 2024). Se supplementation has been reported to improve spermatozoa quality in broilers (Alavi et al., 2020), rams (Stefanov et al., 2018), bucks (Lukusa and Lehloeny, 2017), buffaloes (El-Sharawy et al., 2017), humans (Rezaeian et al., 2016) and boars (Tareq et al., 2010).

Furthermore, based on other studies by Boroujeni et al. (2019) and Zečević et al. (2026), selenium may enhance the expression of genes associated with mitochondrial activity, such as ATP5A1 and ND1, which could contribute to the restoration of ATP production and the reactivation of sperm motility following the post-thawing period, although this mechanism was not measured directly in the present study. Se may help to maintain osmotic and pH balances during extreme temperature transitions and reinforce chromatin structure integrity, potentially reducing the risk of DNA fragmentation associated with post-thaw membrane expansion. Se is hypothesized to exert anti-apoptotic effects at the cellular level by modulating the activity of cell death-related factors, including p53 and caspase-3, thus extending the viability of sperm that survive the freezing process (Kaya et al., 2026). Se revitalizes post-thaw sperm by restoring the internal energy machinery and reinforcing the cellular architecture, which is crucial for successful fertilization (Boroujeni et al., 2019).

The PA levels were very low in both breeds at baseline (Table 1), indicating minimal defects associated with spermatogenesis. In contrast, SA was substantially higher in the Bali bull than in the Aceh bull samples, implying greater susceptibility to cryo- or post-thaw handling-related morphological damage in the Bali bull semen. After storage, the abnormalities exhibited a less uniform treatment response.

Table 2 Aceh bull spermatozoa quality after 60 min of chilled storage (%).

Treatment	Motility	Viability	Abnormality		PMI	AMI
			Primary	Secondary		
T0	36.67±2.50 ^a	67.67±2.61 ^{ab}	0.00±0.00 ^a	7.50±2.17 ^b	64.33±3.53 ^a	97.17±1.09
T1	46.67±6.61 ^{bc}	71.83±1.80 ^b	0.17±0.25 ^b	4.83±0.66 ^a	79.17±1.52 ^c	97.67±1.09
T2	55.00±4.33 ^d	82.17±7.26 ^c	0.00±0.00 ^a	4.50±2.70 ^a	80.17±2.05 ^c	98.17±0.66
T3	48.33±2.50 ^c	79.00±1.15 ^c	0.00±0.00 ^a	12.00±4.26 ^c	70.33±9.15 ^b	97.67±0.90
T4	43.33±6.61 ^b	65.33±7.21 ^a	0.00±0.00 ^a	10.17±1.25 ^c	66.83±3.88 ^{ab}	96.83±1.52
<i>p</i> value	0.00	0.00	0.01	0.00	0.00	0.11

T0 = control (without Se supplementation); T1 = 1 part per million (ppm) Se; T2 = 5 ppm Se; T3 = 25 ppm Se; T4 = 50 ppm Se. PMI: plasma membrane integrity; AMI: acrosome membrane integrity.

Mean ± SD values within the same column followed by different lowercase superscript letters are significantly ($p < 0.05$) different.

In the Aceh bull (Table 2), SA was lowest at T2 but increased at higher doses (T3 and T4) and the difference among the treatments was significant. This pattern was consistent with a dose-dependent redox imbalance at supra-optimal Se exposure and the fact that post-thaw supplementation is unlikely to reverse the structural defects established during freezing-thawing. In the Bali bull (Table 3), SA decreased numerically at T2 compared with T0 but not significantly, likely due to the higher baseline SA and its greater viability. The identified abnormalities were predominantly primary and were more significantly affected by fresh semen characteristics than by the treatment process.

The SA percentages increased in some Se groups of Aceh bull, particularly at higher doses, indicating a dose-related redox imbalance consistent with an antioxidant paradox that disrupts redox homeostasis and compromises membrane or cytoskeletal stability (Dutta et al., 2022). In contrast, Bali bull did not exhibit the same response pattern, reflecting breed-specific cryotolerance and higher baseline viability that obscured treatment-related shifts. Post-thaw Se supplementation did not reverse structural defects formed during freezing and thawing but limited additional oxidative damage and preserved function

in the surviving sperm population. Nevertheless, all treatments showed total abnormality rates below 20%, indicating the key role of Se in preserving sperm morphology during chilled storage for 60 min. Se supplementation effectively controlled morphological defects in post-thaw spermatozoa through selenoprotein components. Morphological abnormalities may result from cryopreservation stress, with temperature fluctuations damaging the sperm structure. Selenoprotein P and phospholipid hydroperoxide glutathione peroxidase (PHGPx) can maintain normal sperm morphology (Bahmyari et al., 2021).

