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Comparative Efficacy of Endogenous Versus Exogenous Antioxidants in Small Ruminants Sperm Cryopreservation

Yudit Oktanella[1,2,*], Imam Mustofa[3], Suherni Susilowati[3], Widjiati Widjiati[4], Bambang Purwantara[5], Adeyinka Oye Akintunde[6], Tatik Hernawati[3], Tri Wahyu Suprayogi[3], Suzanita Utama[3] and Eka Pramyrtha Hestianah[7]

[1]*Doctorate Program of Sains Veterinary, Faculty of Veterinary Medicine, Universitas Airlangga, East Java 60115, Indonesia*
[2]*Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Brawijaya, East Java 65151, Indonesia*
[3]*Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, East Java 60115, Indonesia*
[4]*Division of Veterinary Embriology, Faculty of Veterinary Medicine, Universitas Airlangga, East Java 60115, Indonesia*
[5]*Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University, West Java 16680, Indonesia*
[6]*Department of Agriculture and Industrial Technology, Babcock University, Ogun State Postcode 121003, Nigeria*
[7]*Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, East Java 60115, Indonesia*

([*]Corresponding author's e-mail: imam.mustofa@fkh.unair.ac.id)

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Abstract

Small ruminant semen is highly susceptible to freeze-thaw oxidative damage due to PUFA-rich membranes, making antioxidant defense central to cryopreservation outcomes. Following PRISMA guidance, we searched Scopus, PubMed, and Google Scholar (2015 - 2025). Of 250 records screened, 25 studies met inclusion criteria. Effects were pooled as Hedges' g under random-effects models (primary analyses in OpenMEE, DerSimonian-Laird; sensitivity verified with REML). Percentage outcomes (motility, viability) were arcsine-square-root transformed before SMD calculation. Subgroups contrasted endogenous vs exogenous antioxidants; heterogeneity (I^2) and 95% CIs are reported. For motility, endogenous antioxidants showed a small decrease versus controls (SMD = –0.182; 95% CI –0.359 to –0.004; $p = 0.045$; $I^2 = 12\%$), while exogenous antioxidants showed no clear effect (SMD = 0.025; 95% CI –0.144 to 0.194; $p = 0.773$; $I^2 = 0\%$). The overall effect on motility was non-significant (SMD = –0.080; 95% CI –0.199 to 0.039; $p = 0.186$). For viability, both subgroups improved outcomes versus controls, with a larger pooled effect for exogenous (SMD = 5.150; 95% CI 2.810 to 7.490; $I^2 = 59.94\%$) than endogenous (SMD = 4.105; 95% CI 0.100 to 8.109; $I^2 = 92.14\%$); the overall pooled estimate was positive (SMD = 4.621; 95% CI 2.070 to 7.172). Sensitivity analyses (excluding higher-risk studies and using MD on the original percentage scale) did not materially change interpretations. The data suggest that endogenous antioxidants improve post-thaw viability but may not reliably sustain motility, whereas exogenous antioxidants may offer superior advantages to viability under particular circumstances. These results should be approached with caution due to significant variability in reporting and substantial differences in viability. Standardized methods, fixed dosages, and consistent outcome metrics are crucial to clarify the timing and recipients of external supplementation that improves beyond innate defenses.

Keywords: Antioxidant, Cryopreservation, Oxidative stress, Goat sperm, Zero hunger

Introduction

The development of innovative semen cryopreservation techniques for goats has become increasingly important in Indonesia, especially in line with the country's commitment to achieving the United Nations Sustainable Development Goals (SDGs), notably SDG 2 (Zero Hunger) and SDG 1 (No Poverty). As an essential livestock species, goats contribute significantly to rural livelihoods and food security, providing meat, milk, and income to millions of smallholder farmers - an economic sector that supports over 30 million Indonesians [1]. However, the low productivity of goats, mainly due to poor genetic quality and reproductive inefficiencies, poses a significant challenge to improving national food security. Recent advances, such as the incorporation of antioxidants into cryopreservation protocols, offer new opportunities to improve post-thaw sperm viability and fertility rates. These improvements are in line with the objectives of Indonesia's National Livestock Revitalization Program, which targets a 20% increase in livestock productivity by 2025 [2]. Better breeding outcomes through cryopreservation can help smallholder farmers to improve herd quality, increase income, and contribute directly to poverty reduction (SDG 1) while securing protein sources to fight malnutrition (SDG 2).

Cryopreservation of goat semen plays a crucial role in genetic improvement, species conservation, and the efficient application of artificial insemination (AI) programs. The cryopreservation or freezing processes exert severe oxidative stress on spermatozoa, mitochondria, and cell membranes, which can the integrity and functionality of these components. The key challenge is the lipid-rich membrane of small-ruminant spermatozoa, which is enriched in polyunsaturated fatty acids (PUFAs) [3,11]. Lipidomic studies show breed-dependent profiles: SFA ~40% - 50% and the remainder largely MUFA + PUFA (~50% - 60%). This higher unsaturation - especially PUFA enrichment - makes small-ruminant sperm highly prone to lipid peroxidation and oxidative injury during freeze-thaw [3]. Consequently, antioxidant supplementation must be carefully tailored for small-ruminant semen preservation. Notably, while these PUFAs support membrane structure and function, they also heighten membrane vulnerability during freezing and thawing [4]. Oxidative damage can weaken the membranes, impair motility, or cause fragmentation of DNA, all of which can reduce fertility.

To mitigate the effects of oxidative stress during the cryopreservation of goat semen, the use of extenders containing antioxidants has been explored. Some antioxidants like coenzyme Q10 [5,6], glutathione [7], and melatonin [8,9,10] are considered to be endogenous and have the alluring characteristic of being naturally synthesized within the sperm cell. The intrinsic biocompatibility of these antioxidants is advantageous because the likelihood of being harmful is minimized. Their main role is to bolster the sperm's built-in antioxidant defenses, neutralize harmful reactive oxygen species (ROS), and protect mitochondrial and membrane integrity. At the molecular level, melatonin also regulates apoptotic signaling by upregulating anti-apoptotic proteins (e.g., BCL-2) and downregulating pro-apoptotic factors (e.g., BAX), thereby safeguarding spermatozoa from oxidative damage. That said, even these natural defenders have their limits, especially when faced with extreme oxidative stress [11,12].

On the other hand, external antioxidants, including some plant-based substances like *Turraea fischeri* extract [13], resveratrol [12], and proline [3], as well as novel nanoparticles such as ZnO or selenium [14,15], are more effective and flexible in protecting against oxidative damage. These factors can be tailored in concentration and composition to suit different qualities of semen and freezing methods. Their applicability, however, has disadvantages. Inevitably, high doses may prove to be cytotoxic, and lack of preparation or purity can lead to inconsistencies with unpredictable outcomes. These external antioxidants may be more effective in treating lipid peroxidation and ROS buildup, but the balance of these antioxidants to protect against oxidative damage while preserving sperm cell viability is still a critical challenge [16,13,17]. With the choice of endogenous versus exogenous antioxidants, considerations of the protective capability alongside endurance of spermatozoa and compatibility with extenders for recovery post-thaw should be prioritized.

