Research Article

Omega-Rich Egg Yolk Enhances Membrane and Mitochondrial Preservation of Chilled Ram Semen in a Polyphenol-Rich *Dendrophthoe pentandra* Extract Supplemented Extender

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Abstract | Egg yolk-based extenders are widely used for chilled sheep semen preservation; however, oxidative stress during storage can impair sperm membrane integrity and mitochondrial function. Enrichment of egg yolk with omega fatty acids may improve membrane stability, while supplementation with antioxidant-rich plant extracts such as *Dendrophthoe pentandra* (locally known in Indonesia as duku mistletoe) may further mitigate lipid peroxidation. The objective of this study was to determine whether a skim milk–egg yolk extender containing omega-rich egg yolk and *D. pentandra* leaf extract provides greater protection against oxidative damage and better preserves the functional quality of chilled sheep semen stored at 4–5°C than a conventional egg yolk extender containing the same dose of extract. Paired aliquots of Sapudi ram semen were diluted in either (1) skim milk-omega-rich egg yolk extender or (2) skim milk-conventional egg yolk extender. Both extenders were supplemented with sterile-filtered ethanolic extract of *D. pentandra* leaves (5.4 mg/mL). Samples were stored at 4-5°C for 48 h. Sperm quality was assessed by progressive motility (%), malondialdehyde (MDA; ng/µL) as a lipid peroxidation marker, and mitochondrial membrane potential parameter (MMP; %). The omega-rich egg yolk extender supplemented with *D. pentandra* extract demonstrated superior outcomes compared to the conventional egg yolk extender with the same extract dose. Progressive motility was higher in the omega-rich group (53.39 ± 8.43%) than in the conventional group (35.02 ± 5.18%; p = 0.0000419). MDA levels were lower in the omega-rich group (18.83 ± 4.45 ng/µL) compared to the conventional group (31.30 ± 8.91 ng/µL; p = 0.00244). The mitochondrial membrane parameter was also higher in the omega-rich group (55.30 ± 0.23%) than in the conventional group (41.17 ± 2.18%; p = 0.000488). The combination of omega-rich egg yolk and *D. pentandra* leaf extract in a skim milk–based extender significantly enhances sperm motility, reduces lipid peroxidation, and improves mitochondrial-related parameters during chilled storage, indicating synergistic protection against oxidative stress and supporting its application in short-term sheep semen preservation.

Keywords | Antioxidant, *Dendrophthoe pentandra*, Omega-rich egg yolk, Oxidative stress, Semen extender, Sapudi ram

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INTRODUCTION

Artificial insemination (AI) using chilled semen is a practical reproductive technology for sheep because it avoids liquid-nitrogen logistics and enables short-term distribution of superior genetics to farms located far from semen collection centers (Gangwar *et al*., 2023; Bashawat *et al*., 2021). Despite these advantages, semen storage at 4-5°C remains biologically challenging because sperm quality declines as storage time increases. Previous studies in small ruminants have shown that refrigerated storage can reduce motility and functional competence within 24-48 h, thereby limiting the fertile lifespan of chilled doses and contributing to inconsistent field fertility when semen must be transported or held before insemination.

A major biological challenge in preserving small-ruminant semen is that the sperm plasma membrane contains abundant polyunsaturated lipids, which maintain membrane fluidity but also make spermatozoa highly susceptible to cooling-induced oxidative injury (Fang *et al*., 2016; Evans *et al*., 2020). During refrigeration, reactive oxygen species generated by sperm metabolism and damaged cells can initiate lipid peroxidation, resulting in malondialdehyde (MDA) formation, membrane destabilization, impaired mitochondrial function, and reduced progressive motility (Susilowati *et al*., 2021; Toker, 2023; Mustofa *et al*., 2025). Cooling may also disrupt membrane organization and ion homeostasis, thereby intensifying cold-shock injury. Therefore, chilled semen extenders should protect the plasma membrane while also limiting oxidative damage during storage.

Egg yolk remains one of the most widely used extender components because its low-density lipoprotein and phospholipid fractions can associate with sperm membranes and improve resistance to cold shock (Brito *et al*., 2018). However, conventional egg yolk is biologically variable, and its fatty-acid profile depends on hen diet and egg source. Omega-rich egg yolk may provide a more favorable lipid composition to support membrane stability, maintain mitochondrial function, and preserve motility during low-temperature storage (Swelum *et al*., 2022). This lipid-based protection may be particularly useful for ram spermatozoa, which are vulnerable to cooling stress and oxidative peroxidation during short-term liquid storage.

In addition to lipid protection, antioxidant supplementation may further improve chilled semen preservation. *Dendrophthoe pentandra*, locally known in Indonesia as duku mistletoe when collected from duku trees, contains polyphenols and flavonoids with antioxidant activity. These redox-active compounds may help reduce lipid peroxidation in PUFA-rich sperm membranes and thereby support mitochondrial activity and progressive motility during refrigerated storage. Combining omega-rich egg yolk with *D. pentandra* extract may therefore provide complementary protection through membrane stabilization and antioxidant defense.