The Aceh bull samples tended to exhibit slightly better PMI in the fresh-thawed semen than the Bali bull samples, paralleling their relatively higher motility. After 60 min of storage, PMI declined markedly in the T0 groups of both breeds, indicating that the plasma membrane was highly vulnerable during post-thaw holding. The Se groups significantly improved PMI in both breeds, with the highest PMI consistently observed in the T2 groups. This supported membrane stabilization as a major mechanism underlying the improved motility and viability with optimal Se supplementation. Se addition may help to maintain spermatozoa membrane integrity after thawing by

Table 3 Bali bull spermatozoa quality after 60 min of chilled storage (%).

Treatment	Motility	Viability	Abnormality		PMI	AMI
			Primary	Secondary		
T0	35.00±4.33 ^a	66.33±6.61 ^a	0.67±0.66 ^b	16.50±2.84	58.33±6.61 ^a	98.00±1.56 ^{ab}
T1	45.00±4.33 ^{bc}	78.33±2.82 ^b	0.17±0.25 ^a	16.83±2.88	72.50±6.67 ^{cd}	97.67±1.09 ^{ab}
T2	50.00±4.33 ^d	80.50±1.50 ^b	0.17±0.25 ^a	14.00±3.44	76.50±2.41 ^d	99.17±0.50 ^b
T3	48.33±2.50 ^{cd}	79.33±3.07 ^b	0.00±0.00 ^a	16.00±8.65	69.83±3.88 ^{bc}	96.17±2.54 ^a
T4	41.67±6.61 ^b	70.17±5.17 ^a	0.00±0.00 ^a	16.67±2.22	65.83±5.45 ^b	96.33±2.50 ^a
<i>p</i> value	0.00	0.00	0.00	0.69	0.00	0.01

T0 = control (without Se supplementation); T1 = 1 part per million (ppm) Se; T2 = 5 ppm Se; T3 = 25 ppm Se; T4 = 50 ppm Se. PMI: plasma membrane integrity; AMI: acrosome membrane integrity.

Mean ± SD values within the same column followed by different lowercase superscript letters are significantly ($p < 0.05$) different.

enhancing mitochondrial function, reducing overall survival and preserving lipid bilayer stability. The plasma membrane shields cytosolic physiology, whereas osmotic shifts during cryopreservation can affect PMI and disrupt sperm homeostasis (Shahandeh et al., 2022).

In hypo-osmotic testing, viable spermatozoa with intact membranes had water influx for osmotic balance, causing flagellum coiling within the cell membrane. Membrane fluidity depends on the maintenance of membrane structure by cholesterol, phospholipids and proteins (Anand et al., 2024). This was consistent with other studies demonstrating that Se enhanced mitochondrial activity and membrane integrity, as intact membranes ensure controlled substrate passage (Berridge et al., 2023; Anand et al., 2024).

The percentage of fresh-thawed AMI was very high and comparable in the Aceh and Bali semen samples (Table 1), indicating that the acrosome structure was largely preserved immediately after thawing. After 60 min at 4°C, the average AMI in post-thaw semen varied significantly among the treatment groups. The AMI remained high but differed by breed in its response pattern. In the Aceh bull samples (Table 2), AMI did not differ significantly among treatments and remained consistently high with little fluctuation, suggesting that acrosomal integrity was relatively stable over post-thaw storage. In the Bali bull samples (Table 3), AMI differed significantly among treatments, with the highest value in the T2 group, indicating that optimal post-thaw Se supplementation may also help to preserve acrosomal integrity in more susceptible groups.

The observed upward trend indicated that Se supplementation enhanced acrosomal membrane stability through its antioxidant capacity, effectively acting as a membrane stabilizing agent for both structural and enzymatic components. This protection specifically reduced abnormalities in the sperm head and acrosome by preserving the membrane lipids surrounding the acrosome structure (Lukusa and Lehloeny, 2017). Such stabilization is critical because the acrosome contains enzymes essential for spermatozoa penetration through the cumulus oophorus and ZP. Yet, these enzymes remain highly susceptible to damage during cryopreservation (Lopes et al., 2021). Se supplementation serves as a vital protective mechanism to maintain the functional integrity required for successful fertilization.