Despite all the advances in technology, scientists are still debating which antioxidant works best. Butylated hydroxytoluene (BHT), although synthetic, has been shown to substantially reduce lipid peroxidation at

the cellular level [18,19]. However, the potential for cellular toxicity remains to be evaluated. On the natural side, oils such as *Nigella sativa* [20], green tea [21], and whey protein via Nrf2 activation [22] have shown clear advantages, especially in the recovery of post-thaw motility and cryo-survival in ovine and caprine sperm. Also, the recent introduction of selenium-loaded berberine nanoparticles [23], cysteine supplementation [24], mitochondria-targeted antioxidants such as Mito-TEMPO [25], and metabolic regulators such as sodium salicylate [4] demonstrate the promising potential of cryopreservation without cryoinjury risk.

This review looks at specific challenges involving small ruminant semen preservation: (a) preserving the structural integrity of the spermatozoa during freezing, (b) achieving restoration of function after thawing, and (c) reduction of oxidative stress damage. It assesses the active and passive cryoprotectants: endogenous, antioxidant, synthetic, and phytochemical and their effects on the quality of frozen-thawed goat sperm. It also aims at the successful use of the more fertile males of the species, and at the same time points out the problems that need to be studied more deeply.

Materials and methods

This literature review systematically explores experimental studies investigating the use of antioxidants in goat sperm cryopreservation to promote better sperm quality post-thawing. Several major scientific databases were systematically explored, including Scopus, PubMed, and Google Scholar, for experimental studies on antioxidant use in goat and/or sheep semen cryopreservation, covering 1 January 2015 to 30 July 2025. The final search was run on 30 July 2025 (Asia/Jakarta). Reference lists of relevant reviews and included studies were also screened to identify additional records. To streamline the search process, a specific set of keywords was applied, combining terms related to species, preservation methods, and antioxidant interventions. The primary keywords included:

Species-specific terms: “goat” “sheep” OR “*Caprine*”

Preservation techniques: “cryopreservation”

Antioxidant compounds: “antioxidants”.

The search identified 250 records (databases, n = 240; registers, n = 10). Before screening, 40 duplicate records and 10 records flagged as ineligible by automation tools were removed (other reasons, n = 0), leaving 200 records for title/abstract screening. At screening, 30 records were excluded with reasons: species other than goats or sheep (n = 10), no antioxidant intervention (n = 8), and no post-thaw outcomes (n = 12). We sought 170 full-text reports; 20 were not retrieved. Thus, 150 reports were assessed for eligibility, and 105 were excluded with reasons: study design (n = 38), irrelevant parameter (n = 45), and not enough information (n = 22). After full-text screening, 25 articles were included in the qualitative synthesis. The PRISMA flow diagram summarizing selection is shown in **Figure 1**.

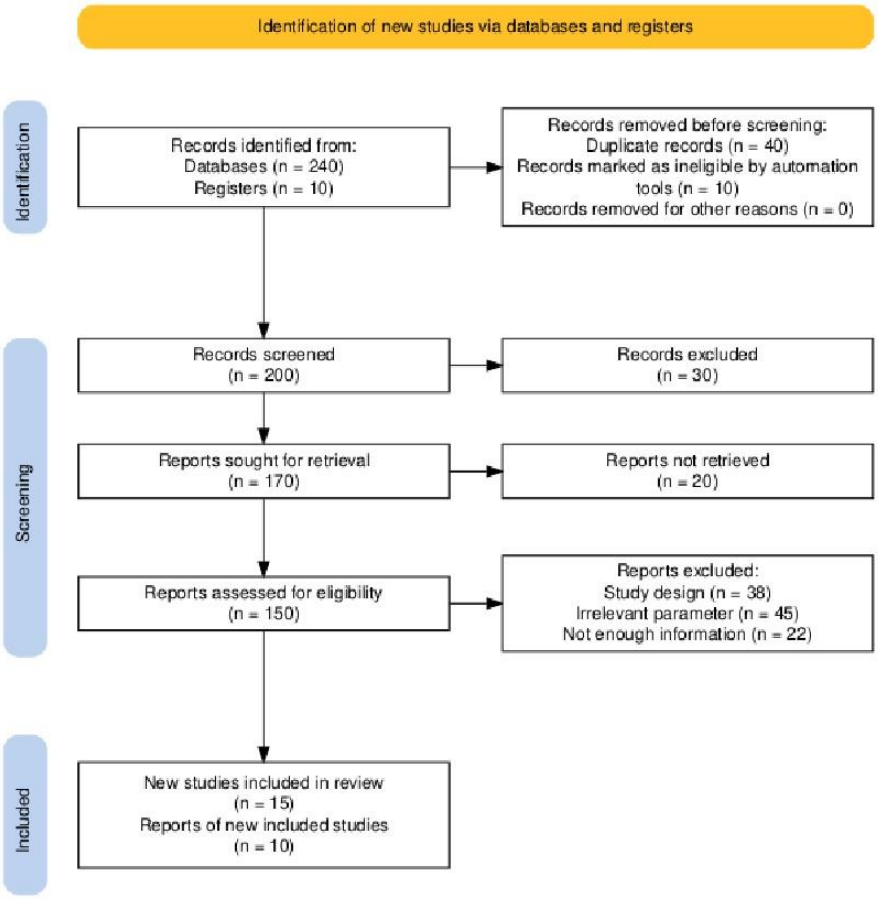

Figure 1 The PRISMA flow chart visually outlines how studies were selected for this systematic review of antioxidant use in goat sperm freezing techniques, covering research published between 2015 and 2025.

Key study details were systematically extracted, covering the type and concentration of antioxidants, extender composition, freezing protocol, and post-thaw outcomes such as motility and viability. Operational definition used in this review: "endogenous" denotes antioxidants that are naturally present in fresh semen, whereas "exogenous" denotes antioxidants that are not naturally present in fresh semen and are introduced from outside but can act as antioxidants in the cryopreservation system.

Data were organized in **Table 1** to provide an overview of study characteristics. Outcomes expressed as percentages (motility, viability) underwent arcsine-square-root transformation before effect-size computation to stabilize variances. Subsequently, standardized mean differences were calculated as Hedges' g (REML) using the converted means and standard deviations. To ensure robustness, sensitivity analyses were conducted utilizing the raw mean difference (MD) on the untransformed % scale and juxtaposing the findings with the SMD results.