Improving chilled semen preservation in Sapudi rams is also important for supporting local sheep genetic resources, AI-based breeding programs, and smallholder livestock productivity in Indonesia. However, evidence comparing omega-rich and conventional egg yolk in a plant-extract-supplemented extender remains limited, particularly when oxidative and mitochondrial indicators are evaluated together with motility. Therefore, this study aimed to compare omega-rich egg yolk and conventional egg yolk in a skim milk-based extender, both supplemented with the same dose of *D. pentandra* leaf extract, for preserving chilled Sapudi ram semen at 4-5°C for 48 h. The study focused on progressive motility, MDA concentration, and mitochondrial membrane potential as complementary indicators of sperm functional quality and oxidative stability.

MATERIALS AND METHODS

Study design

This research employed a paired experimental design. Each ejaculate was divided into two equal aliquots and supplemented with either Omega-rich egg yolk extender (omega-enriched egg yolk) or conventional egg yolk extender (standard egg yolk).

The extended semen was preserved at 4–5°C and evaluated after 48 hours of storage. The experimental unit was the ejaculate, with 12 paired ejaculates collected from a single 3-years old Sapudi ram. Each ejaculate was divided into the respective treatment groups to allow within-ejaculate comparison. While this paired design reduces inter-ejaculate variability and strengthens internal comparison among treatments, the use of ejaculates from only one ram limits the generalizability of the findings to the broader Sapudi ram population.

Initial semen evaluation and inclusion criteria

Fresh semen collected from a Sapudi ram aged 3 years which was evaluated immediately after collection for volume, color, viscosity, and gross contamination. Semen volume from two ejaculates in total 1,5mL, color was normal, and viscosity was very dense (highly viscous), total sperm concentration $3,2\times10^{9}$ mL. Sperm concentration was not determined in this study. Only ejaculates fulfilling the minimum acceptance criteria (≥75% progressive motility, normal macroscopic appearance, and absence of contamination) were included for subsequent dilution, chilled storage at 4–5°C, and analysis.

Preparation of *Dendrophthoe pentandra* (duku mistletoe) extract

Fresh leaves of *Dendrophthoe pentandra* (L.) Miq., locally known in Indonesia as duku mistletoe when collected from duku trees, were collected from duku trees (*Lansium domesticum*). Leaves were washed with running water, rinsed with distilled water, and drained. The material was shade-dried and/or oven-dried at low temperature (e.g., 40 °C) until a constant weight was achieved, then ground into a fine powder and stored in airtight, light-protected containers until extraction.

Leaf powder was extracted using 96% ethanol by maceration as previously described for *D. pentandra* extracts. Briefly, a known amount of powdered sample (250 g) was soaked in 96% ethanol at a 1:10 (w/v) ratio. The mixture was macerated at room temperature for 3 days, and the solvent was replaced every 24 h (re-maceration) with periodic agitation. The combined macerates were filtered (Whatman No. 1), and the filtrate was concentrated under reduced pressure using a rotary evaporator to obtain the crude ethanolic extract. The extract was stored in amber vials at 4°C until use (Ingrid *et al*., 2024).

To ensure microbiological safety for semen handling, the crude *D. pentandra* extract was reconstituted to a concentrated stock solution using a semen-extender-compatible solvent. The stock was sterilized by 0.22 µm membrane filtration and stored at 4°C (protected from light) before extender preparation. The extract was added to extenders to reach a final concentration of 5.4 mg/mL, and the same final dose was applied to all experimental groups.

Extender formulation

A skim milk–egg yolk (SM–EY) extender was prepared using a simple heating method adapted from Puspita *et al*. (2020) for refrigerated semen preservation in small ruminants. Briefly, skim milk powder (10 g) was dissolved in distilled water to a final volume of 100 mL, homogenized, and heated at 92–95°C for 10 min. The milk was then cooled to room temperature (≈20–32°C) and filtered through sterile gauze to remove coagulated particles ("milk scum"). Fresh chicken eggs were cleaned with 70% ethanol, cracked aseptically, and the yolk was separated from the albumen; the vitelline membrane was removed, and yolk volume was measured. Egg yolk was added to the skim milk base at 5% (v/v) (i.e., 5 mL yolk per 100 mL milk) and mixed thoroughly. The extender was supplemented with antibiotics to obtain final concentrations of penicillin 1,000 IU/mL and streptomycin 1 mg/mL, then mixed until uniform.

Two extender variants were prepared identically. Both variants received the same final dose of *D. pentandra* leaf extract (5.4mg/mL), differing only in egg yolk source:

1. Omega-rich egg yolk SM–EY extender: skim milk base + 5% (v/v) omega-rich egg yolk
2. Conventional egg yolk SM–EY extender: skim milk base + 5% (v/v) conventional/regular egg yolk

The conventional egg yolk was obtained from regular commercial chicken eggs. In the present study, the classification of omega-rich versus conventional egg yolk was based on the product source and labeling, and the fatty-acid composition of the yolks was not independently verified by laboratory analysis. This limitation should be considered when interpreting the comparative effect of the two yolk sources. Prepared extenders were used fresh and semen doses were subsequently cooled and stored at 4–5°C for evaluation.