Production of reactive oxygen species in spermatozoa under chilled storage

Se supplementation significantly affected ROS levels in post-thaw spermatozoa of Aceh and Bali bulls ($p < 0.01$). The fresh-thawed semen had low ROS values (10.33% in Aceh and 9.23% in Bali bulls) owing to minimal oxidative exposure. Spermatozoa stored for 60 min without supplementation had increased ROS levels of 50.53% and 43.77% in the Aceh and Bali bulls, respectively, indicating OS due to mitochondrial dysfunction (Fig. 1). Juanpanich et al. (2022) found that post-thaw spermatozoa without antioxidants had elevated ROS levels due to increased mitochondrial and nicotinamide adenine dinucleotide phosphate oxidase 5 activity. Spermatozoa are susceptible to OS during incubation because of the absence of seminal antioxidants (Riley et al., 2021). H_2O_2 treatment was used as a positive control for ROS production, where a 60 min incubation resulted in elevated ROS levels exceeding 67% in both bull breeds. Based on these results, the decreased ROS levels in the treated groups were a direct result of Se supplementation.

Se supplementation mitigated the increase in OS in both breeds. In the Aceh bull samples (Fig. 1A), ROS levels

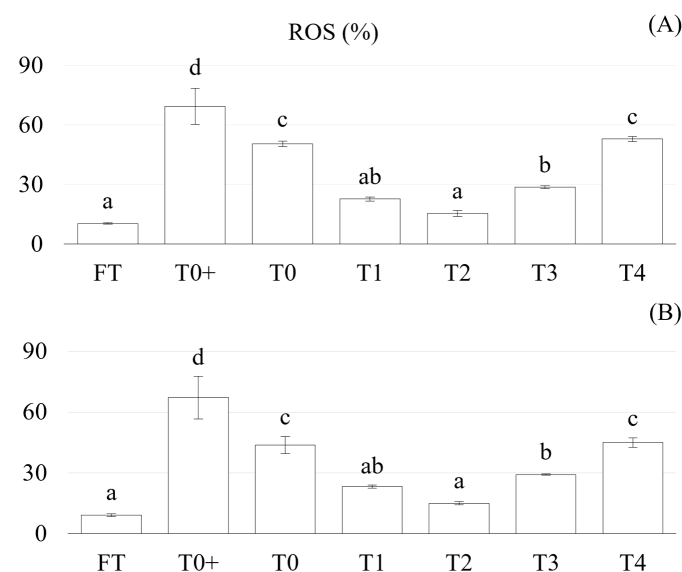


Fig. 1 Spermatozoa reactive oxygen species (ROS) levels in fresh-thawed (FT) and chilled (60 min) semen across treatment groups: (A) Aceh bull samples; (B) Bali bull semen samples. T0+ = positive control; T0 = control (without Se supplementation); T1 = 1 part per million (ppm) Se; T2 = 5 ppm Se; T3 = 25 ppm Se; T4 = 50 ppm Se. Different lowercase letters above columns indicate significant ($p < 0.05$) differences. Error bars = $\pm$ SD.

decreased in the T1, T2 and T3 groups (22.70, 15.40 and 28.70%, respectively). A similar pattern was observed in the Bali bull samples (Fig. 1B), with decreases of 23.33, 15.03 and 29.23%, respectively. The lowest ROS values were in the T2 groups for both the Aceh and Bali bulls, indicating optimal antioxidant protection during storage. However, in the T4 groups, ROS levels increased sharply (52.90% in the Aceh bull and 45.00% in the Bali bull samples). It was suspected that excessive Se contributed to pro-oxidant activity. Extended storage of chilled semen is associated with OS-induced sperm apoptosis, highlighting ROS as a critical determinant of fertility decline (Loureiro et al., 2020). The low intracellular ROS levels could be explained by the DCFH-DA assay characteristics and sperm cell compartmentalization. Intracellular esterases hydrolyze DCFH-DA to produce DCFH, which mitochondrial ROS oxidize to produce fluorescent DCF (Hajnal et al., 2023). Limited mitochondria in the midpiece result in weak fluorescence signals, whereas post-thaw membrane disruption enables ROS efflux through aquaporin channels (Juanpanich et al., 2022).

Sperm membrane lipid composition plays a crucial role in cryopreservation resistance, as it modulates membrane fluidity and stability, making membrane fluidity a marker of sperm freezability (Authaida et al., 2023). Antioxidant use is crucial for mitigating oxidative injury in post-thaw sperm. However, semen freezing impairs endogenous antioxidant defenses (Fleming and Thomson, 2025). Therefore, Chauychu-noo et al. (2021) examined the role of antioxidant supplementation during cryopreservation to improve post-thaw semen quality. Among various antioxidant strategies, Se has received particular attention because of its unique role in the enzymatic defense system against OS (Erlindra et al., 2026). GPx is an antioxidant enzyme that contributes to cellular redox homeostasis, catalyzes H_2O_2 degradation and mediates H_2O_2 -dependent signaling (Zhang et al., 2020). Furthermore, most selenoproteins are involved in redox regulation and signaling pathways (Yuan et al., 2024).