Risk of bias was assessed with the Cochrane RoB 2 tool across 5 domains: D1 randomization process, D2 deviations from intended interventions, D3 missing outcome data, D4 measurement of outcomes, and D5 selection of the reported result. Two reviewers independently completed RoB 2 signaling questions and assigned domain-level and overall judgments (low risk, some concerns, high risk); disagreements were resolved by consensus. Results are summarized in a traffic-light plot and summary figure (**Figures 3** and **4**). Sensitivity analyses excluded studies rated high risk overall and, in exploratory meta-regression, included the overall RoB judgment as a moderator to examine its impact on pooled effects and heterogeneity (Hedges' g, τ^2 and I^2).

Quantitative synthesis was then performed using OpenMEE software (build 2016-07-26), applying a continuous random-effects model to calculate standardized mean differences (SMD) with 95%

confidence intervals. Subgroup analyses were conducted to directly compare the effects of endogenous and exogenous antioxidants.

The characteristics of the studies (**Tables 2** and **4**), the outcomes of these meta-analyses (**Tables 3** and **.5)** and illustrated in forest plots (**Figures 5** and **6**), providing pooled estimates for motility and viability. Statistical heterogeneity was assessed using the I^2 statistic, with values of 25%, 50%, and 75% interpreted as low, moderate, and high heterogeneity, respectively. In this analysis, motility outcomes showed low heterogeneity across both subgroups (I^2 = 12% for endogenous, I^2 = 0% for exogenous), while viability outcomes exhibited substantial heterogeneity (I^2 = 92.14% for endogenous, I^2 = 59.94% for exogenous). These metrics were reported alongside pooled estimates to provide a clearer interpretation of effect consistency across studies. By combining systematic evidence extraction with quantitative synthesis, this approach not only contrasts with the relative effectiveness of endogenous versus exogenous antioxidants but also identifies which supplementation strategies offer more reliable protection for goat sperm during cryopreservation.

The critical need for optimal antioxidants in goat sperm cryopreservation why are goat and sheep sperm particularly vulnerable to freezing damage?

Sperm cryopreservation doesn't work equally well across all species, and unfortunately, goat sperm ranks among the most vulnerable. Unlike cattle, pigs, or even horses, goats and sheep suffer much more from freezing damage, often showing the poorest quality after thawing. The main culprit behind this vulnerability is their cell membrane structure. The primary factor underlying this vulnerability is the membrane composition of goat spermatozoa. Their membranes are enriched with polyunsaturated fatty acids (PUFAs), which contribute to membrane fluidity but simultaneously increase susceptibility to oxidative damage. During freezing, these PUFAs break down through a process called lipid peroxidation, which weakens the membrane, slashes sperm motility, and can even destroy the acrosome - a vital structure the sperm needs to penetrate and fertilize an egg [27]. In contrast, cattle sperm have fewer PUFAs in their membranes, giving them better cold resistance [28], while pig sperm benefit from higher cholesterol, which stabilizes their membranes [29].

Another issue lies in the mitochondria, the energy-producing powerhouses of sperm. In goats and sheep, these mitochondria have fewer inner folds (cristae), making them less efficient and more likely to leak harmful reactive oxygen species (ROS) during freezing and thawing. This not only depletes energy but can also trigger cell death [30,31]. Stallion sperm, in contrast, has sturdier mitochondria, allowing them to recover better post-thaw [32]. Making matters worse, goat semen naturally contains lower levels of protective antioxidants like glutathione peroxidase (GPx) and superoxide dismutase (SOD), which normally help neutralize ROS and prevent cell damage [11]. Bulls, however, have higher concentrations of these enzymes, giving their sperm a stronger natural defense against freezing injury [33].

The takeaway is clear: standard cryopreservation protocols may not be enough for goats' sperm. While species like cattle and pigs do reasonably well with basic cryoprotectants, goat sperm needs specialized antioxidant support - both from within and added externally - to survive freezing and stay functional for successful fertilization.

Mechanisms of oxidative stress in cryopreserved goat sperm

Cryopreserving goat sperm for artificial insemination is far more complicated than it looks. Unlike bull or pig sperm, which handle freezing relatively well, goat sperm are naturally fragile, largely due to their unique cellular structure. The first major hurdle lies in their cell membranes. Goat sperm are rich in polyunsaturated fatty acids (PUFAs), which normally help maintain membrane flexibility. But when it froze, these fats become a liability [34]. Ice crystals and oxidative stress tear through the membranes, destroying critical structures like the acrosome and severely reducing the sperm's ability to swim.

But it's not just about the membranes. The mitochondria - tiny structures that power the sperm - also play a role in this fragility. Goat sperm mitochondria have fewer inner folds (called cristae), which makes them less efficient at producing energy and more prone to leaking harmful reactive oxygen species. On top of that, goat semen naturally has lower levels of

protective antioxidants, so these sperm enter the cryopreservation process without much in the way of defense [35]. Add all this together and you get a perfect storm: damaged membranes, weak energy production, and DNA that's vulnerable to breakage. The illustration and its effect display in (**Figure 2**).