Progressive motility assessment

Progressive motility was evaluated using a Computer-Assisted Sperm Analysis (CASA) system (Open CASA®; WI-LI New Century Technical Development). Because CASA output is highly dependent on species-specific settings, the analysis parameters (e.g., particle size limits, velocity thresholds, and tracking settings) were adjusted to match small ruminant/ram semen characteristics, following published guidance for species-adapted CASA configuration (Alquézar-Baeta *et al*., 2019). Prior to analysis, chilled semen samples were equilibrated to 37 °C for 3 min. Progressive motility was expressed as the percentage of progressively motile spermatozoa. The detection settings were adjusted to exclude debris and non-sperm particles based on head-size and contrast thresholds appropriate for ram spermatozoa. Progressive motility was defined using kinematic thresholds, with sperm classified as progressively motile when they showed an average path velocity (VAP) of ≥45 µm/s and a straightness (STR) of ≥45%. Progressive motility was expressed as the percentage of spermatozoa meeting these predefined criteria.

MDA quantification

Malondialdehyde (MDA), serving as an indicator of lipid peroxidation and oxidative stress, was measured utilizing a thiobarbituric acid reactive substances (TBARS) spectrophotometric technique. Chilled semen (100 µL) was transferred into a microtube and combined with 550 µL of distilled water and 100 µL of 10% trichloroacetic acid (TCA). The resultant mixture was homogenized employing a vortex mixer, succeeded by the introduction of 250 µL of 1 N HCl and 100 µL of 1% Na-thiobarbituric acid (TBA), and subsequently vortexed once more until a homogeneous solution was achieved. The solution was then subjected to centrifugation at 500 rpm for a duration of 10 minutes, and the supernatant (100 µL) was heated in a water bath at 100°C for a period of 20–30 minutes. The

TBA reaction and absorbance were recorded at 532 nm utilizing a Bio-Rad iMark™ microplate reader. The results were expressed as ng/µL.

Mitochondrial membrane potential (MMP) by Rhodamine 123 staining and confocal microscopy

Mitochondrial membrane potential (MMP) was assessed using Rhodamine 123 staining and confocal microscopy (Leica DMi8 STELLARIS 5). Rhodamine 123 is a fluorescent dye that accumulates in mitochondria; therefore, sperm cells with stronger fluorescence are considered to have higher/optimal MMP. Briefly, post-thaw semen was washed with phosphate-buffered saline (PBS) and adjusted to a suitable concentration. Samples were incubated with freshly prepared Rhodamine 123 in PBS at 37°C in the dark. After incubation, spermatozoa were gently washed to remove excess dye, placed on microscope slides, and observed under the confocal microscope.

Mitochondrial membrane potential (MMP) was assessed from confocal fluorescence images based on signal intensity in the Leica pseudocolor display. Spermatozoa exhibiting strong red/high-intensity fluorescence were classified as having optimal MMP (yellow arrows), whereas spermatozoa with weak fluorescence were classified as having low/reduced MMP (white arrows). For each sample, sperm cells were evaluated across ten microscopic fields, with a total of twenty spermatozoa counted per sample. The percentage of spermatozoa with optimal MMP was calculated relative to the total number of observed sperm cells.

Statistical analysis

Data were analyzed using SPSS version 25 (IBM Corp., Armonk, NY, USA). Given the paired study design, comparisons were performed between paired aliquots derived from the same ejaculate. The normality of paired differences for each outcome variable, including progressive motility, malondialdehyde (MDA), and mitochondrial membrane potential (MMP), was assessed using the Shapiro–Wilk test. Variables with normally distributed paired differences were analyzed using the paired t-test and are reported as mean difference with 95% confidence interval (CI), whereas variables with non-normally distributed paired differences were analyzed using the Wilcoxon signed-rank test and are reported with the corresponding effect size (r). For parametric comparisons, effect size was expressed as Cohen's d for paired samples. Data are presented as mean ± standard deviation (SD) unless otherwise indicated, and a p value of < 0.05 was considered statistically significant.

RESULTS

Fresh semen collected from a Sapudi ram was first evaluated macroscopically to ensure suitability for the chilled-storage experiment. The ejaculates showed a volume of 1-2 mL, normal semen colour, and a very dense/highly viscous consistency, with no visible signs of contamination. Only ejaculates meeting the minimum inclusion criterion of ≥75% progressive motility were processed further. Each accepted ejaculate was then divided into paired aliquots and diluted with either an omega-rich egg yolk extender or a conventional egg yolk extender, followed by storage at 4-5 °C. The effects of extender type on semen quality after chilled storage were subsequently assessed using progressive motility, MDA (ng/µL), and mitochondrial membrane parameter (MMP, %), as summarized in Figure 1.

Progressive motility

Chilled semen stored at 4-5°C in the omega-rich egg yolk extender maintained a significantly higher progressive motility than semen stored in the conventional egg yolk extender (Figure 1A). The paired plot shows that almost all ejaculates experienced a decline in motility after storage, but the decrease was consistently less pronounced in the omega-rich group, resulting in a clearly higher post-storage motility compared with the conventional group ($p = 0.0000419$).

Malondialdehyde (MDA)

Lipid peroxidation, measured as MDA (ng/µL), was significantly lower in semen preserved with the omega-rich egg yolk extender compared with the conventional egg yolk extender ($p= 0.0024414$). The paired lines demonstrate that most ejaculates showed an increase in MDA in the conventional group, while the omega-rich group generally remained at lower MDA values, indicating reduced oxidative damage during cold storage (Figure 1B).