Weaver and Skouta (2022) indicated that GPx4 was uniquely capable of reducing complex lipid hydroperoxides, including phospholipid hydroperoxides (even when embedded in biological membranes) by converting these hydroperoxides into nontoxic lipid alcohols using glutathione as a cofactor, thereby interrupting the lipid peroxidation chain reaction and protecting membrane integrity. Se availability can enhance GPx4 oxidative efficacy (Kaya et al., 2026). The parallel response observed in the Aceh and Bali bull samples in the present study indicated that Se supplementation exerted

consistent protective effects across both breeds. However, minor differences in basal ROS levels may reflect breed-specific variations in sperm antioxidant capacity (Samanta et al., 2024).

Pearson's correlation between post-thaw spermatozoa parameters

Pearson's correlation analysis was performed to examine the relationships among the post-thaw semen quality parameters in the Aceh (Fig. 2A) and Bali (Fig. 2B) bulls. In both breeds, sperm quality parameters, such as motility, viability and PMI, were correlated strongly ($p < 0.01$). Furthermore, in the Aceh bull samples, motility was strongly and positively correlated with AMI ($p < 0.01$). Based on these results, post-thaw motility could be closely associated with sperm survival and membrane status (Fitriana et al., 2025; Erlindra et al., 2026). However, the Bali bull samples had tighter membrane survival coupling, as evidenced by a stronger PMI-viability correlation than the moderate correlation observed in the Aceh bull samples. Additionally, ROS exhibited consistent inverse relationships with sperm function in both breeds, showing strong negative correlations with motility, viability and PMI ($p < 0.01$), as well as a negative correlation with AMI ($p < 0.05$). These findings supported OS as a key constraint on post-thaw quality.

Morphological defects had a clearer negative functional impact in the Bali bull samples, where PA was strongly and negatively correlated with motility, viability and PMI ($p < 0.01$). In contrast, PA in Aceh bull samples was only correlated with motility and viability ($p < 0.05$). Additionally, SA in the Aceh bull samples had a strong negative correlation with AMI and ROS ($p < 0.01$), whereas SA in the Bali bull samples had no significant correlation with any of these parameters. Overall, the Pearson's heatmap strengthened the biological interpretation of the Se supplementation results by showing that post-thaw sperm function was organized around a PMI, viability and motility axis in both breeds, whereas ROS acted as an inverse driver of functional competence. In support of these observations, Neamah (2022) demonstrated a moderate correlation between motility and viability. Furthermore, based on the present results, membrane quality was positively associated with both motility and viability.

This pattern was consistent with oxidative damage during cryostorage, where peroxidation of membrane lipids and loss of membrane stability reduce the ability of sperm to maintain motility and normal function (O'Flaherty and Scarlata, 2022). Specifically, an intact plasma membrane maintains cellular

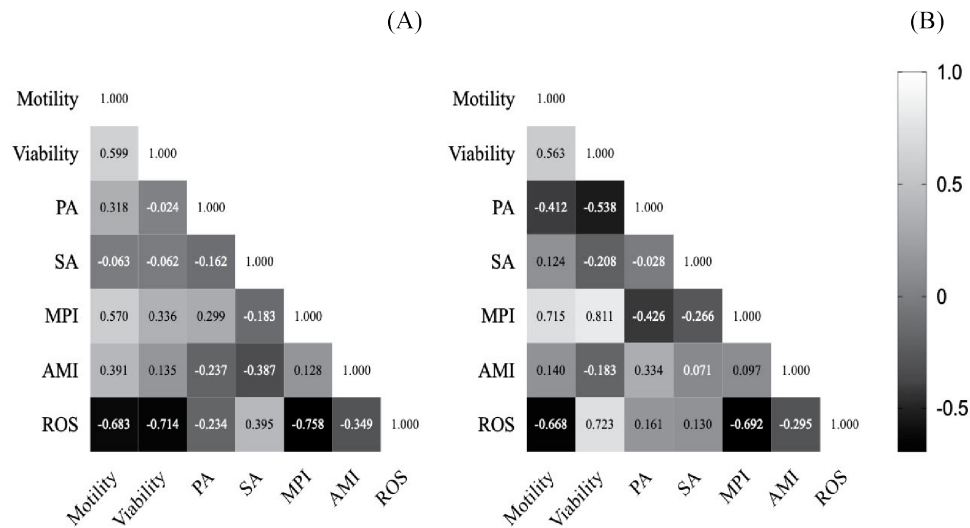


Fig. 2 Heatmap of Pearson's correlation coefficients among post-thaw spermatozoa quality parameters after chilled storage (60 min) for: (A) Aceh bull sample; (B) Bali bull sample. PA = primary abnormality; SA = secondary abnormality; PMI = plasma membrane integrity; AMI = acrosome membrane integrity; and ROS = reactive oxygen species.