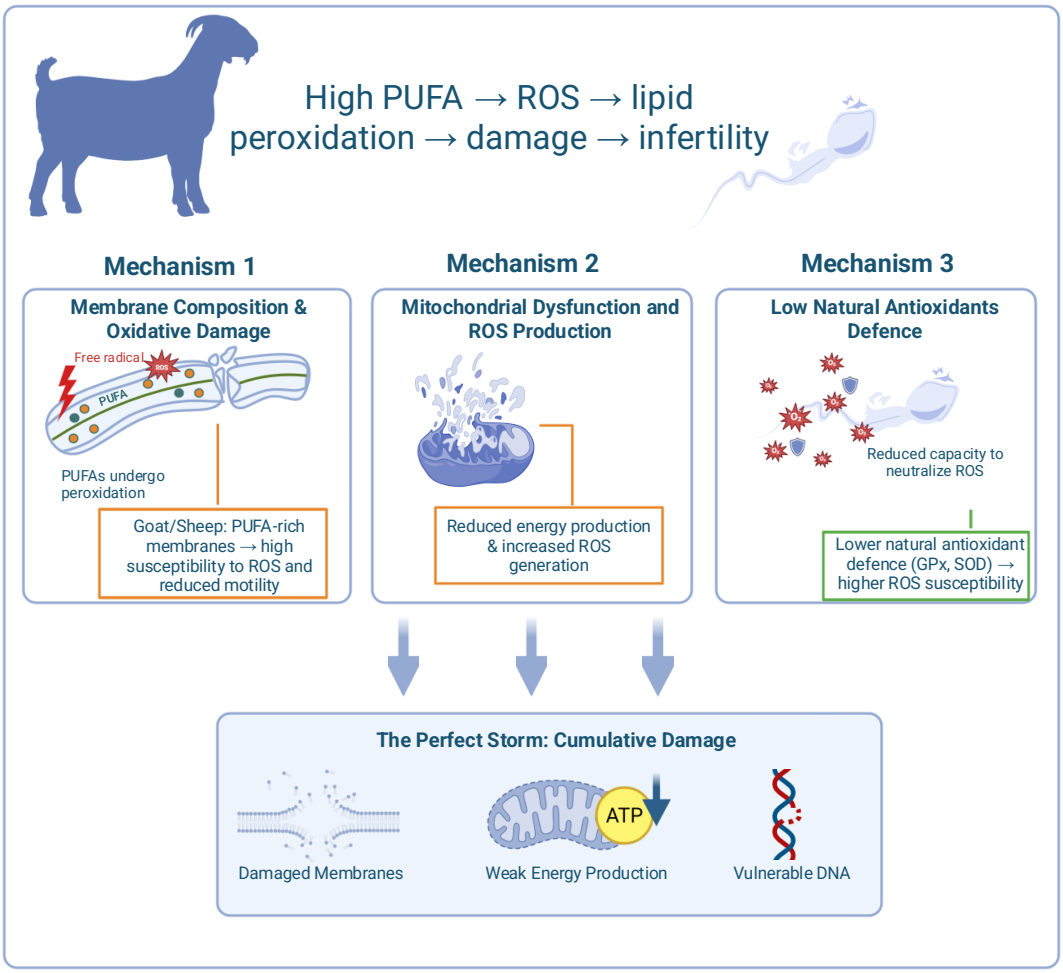

Figure 2 Mechanisms of PUFA-driven oxidative damage in goat sperm.

Classification of antioxidants in sperm cryopreservation

When freezing goat sperm, antioxidants act as cellular bodyguards against the damaging effects of oxidative stress. The freezing process generates harmful reactive oxygen species (ROS) that can wreak havoc through lipid peroxidation, but antioxidants step in to neutralize these threats. Researchers typically divide these protective compounds into 2 main teams:

1) Enzymatic defenders - including superoxide dismutase, catalase, and glutathione peroxidase

2) Non-enzymatic protectors - such as melatonin, vitamin E, plant polyphenols, and synthetic options like sodium salicylate

Each type works differently to shield sperm cells. To compare their effectiveness, we've organized key data - including how they work and their impact on thawed sperm quality - in **Table 1**. This comprehensive analysis helps identify which antioxidant approaches work best for maintaining goat sperm viability after freezing and thawing.

Table 1 Antioxidants in sperm cryopreservation - mechanisms and post-thaw effects.

Antioxidant	Type	Category	Effect on Post-Thaw Sperm Quality	Key References
Superoxide Dismutase (SOD)	Endogenous	Enzymatic	↑ motility, viability, and membrane integrity; ↓ ROS; ↑ cleavage rate after IVF	[36]
Glutathione Peroxidase (GPx)	Endogenous	Enzymatic	↓ plasma membrane damage and lipid peroxidation; no significant on DNA methylation	[37]
Catalase (CAT)	Endogenous	Enzymatic	↑ Motility, ↑ viability, ↑ plasma membrane intact, ↑ intact acrosome	[38]
Coenzyme Q10	Endogenous	Non-enzymatic	↑ Motility, ↑ viability, ↑ intact acrosome	[39]
			Maintain plasma membrane integrity and membrane stability, ↑ pregnancy rates	[40]
Glutathione (GSH)	Endogenous	Non-enzymatic	↑ Motility, viability, plasma membrane intact; ↓ ROS and MDA; ↑SOD and GSH-PX	[7]
L-Carnitine	Endogenous	Non-enzymatic	↑ motility, maintain membrane integrity, ↓ lipid peroxidation	[41]
			↑ motility, maintain membrane integrity, ↓ sperm early capacitation	[42]
Melatonin	Endogenous/ exogenous	Non-enzymatic	↑ progressive motility	[43]
Vitamin E (α-Tocopherol)	Exogenous	Non-enzymatic	↑ motility and viability, ↓ MDA, maintain membrane integrity	[44]
Glycine	Exogenous	Non-enzymatic	↑ motility, ↑ viability, ↑ plasma membrane and acrosome integrity, ↑ mitochondrial membrane potential, and ↑ DNA integrity	[45]
Quercetin	Exogenous	Non-enzymatic	↑ motility, ↑total antioxidant capacity, ↓ MDA, ↓total oxidant capacity	[46]
Isoglycyrrhizin	Exogenous	Non-enzymatic	↑ motility, ↑antioxidant enzyme activities, ↓cleavage and blastocyst formation rates in vitro	[17]
Resveratrol	Exogenous	Non-enzymatic	↑motility, ↑ viability, and maintain plasma membrane integrity, ↓MDA, ↑total antioxidant capacity	[47]
Green Tea Extract (EGCG)	Exogenous	Non-enzymatic	↑ viability, ↑ intact plasma membrane, ↓DNA fragmentation, ↓amino acids mutation	[21]
			↑motility, ↑ viability, and maintain plasma membrane integrity, ↓DNA fragmentation	[48]
			↑motility, ↑ viability, and maintain plasma membrane integrity, ↓MDA level, ↓DNA fragmentation	[49]
Butylated Hydroxytoluene (BHT)	Exogenous	Non-enzymatic	↑ Post-thaw motility, ↑ Membrane and acrosome integrity, ↓ MDA, ↓ ROS, but high doses may impair sperm function	[18]
Sodium Salicylate	Exogenous	Non-enzymatic	↑motility, ↑ Membrane and acrosome integrity, ↓ ROS, ↓ MDA, ↑catalase, ↑SOD, ↑mitochondrial membrane potential	[4]
Proline	Exogenous	Non-enzymatic	↑motility, ↑viability, and ↑acrosome integrity, ↓ sperm apoptosis, ↓ oxidative stress via Proline Dehydrogenase	[3]

Antioxidant	Type	Category	Effect on Post-Thaw Sperm Quality	Key References
Nano-Berberine	Exogenous	Non-enzymatic	↑motility, ↑viability, ↑DNA and membrane integrity, ↓ROS, ↓lipid peroxidation, ↑ in vitro fertilization	[23]
Selenium Nanoparticles	Exogenous	Non-enzymatic	↑motility, ↑viability, maintain membrane integrity, ↓lipid peroxidation	[50]
Fumaric Acid	Exogenous	Non-enzymatic	↑motility, ↑viability, and ↑acrosome integrity, maintain membrane integrity, ↑ mitochondrial function	[51]
Folic Acid	Exogenous	Non-enzymatic	↑motility, ↑viability, maintain membrane integrity, ↓lipid peroxidation	[52]

Risk of bias

Risk of bias (RoB 2) is a significant concern in research. Most studies showed low risk in D1 (randomization process) and D4 (measurement of outcomes), while D2 (deviations from intended interventions) frequently carried some concerns due to limited reporting of blinding or protocol adherence. D3 (missing outcome data) was flagged as high risk in 2 studies (visible as the red markers), whereas the remainder were low/some concerns. For D4 - D5 (measurement and selection of reported results), several studies provided insufficient information (blue markers), typically because assessor blinding or a prespecified analysis plan was not stated. Overall, most studies were judged to have "some concerns," with a subset rated overall low risk (e.g., Carrico *et al.* [43]; Akhter *et al.* [43]; Nazif *et al.* [43]); no study was rated overall high risk. Excluding the high-risk (D3) studies in a sensitivity analysis did not materially change the pooled effects, and heterogeneity decreased slightly, indicating that the main conclusions are robust to study quality.