Mitochondrial membrane parameter/ MMP

The mitochondrial membrane parameter (%), reflecting mitochondrial functional status, was significantly higher in semen stored in the omega-rich egg yolk extender than in the conventional egg yolk extender ($p = 0.000488$; Figure 1C). The paired pattern indicates that mitochondrial function declined after storage in both treatments, but semen extended with omega-rich egg yolk retained better mitochondrial integrity/function. This is supported by representative confocal images (Figure 1D), where spermatozoa with strong Rhodamine 123 fluorescence (optimal MMP; yellow arrows) are more evident, whereas spermatozoa with low fluorescence (reduced MMP; white arrows) represent impaired mitochondrial potential after chilling.

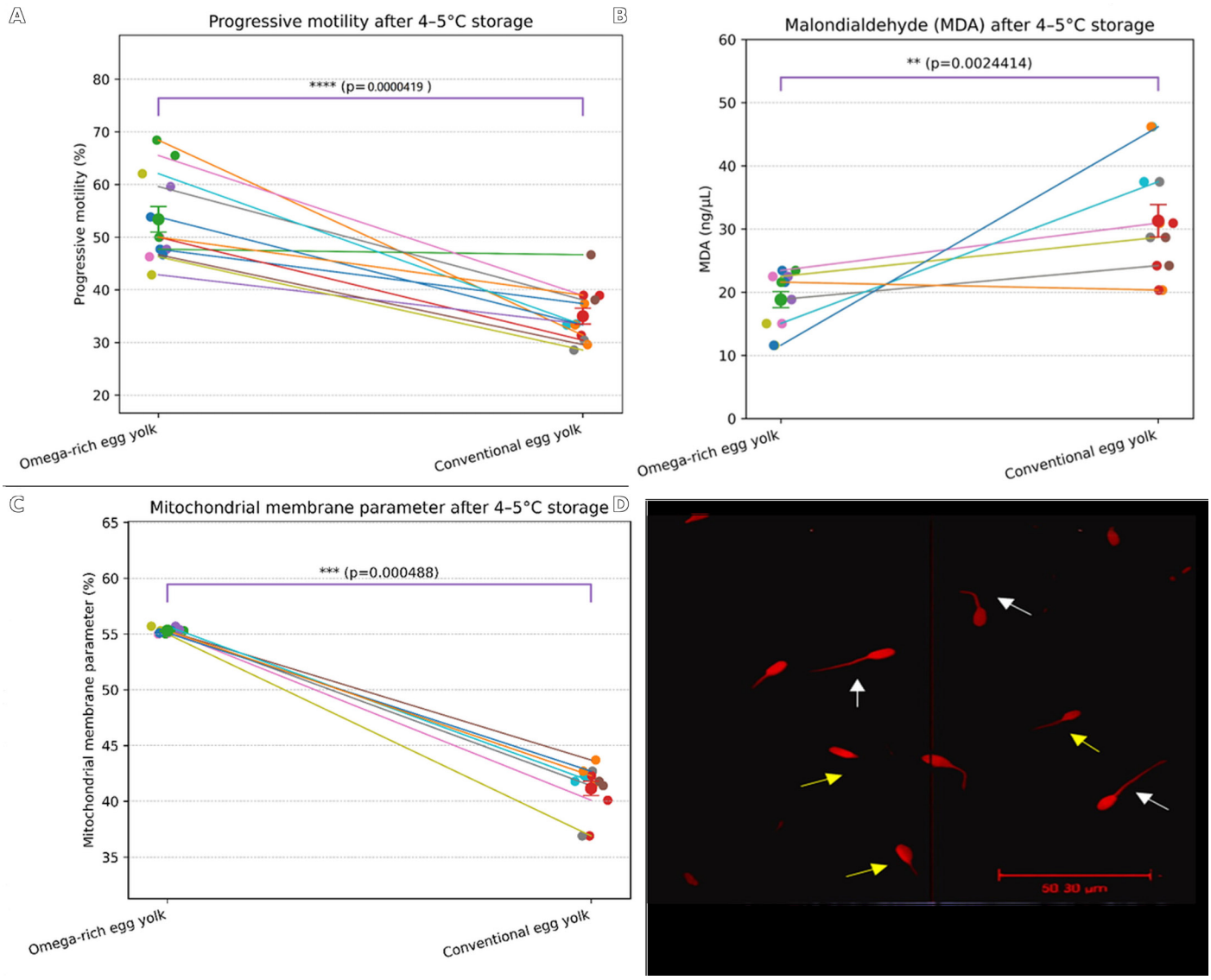

Figure 1: Paired comparison of chilled Sapudi ram semen quality after storage at 4-5˚C in extenders containing omega-rich egg yolk versus conventional egg yolk. (A) Progressive motility (%). (B) Malondialdehyde (MDA; ng/µL) as an index of lipid peroxidation. (C) Mitochondrial membrane parameter (%). (D) Representative confocal images of Rhodamine 123-stained spermatozoa showing mitochondrial membrane potential (MMP); spermatozoa with low fluorescence (reduced MMP) are indicated by white arrows, while spermatozoa with strong fluorescence (optimal MMP) are indicated by yellow arrows (×1000; scale bar = 50.30 µm). For panels A-C, each dot represents one ejaculate and colored lines connect paired aliquots from the same ejaculate; error bars indicate mean ± SEM. Significance is reported using exact p-values: A, = 0.0000419; B, p= 0.0024414; C, p= 0.000488.