function to allow progressive motility (Maulana et al., 2024), while acrosome integrity facilitates oocyte penetration and governs spermatozoa viability (Khan et al., 2021). Therefore, the heatmap provides mechanistic support for the finding that 5 ppm Se supplementation produced the most consistent improvement in post-thaw sperm quality by suppressing ROS accumulation. By protecting the membranes, Se maintained the linkage between viability and motility captured in the correlation structure. Similar antioxidant-based improvements in post-thaw motility and membrane integrity following selenium-related supplementation have been reported in other cryopreservation studies, further supporting this interpretation (Salimi et al., 2024; Erlindra et al., 2026).

Study limitations and future perspectives

Acknowledging certain methodological constraints in the present study is important to ensure a comprehensive interpretation of the findings. The use of conventional staining was aligned with standard laboratory practices for the assessment of acrosome integrity, although it carried inherent limitations such as susceptibility to interobserver variability and lower resolution compared with fluorescence-based or flow cytometric techniques (Kumar et al., 2019). Additionally, while the use of three independent batches from a single bull per breed ensured high measurement consistency, this design focused on individual responses rather than broad population variability. Therefore, the findings should be interpreted as preliminary

observations specific to the individual Aceh and Bali bulls included in this study rather than as being representative of the broader biological response of these breeds. Further studies are required involving a larger number of bulls per breed to confirm the consistency of the observed effects and support broader breed-level interpretations.

Individual differences in genetic background and physiological status are well documented in livestock reproduction (Lopes et al., 2021), reflecting that different bulls may have variable responses to Se supplementation. Although breed-specific analysis and standardized conditions partially minimized this variation, primarily, the present results offer a precise baseline for treatment-associated responses. Further validation in larger, multi-animal trials is warranted to extend these biological inferences to a wider population. Furthermore, as fertility validation through *in vivo* insemination trials or IVF assays was beyond the scope of the present study, it remains unclear whether the observed improvements in acrosomal integrity confer measurable gains in fertilizing capacity.

Despite these limitations, post-thaw Se supplementation could serve as a complementary strategy to conventional antioxidant incorporation. Most current approaches focus on adding antioxidants directly into the extender before freezing to protect sperm during cryoinjury (Shahandeh et al., 2022). Oxidative damage may continue or even intensify after thawing, particularly during handling and temporary storage before AI or IVF (Anand et al., 2024). In this context, post-thaw supplementation provides an additional protective window by

mitigating residual OS that is not fully addressed during the freezing process. This approach may be especially beneficial under field conditions, where strict control over handling time and temperature is often limited. Nevertheless, improvements in motility, viability, membrane integrity and oxidative status remain laboratory-based indicators of sperm quality and should not be directly extrapolated to enhance fertility, AI success, IVF performance, or reproductive outcomes. Therefore, further studies are required involving direct reproductive outcome assessments to confirm the practical relevance of this approach.

Conclusion

Se supplementation was effective at preserving post-thaw semen quality in Aceh and Bali bull samples by enhancing motility, viability, PMI and AMI, while mitigating ROS levels and maintaining abnormalities below 20%. Although correlations between quality parameters varied by breed, the consistent negative correlation between ROS and nearly all parameters confirmed that OS is a universal constraint. The 5 ppm concentration was identified as the optimal dose for improving post-thaw spermatozoa quality and suppressing storage-induced ROS accumulation. These findings suggest that Se is a promising antioxidant additive for semen extenders to optimize the preservation of frozen-thawed sperm quality in indigenous breeds. However, the observed improvements remain limited to laboratory assessments of sperm quality and should not be directly interpreted as evidence of fertility outcomes. Further studies are required involving direct reproductive outcome evaluations to confirm the practical reproductive relevance of Se supplementation.

Conflict of Interest

All authors declare no known conflicts of interest associated with this article.

Acknowledgements

The Ministry of Higher Education, Science and Technology of the Republic of Indonesia provided funding support through the *Pendidikan Magister menuju Doktor untuk Sarjana Unggul* (PMDSU) Program (Grant Nos. 048/E5/PG.02.00.PL/2024; 2813/UN1/DITLIT/PT.01.03/2024). The Faculty of Animal

Science, Universitas Gadjah Mada, Yogyakarta, Indonesia provided access to laboratory facilities during this study.

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