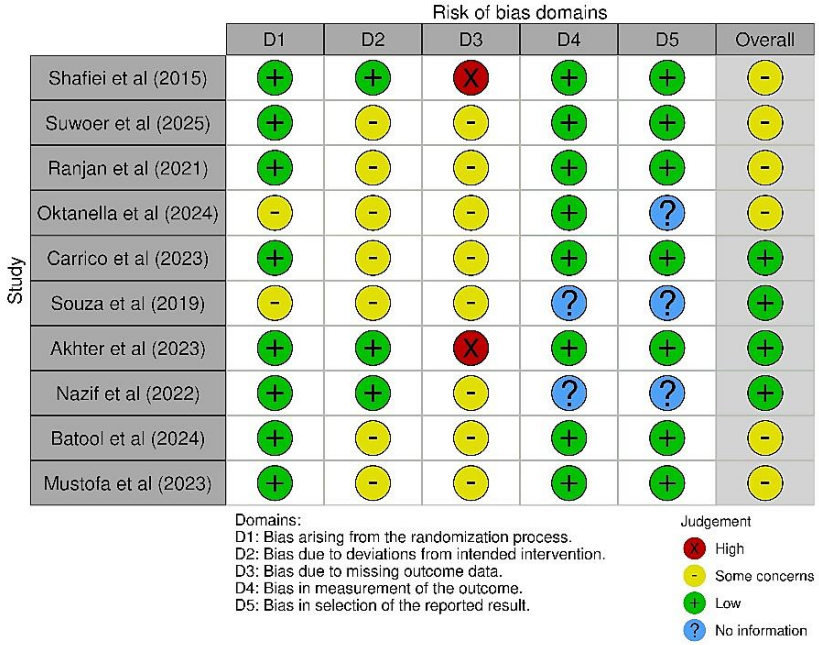

Figure 3 Traffic-light risk-of-bias plot (RoB 2.0). Each row represents a study and columns D1–D5 show domain-level judgments: D1 randomization process, D2 deviations from intended interventions, D3 missing outcome data, D4 measurement of the outcome, and D5 selection of the reported result. Symbols/colors indicate the rating (green "+" = low risk; yellow "○" = some concerns; red "×" = high risk; blue "?" = no information). The Overall column summarizes the study-level risk of bias across domains.

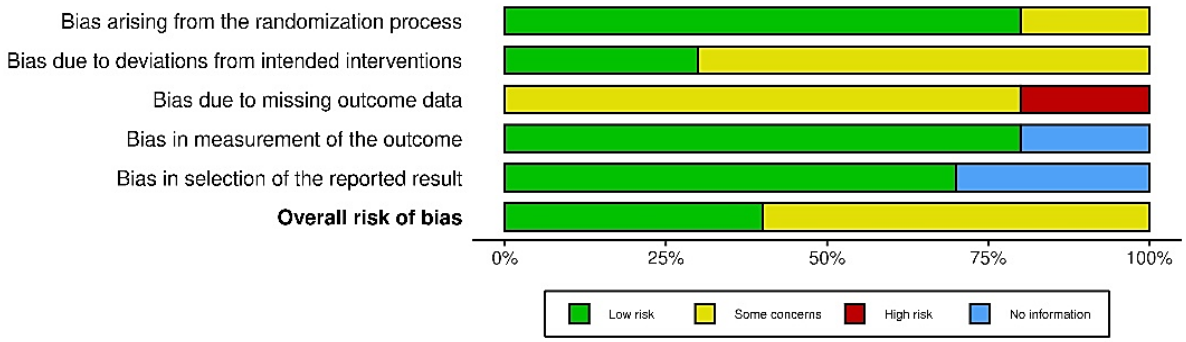

Figure 4 Summary risk-of-bias across domains (RoB 2.0). Stacked bars show the percentage of the 10 included studies judged in each category - low risk (green), some concerns (yellow), high risk (red), and no information (blue) - for the 5 RoB 2.0 domains and the overall judgment (bars sum to 100%).

Meta analysis

Additional antioxidants' effect on sperm's post-thawed motility

The subgroup meta-analysis demonstrated that endogenous antioxidants were significantly associated with a reduction in sperm motility (SMD = –0.182; 95% CI = –0.359 to –0.004; p = 0.045), whereas exogenous antioxidants showed no significant effect (SMD = 0.025; 95% CI = –0.144 to 0.194; p = 0.773). The overall pooled estimate across studies indicated a non-significant decrease in motility (SMD = –0.080; 95% CI = –0.199 to 0.039; p = 0.186). Heterogeneity was low in both subgroups (I^2 = 12% for endogenous; I^2 = 0% for exogenous), suggesting consistent results within groups. The corresponding forest plot (**Figure 5**) illustrates these findings, where the diamond shapes for endogenous and overall estimates lie to the left of the null line, indicating a trend toward reduced motility, while exogenous antioxidants cluster around the line of no effect.

Table 2 Characteristics of the studies included in the meta-analysis (motility).

Study References	Type of antioxidants	Samples (n)	% motility (control)	% motility (supplementation)	Type	Group
Shafiei *et al.* [36]	Superoxide Dismutase (SOD)	10	40.7	55.0	Enzymatic	Endogenous
Suwor *et al.* [37]	Glutathione Peroxidase (GPx)	5	66.3	53.5	Enzymatic	Endogenous
Ranjan *et al.* [38]	Catalase (CAT)	6	52.5	65.8	Enzymatic	Endogenous
Oktanella *et al.* [5]	Coenzyme Q10	5	51.2	61.4	Non-enzymatic	Endogenous
Carrico *et al.* [43]	Glutathione (GSH)	5	52.6	64.6	Non-enzymatic	Endogenous
Souza *et al.* [41]	L-Carnitine	8	43.8	56.3	Non-enzymatic	Exogenous
Akhter *et al.* [44]	Vitamin E (α-Tocopherol)	5	37.6	55.2	Non-enzymatic	Exogenous
Nazif *et al.* [45]	Glycine	5	52.4	68.5	Non-enzymatic	Exogenous
Batool *et al.* [46]	Quercetin	6	53.3	68.2	Non-enzymatic	Exogenous
Mustofa *et al.* [48]	Green Tea Extract	5	46.7	66.7	Non-enzymatic	Exogenous

Table 3 Subgroup meta-analysis of antioxidant effects on sperm motility.