DISCUSSION

This study demonstrates that replacing conventional egg yolk with omega-rich egg yolk in a chilled-semen extender provides measurable protection to Sapudi ram spermatozoa during storage at 4–5˚C, as shown by (i) higher progressive motility, (ii) lower MDA (ng/µL), and (iii) higher mitochondrial membrane potential/parameter (MMP, %). These three endpoints together indicate that omega-rich egg yolk helped preserve both functional performance (motility) and the underlying cellular bioenergetics and oxidative stability that support sperm survival during refrigeration.

The 48 h storage window is a critical "stress test" for chilled ram semen. Practical references note that ram semen can be cooled and held at 2–5˚C for ~24 h, but fertility decreases rapidly and is low by 48 h after collection (Ebner *et al.*, 2012). Experimental work also shows that cooling and storage can produce measurable cellular damage by 48 h, including reduced viability and other signs of cold-stress injury. Therefore, the significant differences observed in this study after 48 h indicate that the omega-rich egg yolk extender did not merely delay minor quality loss, but provided biologically relevant protection during a timepoint when ram semen typically becomes less reliable for AI.

A major practical limitation of ram semen preservation is the rapid decline in motility during liquid storage (Carro *et al*., 2020), which can occur even within a short storage window and tends to worsen progressively with time at low temperature. Studies on liquid-stored ram semen have repeatedly documented time-dependent decreases in motility under 4–5°C storage (O'Hara *et al*., 2010; Kasimanickam *et al*., 2011), making motility one of the earliest and most sensitive indicators of preservation failure. Our data are consistent with this general pattern: motility dropped after chilled storage in both extenders, but the omega-rich egg yolk group retained motility at a substantially higher level than the conventional egg yolk group (Figure 1A).

Importantly, several applied studies in sheep liquid semen preservation use motility thresholds (often around ≥40%) as a practical benchmark for "insemination-eligible" chilled semen. For example, thin-tailed sheep semen stored at 5°C maintained acceptable motility when antioxidant supplementation was used (e.g., noni extract), and a Sapudi sheep study reported that extender supplementation could keep motility above ~40% even after several days of storage (Bintara *et al*., 2022). In the present work, the omega-rich egg yolk extender kept post-storage motility clearly above the conventional egg yolk group, suggesting a meaningful practical advantage for chilled semen handling in sheep, where motility loss is often a key bottleneck.

Because sheep semen is highly susceptible to chilling injury, motility typically declines with storage time, and the 48 h point is often where performance drops to less desirable levels for field AI. In this study, the omega-rich egg yolk group retained significantly higher progressive motility after 48 h, suggesting better preservation of membrane function and energy supply. This is consistent with prior sheep studies showing that improving extender "protection capacity" often via antioxidants or optimized egg-yolk systems helps maintain post-storage motility (Zarei *et al*., 2018). For example, adding α-tocopherol into a skim milk–egg yolk extender improved quality of Sapudi ram sperm stored at 5°C (Puspita *et al*., 2020).

Dendrophthoe pentandra (Indonesian mistletoe; including specimens growing on *Lansium domesticum*/duku) is consistently reported to contain polyphenols and flavonoids (including flavonoid isolates such as quercitrin/quercetin-related compounds), as well as other secondary metabolites such as tannins, saponins, terpenoids/steroids, and alkaloids, which collectively underpin its bioactivity profile (Awang *et al*., 2023). Ethanolic leaf extracts show measurable free-radical scavenging/antioxidant activity in standard assays (e.g., DPPH) and are also reported to have antibacterial activity, supporting dual functionality (oxidative control + microbial suppression) that is relevant to semen handling and chilled storage (Haryono *et al*., 2024). Mechanistically, polyphenol/flavonoid-rich extracts can act as chain-breaking antioxidants and ROS scavengers, limiting the propagation of lipid peroxidation in PUFA-rich sperm membranes; this provides a biologically plausible rationale for using *D. pentandra* as a natural extender additive to reduce MDA formation, preserve membrane integrity, and indirectly support mitochondrial function and progressive motility during chilling. Additionally, mistletoe extracts have been described to exert broader bioactivities including anti-inflammatory signalling modulation in biomedical models, which while not directly tested in semen reinforces the concept that *D. pentandra* contains redox-active phytochemicals capable of influencing oxidative–inflammatory cascades that commonly accompany storage stress (Kong *et al*., 2023).

MDA is a widely used proxy for lipid peroxidation, and increased MDA during storage indicates that sperm membrane lipids are undergoing oxidative degradation (Yonny *et al*., 2015). Liquid storage of ram semen at 4°C has been shown to alter oxidative stress parameters over time, including changes consistent with progressive oxidative injury as storage continues (Ben moula *et al*., 2024). Lower MDA in the omega-rich egg yolk group indicates reduced lipid peroxidation during 48 h storage. This direction matches the broader ram semen literature where antioxidant fortification (propolis extract) is tested specifically to reduce lipid peroxidation (including MDA) during 48 h chilling at 5°C (El-Harairy *et al*., 2018). Mechanistically, the egg yolk LDL fraction and yolk antioxidants plausibly reduce oxidative chain reactions and protect PUFA-rich sperm membranes from peroxidation (El-Harairy *et al*., 2018).