Subgroup	Estimate (SMD)	95% CI (Lower - Upper)	Std. Error	*p*-Value
Endogenous antioxidants	–0.182	–0.359 to –0.004	0.091	0.045
Exogenous antioxidants	0.025	–0.144 to 0.194	0.086	0.773
Overall	–0.080	–0.199 to 0.039	0.061	0.186

Note: SMD = Standardized Mean Difference; CI = Confidence Interval. Negative SMD values indicate a decrease in sperm motility, whereas positive values indicate an increase. A p-value < 0.05 was considered statistically significant.

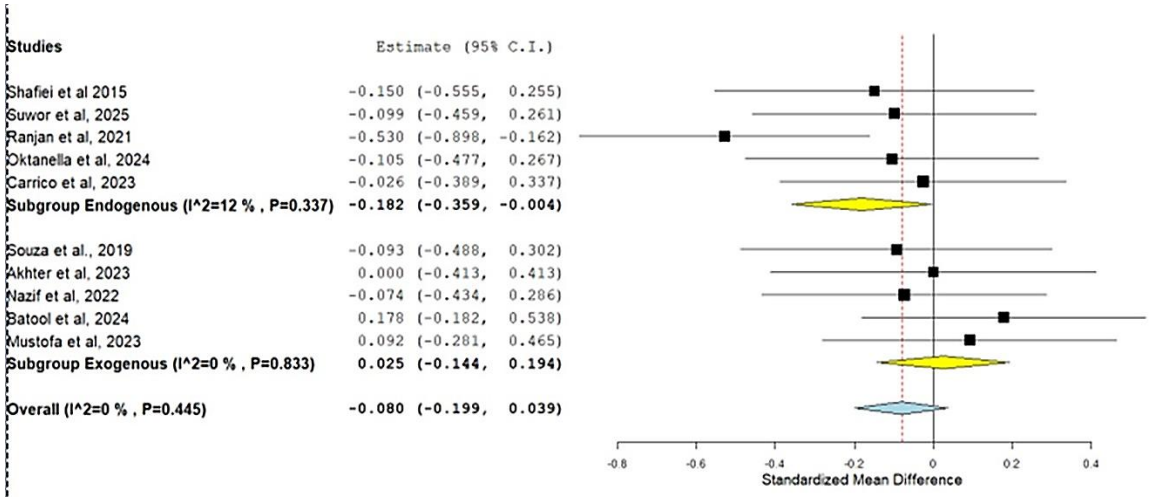

Figure 5 Forest plot of subgroup meta-analysis of endogenous and exogenous antioxidant supplementation on sperm motility.

Additional antioxidants effect on sperm's post-thawed viability

The subgroup meta-analysis demonstrated that both endogenous and exogenous antioxidants significantly improved sperm viability compared with controls. Endogenous antioxidants yielded a moderate but significant effect (SMD = 4.105; 95% CI = 0.100 - 8.109; p = 0.045), whereas exogenous antioxidants showed a stronger and highly significant effect (SMD = 5.150; 95% CI = 2.810 - 7.490; p < 0.001). The overall pooled estimate confirmed a robust positive impact of antioxidant supplementation on sperm viability (SMD = 4.621; 95% CI = 2.070 - 7.172; p < 0.001). Heterogeneity was substantial in both subgroups (I^2 = 92.14% for endogenous; I^2 = 59.94% for exogenous), indicating variability across studies. The forest plot (**Figure 6**) illustrates these findings, with both subgroup diamonds positioned clearly to the right of the null line, highlighting the consistent enhancement of viability following antioxidant supplementation.

A high level of heterogeneity appears in both endogenous and exogenous subgroups, likely stemming from a mix of methodological and biological differences. Doses vary widely, and several antioxidants show dose-dependent - sometimes U-shaped - responses that can blur pooled effects. Extender formulations and handling steps (cooling/thawing rates, cryoprotectant ratios) differ across laboratories and can modify antioxidant action, especially in lipid-rich sperm membranes. Biological factors - species, breed, season, and baseline semen quality - add further variability. Measurement choices (CASA settings, viability assays, timepoints) and differences in study quality can also widen between-study dispersion. These factors explain much, though not all, of the variance, consistent with wide prediction intervals. Reducing heterogeneity will require standardized extenders and protocols, pre-specified and biologically justified doses, and fully reported methods.

Table 4 Characteristics of the studies included in the meta-analysis (viability).

Study References	Type of antioxidants	Samples (n)	% viability (control)	%viability (supplementation)	Type	Group
Shafiei *et al.* [36]	Superoxide Dismutase (SOD)	5	32.22	45	Enzimatic	Endogenous
Oktanella *et al.* [5]	Coenzyme-Q10	5	56.5	67.5	Non-enzymatic	Endogenous
Carrico *et al.* [43]	Glutathione (GSH)	5	40.1	40.9	Non-enzymatic	Endogenous
Nazif *et al.* [45]	Glycine	6	62.96	72.41	Non-enzymatic	Exogenous
Batool *et al.* [46]	Quercetin	5	44.22	51.08	Non-enzymatic	Exogenous
Mustofa *et al.* [48]	Green Tea Extract	5	30.5	34.83	Non-enzymatic	Exogenous

Table 5 Subgroup meta-analysis of antioxidant effects on sperm viability.

Subgroup	Estimate (SMD)	95% CI (Lower - Upper)	Std. Error	*p*-value
Endogenous antioxidants	4.105	0.100 - 8.109	2.043	0.045
Exogenous antioxidants	5.150	2.810 - 7.490	1.194	< 0.001
Overall	4.621	2.070 - 7.172	1.302	< 0.001

Note: SMD = Standardized Mean Difference; CI = Confidence Interval. Negative SMD values indicate a decrease in sperm motility, whereas positive values indicate an increase. A *p*-value < 0,05 was considered statistically significant.