This finding aligns with the broader literature showing that adding antioxidant support during liquid storage can reduce oxidative stress and improve semen quality (Hungerford *et al*., 2024). For example, resveratrol supplementation in ram semen stored at 5 °C reduced oxidative stress and improved motility-related parameters and even in vitro fertilizing ability, while alpha-lipoic acid has also been shown to improve ram sperm quality during liquid storage at 4°C through oxidative-stress mitigation (Hungerford *et al*., 2024). Likewise, chilled Sapudi sheep semen quality in skim milk–egg yolk extender with α-tocopherol able to maintain chilled semen function (Aktar *et al*., 2024).

Mechanistically, the advantage of omega-rich egg yolk may reflect better membrane stabilization (limiting ROS propagation and membrane destabilization) and/or a more favorable lipid milieu that reduces peroxidation chain reactions (Aktar *et al*., 2024). This is particularly relevant because sperm membranes especially in small ruminants contain high proportions of unsaturated lipids, which are

inherently more prone to peroxidation under stress (Jakop *et al*., 2022).

Mitochondria drive ATP production required for sperm movement, and mitochondrial injury is a recognized outcome of semen handling steps associated with cooling and preservation (Henning *et al*., 2022). Classic work in ruminant sperm has shown that procedures used in semen preparation and cooling can directly impair mitochondrial function, and probes such as Rhodamine 123 can detect mitochondrial membrane potential disruptions linked to cold-shock and processing injury (Llavanera *et al*., 2022). In the present study, the omega-rich egg yolk extender resulted in a significantly higher MMP (%) than the conventional group (Figure 1C), and representative confocal images (Figure 1D) illustrate the contrast between low-fluorescence (reduced MMP) and strong-fluorescence (optimal MMP) spermatozoa. MMP is tightly linked to mitochondrial function and ATP generation for motility. After 48 h chilling, mitochondria are vulnerable to oxidative stress and membrane injury. The significantly higher MMP (%) in the omega-rich egg yolk group supports the interpretation that mitochondrial function was better preserved, likely because membrane-stabilizing lipids (phospholipids/cholesterol) and LDL-mediated protection reduce membrane disruption and lipid loss, indirectly supporting mitochondrial integrity.

Biologically, this is important because mitochondrial depolarization and oxidative stress often reinforce each other: oxidative damage can impair mitochondrial membranes, and dysfunctional mitochondria can generate additional ROS, accelerating lipid peroxidation and motility failure (Aitken, 2017; 2020). Therefore, the concurrent pattern we observed lower MDA + higher MMP + higher motility is internally consistent with a protective mechanism that stabilizes membranes and preserves bioenergetic capacity during cold storage.

Sheep spermatozoa are well known to be highly susceptible to chilling injury (cold shock), in part because membrane composition especially the PUFA: Saturated fatty acid ratio and cholesterol: phospholipid balance influences how membranes behave during rapid temperature transitions (Evans *et al*., 2020). Sperm with high unsaturated lipid content and relatively low cholesterol are more prone to cold-shock–related membrane phase changes, increased permeability, and functional decline (Evans *et al*., 2020). This provides a strong biological rationale for interventions targeting membrane lipid protection during refrigeration. Egg yolk has long been used as a non-penetrating protectant because its lipoproteins/phospholipids can associate with sperm membranes, improving resistance to cold shock; optimizing the egg yolk lipid profile toward omega-rich content may further strengthen this membrane protection (Anzar *et al*., 2019).

From a practical perspective, chilled semen is attractive because it avoids liquid nitrogen logistics and can support more flexible AI distribution. Yet, maintaining sperm quality at 4–5°C remains a key limitation in sheep, where motility can fall rapidly and oxidative injury accumulates. The present findings are significant because they show that a simple extender modification (switching to omega-rich egg yolk) produced statistically robust improvements across three mechanistically linked endpoints in a paired design. This positions omega-rich egg yolk as a promising, low-barrier strategy that can complement (or potentially reduce reliance on) adding purified antioxidants although direct comparisons with antioxidant-supplemented controls should be tested in future work.

Limitations and future directions

This study focused on key functional/biochemical endpoints but did not measure fertility outcomes. Because post-storage motility and membrane/mitochondrial metrics are predictive but not definitive of fertilization success, future studies should include in vivo AI trials (pregnancy/lambing rate) and additional functional assays (e.g., acrosome integrity, DNA fragmentation). In addition, confirming the fatty-acid profile of omega-rich vs conventional egg yolk used would strengthen mechanistic interpretation, because prior work suggests that PUFA composition and oxidative balance are central to chilling sensitivity and post-storage performance.

CONCLUSION

In conclusion, supplementation of a skim milk-egg yolk extender with omega-rich egg yolk combined with *Dendrophthoe pentandra* leaf extract significantly improved the quality of chilled Sapudi ram semen after 48 hours of storage at 4-5°C. The omega-rich formulation demonstrated higher progressive motility, lower malondialdehyde (MDA) levels, and improved mitochondrial membrane parameters compared to the conventional egg yolk extender receiving the same extract dose. These results imply that omega-rich egg yolk offers superior protection against oxidative damage and mitochondrial dysfunction associated with cold storage and may represent a viable strategy to improve short-term preservation of chilled semen for sheep artificial insemination programs.

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NOVELTY STATEMENT

This study provides novel evidence that omega-rich egg yolk, when combined with Dendrophthoe pentandra leaf extract in a skim milk-based extender, offers superior protection for chilled Sapudi ram semen compared with conventional egg yolk containing the same extract dose. The novelty lies in the simultaneous improvement of progressive motility, reduction of lipid peroxidation, and preservation of mitochondrial membrane potential after 48 h storage at 4-5°C. These findings support a practical lipid-antioxidant extender strategy to improve short-term preservation of ram semen for artificial insemination programs.

AUTHOR'S CONTRIBUTION

Imam Mustofa conceptualized and designed the study, conducted the laboratory experiments, performed data analysis, and drafted the original manuscript. Suherni Susilowati contributed to study supervision, methodology refinement, data interpretation, and critical revision of the manuscript for important intellectual content. Yudit Oktanella assisted in experimental procedures, sample preparation, and statistical analysis, and contributed to manuscript editing. Tri Wahyu Suprayogi contributed to methodological validation, laboratory resources, and interpretation of semen quality parameters. Adeyinka Oye Akintunde contributed to data analysis, scientific interpretation of oxidative stress mechanisms, language editing, and final manuscript review. All authors read and approved the final manuscript and agree to be accountable for the content of the work.

Ethical approval

All procedures involving animals were conducted in accordance with institutional guidelines for animal care and use and were approved by the Animal Ethics Committee of 1.KEH.30.02.2026.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aitken RJ (2020). Impact of oxidative stress on male and female germ cells: implications for fertility. Reproduction, 159(4): R189–R201. https://doi.org/10.1530/REP-19-0452

Aitken RJ (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol. Reprod. Dev., 84(10): 1039–1052. https://doi.org/10.1002/mrd.22871

Aktar A, Toker MB, Koca D, Uzun UC, Alçay S (2024). The effects of supplementation of vitamin D to the egg-yolk extender on cryopreservation of ram semen. Vet. Med. Sci., 10(3): e1526. https://doi.org/10.1002/vms3.1526

Alquézar-Baeta C, Gimeno-Martos S, Miguel-Jiménez S, Santolaria P, Yániz J, Palacín I, Casao A, Cebrián-Pérez JA, Muiño-Blanco T, Pérez-Pé R (2019). OpenCASA: A new open-source and scalable tool for sperm quality analysis. PLoS Comput. Biol., 15(1): e1006691. https://doi.org/10.1371/journal.pcbi.1006691

Anzar M, Rajapaksha K, Boswall L (2019). Egg yolk-free cryopreservation of bull semen. PLoS One, 14(10): e0223977. https://doi.org/10.1371/journal.pone.0223977

Awang MA, Nik Mat Daud NNN, Mohd Ismail NI, Abdullah FI, Benjamin MAZ (2023). A review of *Dendrophthoe pentandra* (mistletoe): Phytomorphology, extraction techniques, phytochemicals, and biological activities. Processes, 11(8): 2348. https://doi.org/10.3390/pr11082348

Bashawat M, Hensel B, Müller K, Schulze M (2021). Cooled storage of semen from livestock animals (Part II): Camelids, goats, and sheep. Anim. Reprod. Sci., 234: 106855. https://doi.org/10.1016/j.anireprosci.2021.106855

Ben Moula A, Hamidallah N, Badi A, El-Fadili M, El-Amiri B (2024). Adjusting ram semen preservation: Exploring the impact of oxygen exposure during liquid storage. Reprod. Domest. Anim., 59(5): e14618. https://doi.org/10.1111/rda.14618

Bintara S, Ismaya I, Widayati DT, Aji RN, Asmarawati W (2022). Storage period of liquid semen eligible for insemination in thin tail sheep semen diluted with egg yolk citrate with the addition of noni (*Morinda citrifolia* Linn.) fruit extract. Adv. Biol. Sci. Res., 17: 291–296. https://doi.org/10.2991/absr.k.220207.051

Brito MF, Neves BP, Andrade GO, Gouvêa RR, Almeida J, Morais CL, Filho OAM, Araújo MSS, Melo MIV, Henry M (2018). Low density lipoproteins at 2% concentration can replace whole egg yolk in TES-Tris-milk extender for freezing buffalo sperm cells. Acta Sci. Vet., 46: 1532. https://doi.org/10.22456/1679-9216.81815

Carro M de LM, Peñalva DA, Antollini SS, Hozbor F, Buschiazzo J (2020). Cholesterol and desmosterol incorporation into ram sperm membrane before cryopreservation: Effects on membrane biophysical properties and sperm quality. Biochim. Biophys. Acta Biomembranes, 1862(8): 183357. https://doi.org/10.1016/j.bbamem.2020.183357

Ebner A, Poitz DM, Augstein A, Strasser RH, Deussen A (2012). Functional, morphologic, and molecular characterization of cold storage injury. J. Vasc. Surg., 55(5): 1361–1370.