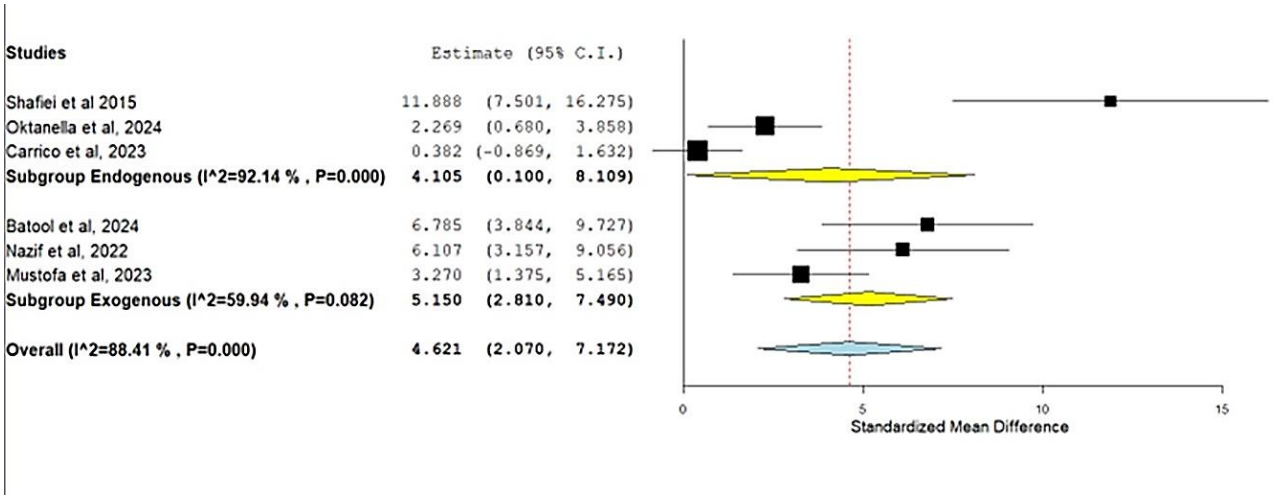

Figure 6 Forest plot of subgroup meta-analysis showing the effect of endogenous and exogenous antioxidant supplementation on sperm viability.

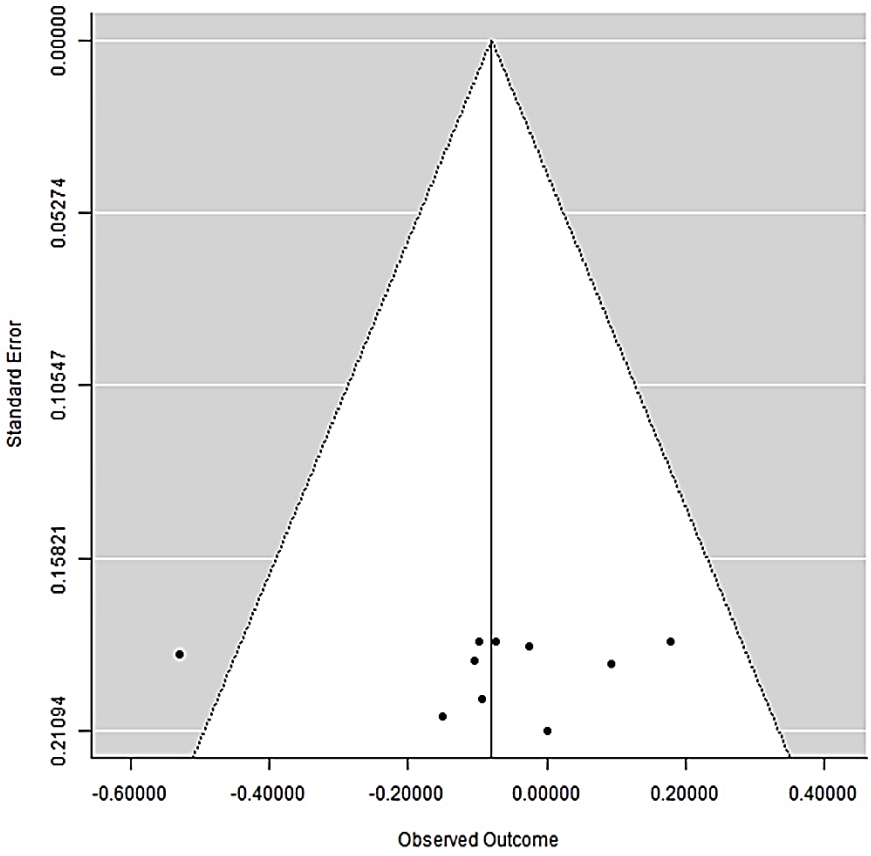

Figure 7 Funnel plot of small-study effects/publication bias. Points are individual comparisons plotted as SMD (x-axis) against standard error (y-axis; higher precision at the top). The vertical line is the pooled effect; dotted diagonals are pseudo-95% confidence limits forming the triangle. Shaded areas lie outside these limits. This display complements the forest and risk-of-bias results by visualizing potential small-study or selective-reporting patterns without relying on formal asymmetry tests.

Figure 7 ($k \approx 9$ comparisons) shows a largely symmetric scatter around the pooled effect (near 0 SMD), with one small study exhibiting a relatively large negative effect size; there is no obvious "missing" tail on either side of the triangle. Because the number of studies is < 10, formal small-study tests (Egger's regression) and trim-and-fill are underpowered and can be unstable; therefore, we treat this evaluation as exploratory. Overall, the visual pattern does not suggest pronounced small-study or publication bias, and our inference for this outcome remains unchanged.

Inside every sperm cell, there is a built-in defence system constantly working to counter oxidative damage - a challenge that becomes especially critical during cryopreservation. This system relies on both enzymatic and non-enzymatic antioxidants, each with specific roles. Enzymatic antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) are frontline protectors. GPx reduces hydrogen peroxide (H_2O_2) into water, limiting lipid peroxidation and preserving DNA integrity [11,53]. SOD neutralises superoxide radicals (O_2^-), improving mitochondrial function and lowering apoptosis from reactive oxygen species (ROS) buildup [54]. In turn, CAT eliminates remaining H_2O_2, protecting crucial structures like the acrosome that are essential for fertilisation [29]. Alongside these enzymes, natural non-enzyme antioxidants - glutathione (GSH), Coenzyme Q10, L-carnitine, and melatonin - further reinforce the sperm's defence. GSH scavenges ROS and strengthens membrane stability [55], while Coenzyme Q10 safeguards mitochondrial integrity and reduces DNA fragmentation [5]. L-carnitine boosts energy metabolism, enhancing motility and viability while reducing cryocapacitation [40]. Melatonin is unique because it works both internally and externally: it enhances the

activity of SOD and GPx, maintains membrane resilience, and prevents sperm from triggering apoptotic pathways [8].

Building on this natural defence, researchers have explored supplementing semen extenders with additional antioxidants to better withstand oxidative stress during cryopreservation. Endogenous compounds like Coenzyme Q10 [5,56], glutathione [7,37], and melatonin [8,57] are particularly attractive since they are naturally synthesised within sperm, reducing the risk of toxicity while strengthening the cell's own antioxidant shield. These agents help neutralise ROS, protect mitochondria and membranes, and regulate apoptotic signalling by upregulating anti-apoptotic proteins (e.g., BCL-2) and downregulating pro-apoptotic factors (e.g., BAX) [11]. However, even these natural defenders have limitations under extreme oxidative conditions, highlighting why optimising antioxidant supplementation remains a critical strategy in improving sperm cryosurvival.