El-Harairy MA, Khalil WA, Khalifa EI, Saber AA (2018). Effect of propolis ethanolic extract supplementation to ram semen extenders on sperm characteristics, lipid peroxidation and some enzymatic activities in seminal plasma in chilled semen. J. Anim. Poult. Prod., 9(12): 543–550. https://doi.org/10.21608/jappmu.2018.41098

Evans HC, Dinh T, Ugur MR, Hitit M, Sajeev D, Kaya A, Topper

E, Nicodemus M, Smith GD, Memili E (2020). Lipidomic markers of sperm cryotolerance in cattle. Sci. Rep., 10: 20100. https://doi.org/10.1038/s41598-020-77089-9

Fang Y, Blair HT, Zhong R, Sun H, Zhou D (2016). Optimizing the freezing rate for ovine semen cryopreservation: Phospholipid profiles and functions of the plasma membrane and quality and fertilization of spermatozoa. Small Rumin. Res., 139: 46–51. https://doi.org/10.1016/j.smallrumres.2016.04.012

Gangwar C, Ranjan R, Kharche SD, Pourouchottamane R, Rai B (2023). Success of artificial insemination in goats: An overview. Indian J. Small Rumin., 29(1): 1–10. https://doi.org/10.59317/9789395763172

Haryono SE, Aditiyarini D, Restiani R (2024). Analysis of secondary metabolites and antioxidant activities of ethanol extract of *Dendrophthoe pentandra* (L.) Miq. in Sapuran, Central Java. Biogenesis: J. Ilmiah Biol., 12(1): 56–65. https://doi.org/10.24252/bio.v12i1.40323

Henning H, Nguyen QT, Luther AM, Wallner U, Beyerbach M, Waberski D (2022). *In vitro* storage of boar spermatozoa increases the demand of adenosine triphosphate for reactivation of motility. Andrology, 10(5): 1018–1028. https://doi.org/10.1111/andr.13226

Hungerford A, Bakos HW, Aitken RJ (2024). Addition of vitamin C mitigates the loss of antioxidant capacity, vitality and DNA integrity in cryopreserved human semen samples. Antioxidants, 13(2): 247. https://doi.org/10.3390/antiox13020247

Ingrid AM, Aditiyarini D, Prakasita VC (2024). Antibacterial activity of *Dendrophthoe pentandra* (L.) leaves extract against *Staphylococcus aureus* and *Escherichia coli*. J. Biodjati, 9(2): 280–293. https://doi.org/10.15575/biodjati.v9i2.30436

Jakop U, Engel KM, Hürland M, Müller P, Osmers JH, Jung M, Schulze M (2023). Lipid alterations by oxidative stress increase detached acrosomes after cryopreservation of semen in Holstein bulls. Theriogenology, 196: 74–83. https://doi.org/10.1016/j.theriogenology.2022.11.036

Kasimanickam RK, Kasimanickam V, Tibary A, Pelzer KD (2011). Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4 °C. Small Rumin. Res., 99(1): 64–70. https://doi.org/10.1016/j.smallrumres.2011.03.057

Kong D, Wang L, Niu Y, Cheng L, Sang B, Wang D, Tian J, Zhao W, Liu X, Chen Y, Wang F, Zhou H, Jia R (2023). *Dendrophthoe falcata* (L.f.) Ettingsh. and *Dendrophthoe pentandra* (L.) Miq.: A review of traditional medical uses, phytochemistry, pharmacology, toxicity, and applications. Front. Pharmacol., 14: 1096379. https://doi.org/10.3389/fphar.2023.1096379

Mustofa I, Susilowati S, Suprayogi TW, Akintunde AO, Oktanella Y, Purwanto DA (2025). Epigallocatechin-3-gallate chitosan nanoparticles in an extender improve the antioxidant capacity and post-thawed quality of Kacang goat semen. F1000Research, 12: 32. https://doi.org/10.12688/f1000research.127744.3

O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans ACO, Lonergan P (2010). Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology, 73(4): 541–549. https://doi.org/10.1016/j.theriogenology.2009.10.009

Puspita SAC, Susilowati S, Sardjito T, Samik A, Triana IN, Soeharsono S (2020). Penambahan alfa-tokoferol dalam pengencer susu skim-kuning telur terhadap kualitas spermatozoa domba Sapudi yang disimpan pada suhu 5 °C. Ovozoa, 9(3): 69–76. https://doi.org/10.20473/ovz.v9i3.2020.69-76

Susilowati S, Mustofa I, Wurlina W, Hernawati T, Oktanella Y (2021). Maintaining the quality of Kacang buck semen in chilled storage with the addition of green tea extract in extender. Trop. Anim. Sci. J., 44(4): 408–414. https://doi.org/10.5398/tasj.2021.44.4.408

Swelum AA, Ba-Awadh H, Olarinre IO, Saadeldin IM, Alowaimer AN (2022). Effects of adding mixed chicken and quail egg yolks to the cryodiluent on the quality of ram semen before and after cryopreservation. Front. Vet. Sci., 9: 1013533. https://doi.org/10.3389/fvets.2022.1013533

Toker M (2023). Lipid peroxidation in sperm cells and the creation of reactive oxygen species: A review. J. Environ. Agric. Sci., 5(1): 000134. https://doi.org/10.23880/jeasc-16000134

Yonny ME, Reineri PS, Palma GA, Nazareno MA (2015). Development of an analytical method to determine malondialdehyde as an oxidative marker in cryopreserved bovine semen. Anal. Methods, 7(19): 8101–8106. https://doi.org/10.1039/C5AY00950B

Zarei M, Rostami B, Masoumi R, Sharafi M, Shahir MH, Stear MJ, Catt S (2018). Egg yolk enriched with polyunsaturated fatty acids (PUFAs) improves the shelf life of ram semen in liquid storage. Small Rumin. Res., 166: 34–41. https://doi.org/10.1016/j.smallrumres.2018.05.002