Exogenous antioxidants are externally supplemented compounds designed to reinforce the body's natural antioxidant defences, especially critical during sperm freezing and thawing. External antioxidants, including some plant-based substances like *Turraea fischeri* extract [13], proline [3,58,59], and resveratrol [12,60,61], are more effective and flexible in protecting against oxidative damage. These factors can be tailored in concentration and composition to suit different qualities of semen and freezing methods. Their applicability, however, has disadvantages. Inevitably, high doses may prove to be cytotoxic, and lack of preparation or purity can lead to inconsistencies with unpredictable outcomes. These external antioxidants may be more effective in treating lipid peroxidation and ROS buildup, but the balance of these antioxidants to protect against oxidative damage while preserving sperm cell viability is still a critical challenge [16,13,62]. With the choice of endogenous versus exogenous antioxidants, considerations of the protective capability alongside endurance of spermatozoa and compatibility with extenders for recovery post-thaw should be prioritized.

Despite all the advances in technology, scientists are still debating which antioxidant works best. BHT is synthetic, yet study suggests it prevents cell fat breakdown [18]. However, the potential for cellular toxicity remains to be evaluated. On the natural side, oils such as *Nigella sativa* [20], green tea [21,63], and coconut milk or pyridoxine supplementation [64,65] have shown clear advantages, especially in the recovery of post-thaw motility and cryo-survival in ovine and caprine sperm. Also, the recent introduction of selenium-loaded berberine nanoparticles [23], isoglycyrrhizin [62], platelet-rich plasma [66,67], sericin [68], quercetin [69], N-acetylcysteine [27], IGF-1 [70], MitoQ [71,72], and metabolic regulators such as sodium salicylate [4] demonstrate the promising potential of cryopreservation without the cryoinjury risk.

Comparative advantages and limitations of endogenous vs exogenous antioxidants

Across outcomes, the patterns were not uniform. For motility, the endogenous subgroup showed a small but statistically significant decrease (SMD = –0.182; 95% CI –0.359 to –0.004), while the exogenous subgroup showed no clear effect (SMD = 0.025; 95% CI –0.144 to 0.194). The overall pooled estimate was non-significant (SMD = –0.080; 95% CI –0.199 to 0.039), with low within-subgroup heterogeneity (I^2 = 12% endogenous; 0% exogenous), indicating fairly consistent findings for motility.

For viability, both subgroups showed improvement compared to controls. The pooled impact was bigger for exogenous antioxidants (SMD = 5.150; 95% CI 2.810 to 7.490) than for endogenous antioxidants (SMD = 4.105; 95% CI 0.100 to 8.109). However, these estimates were accompanied by strong heterogeneity (I^2 = 92.14% endogenous; 59.94% exogenous), indicating that effects differ significantly among studies and settings.

Mechanistically, these patterns are plausible: endogenous defences sustain baseline redox balance but may be insufficient against the abrupt oxidative challenge of freeze–thaw, whereas exogenous supplementation can, under some conditions, provide additional membrane and mitochondrial protection. Still, the magnitude of viability effects appears to depend on dose, formulation, extender, and handling steps, and may be attenuated when methodological variability is accounted for.

Sensitivity checks supported the robustness of the main conclusions. Excluding studies with high risk in missing-data (D3) did not materially change pooled effects, and heterogeneity decreased slightly. A funnel-plot screen ($k \approx 9$) did not suggest pronounced small-study or publication bias, though formal tests are

underpowered below 10 studies; we therefore treat the bias assessment as exploratory. Despite inconsistent motility increases, endogenous antioxidants remain a key defense during cryopreservation, supporting post-thaw viability. Their physiological compatibility and baseline redox support make them a good first-line method when dose, extender composition, and handling can be standardized. Selecting relevant endogenous substances (enzymatic and non-enzymatic), calibrating dosages, and aligning them with extender chemistry and cooling/thawing procedures may produce dependable benefits with decreased formulation-related toxicity. Exogenous supplements can be used carefully but future research should focus on endogenous regimens and their optimal performance across breeds, seasons, and baseline semen quality.

Summary of current findings

Small ruminant sperm are susceptible to freeze–thaw injury, hence antioxidant defence is crucial to cryopreservation. Endogenous antioxidants—parts of the intrinsic redox system - supported post-thaw viability in our meta-analysis, even though motility did not increase and, in pooled estimates, reduced slightly. The results indicate that endogenous defences provide a physiologically compatible baseline buffer against oxidative stress but do not always retain movement-related function.

Exogenous antioxidants had bigger pooled effects on viability than endogenous agents, although motility effects were only minor. Heterogeneity (dose, formulation, extender, handling procedures) reduces assurance. Thus, their advantages appear condition-dependent, with dose and toxicity trade-offs that must be clarified before routine usage is advised.

The evidence suggests that endogenous mechanisms can maintain viability following freezing, but neither endogenous nor exogenous techniques improve motility. Future work should standardize extenders and protocols, pre-specify biologically justified doses, and report methods in full to determine when adjunct exogenous supplementation adds value to the endogenous baseline and its net effect on fertility-relevant outcomes.

Conclusions

Based on the evidence, endogenous antioxidants, which are part of the intrinsic redox system, consistently support post-thaw viability but do not reliably maintain motility, suggesting functional limitations under freeze-thaw stress. Exogenous antioxidants can provide greater improvements in viability; however, their effects seem to be contingent upon specific conditions (dose, formulation, extender, handling) and are influenced by significant variability among studies and uncertainties regarding study quality. The data collectively endorse endogenous strategies as a physiologically compatible foundation, with selective, cautiously optimized exogenous supplementation regarded as a potential complementary rather than an entirely superior treatment.

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Declaration of generative AI in scientific writing

The authors acknowledge the use of generative AI tools in the preparation of this manuscript. ChatGPT (OpenAI) was employed for language editing and grammar refinement, while BioRender was used to generate illustrative figures. No content generation, data interpretation, or scientific conclusions were produced by AI. The authors take full responsibility for the accuracy, integrity, and conclusions of this work.

CRediT author statement

Yudit Oktanella: Conceptualization, Methodology, Writing – Original Draft. **Imam Mustofa:** Data Curation, Funding Acquisition. **Suherni Susilowati:** Screening, Data Curation. **Widjiati Widjiati:** Supervision, Validation, Resources. **Bambang Purwantara:** Supervision, Writing - Review & Editing. **Adeyinka Oye Akintunde:** Software, Visualization. **Tatik Hernawati:** Screening, Validation. **Tri Wahyu Suprayogi:** Formal Analysis, Methodology. **Suzanita Utama:** Supervision, Writing - Review & Editing. **Eka Pramyrtha Hestianah:** Writing - Review & Editing.

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