Research Article

Influence of Quercetin Supplementation in Tris-Aminomethane Extender on the Semen Quality of Dorper Ram During Cold Storage

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Abstract | Quercetin is a polyphenolic antioxidant with free-radical–scavenging, metal-chelating, and membrane-stabilizing properties that can mitigate oxidative-stress–driven deterioration of sperm function during chilled storage. This study aimed to determine the optimal quercetin dose in a Tris–egg-yolk extender for preserving Dorper ram semen quality during 72 hours of storage at 3–5 °C. Fresh ejaculates collected using an artificial vagina and meeting preset quality thresholds were diluted with Tris–egg yolk supplemented with quercetin (0, 30, 60, 90 µM). Samples were stored for 0, 24, 48, and 72 hours and evaluated for progressive motility, viability, morphological abnormalities, and plasma-membrane integrity (HOST) under a completely randomized design (n = 5 per treatment). Quercetin exhibited time-dependent effects, where storage duration significantly influenced progressive motility and plasma-membrane integrity ($P < 0.01$). Although the main effect of quercetin dosage was not statistically significant for motility and viability ($P > 0.05$), the 30 µM group descriptively yielded the highest grand-mean motility. All groups maintained ≥40% motility through 72 hours, indicating acceptable operational quality for artificial insemination. In conclusion, supplementation with 30–60 µM quercetin is recommended for short-term liquid storage.

Keywords | Quercetin, Dorper ram, Reactive oxygen species, Semen diluent, Semen quality

Received | December 31, 2025; **Accepted** | March 10, 2026; **Published** | May 06, 2026
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Citation | Vidyaningrum AN, Nurwahyudin MS, Nugraha CD, Sriwahyuni D, As A, Septian WA, Ardiantoro A, Wahjuningsih S, Novianti I, Rachmawati A, Suyadi S (2026). Influence of quercetin supplementation in tris-aminomethane extender on the semen quality of dorper ram during cold storage. J. Anim. Health Prod. 14(2): 759-767.
DOI | https://dx.doi.org/10.17582/journal.jahp/2026/14.2.759.767
ISSN (Online) | 2308-2801

INTRODUCTION

Artificial insemination (AI) is a cornerstone of genetic improvement in small ruminants because it multiplies the use of superior sires, enables rapid gene flow across flocks, and lowers disease-transmission risk provided that semen quality is preserved from collection to deposition (El-Amiri and Rahim, 2024; Spanner *et al.*, 2024). In practice, program success is tightly constrained by semen handling and storage, since ram spermatozoa are highly susceptible to cold shock and sub-lethal chilling injuries that depress motility, plasma membrane, acrosome integrity, and ultimately pregnancy rates. Short-term liquid storage at 3–5 °C using Tris-based extenders is therefore pivotal for field AI, especially in tropical supply chains where insemination windows and logistics are narrow, yet quality frequently declines over 24–72 h due to oxidative and osmotic stress (Rizkallah *et al.*, 2022). Operational evidence from Indonesia similarly underscores that fertility outcomes hinge on rigorous semen-handling protocols and

extender optimization, reinforcing the need for antioxidant strategies aligned with field constraints (Suyadi *et al.*, 2020).

During chilled storage, sperm metabolism persists mitochondrial respiration and basal substrate use continue at low rates and generate endogenous reactive oxygen species (ROS). When ROS production exceeds the antioxidant capacity of the extender, lipid peroxidation, mitochondrial dysfunction, and acrosomal damage ensue, manifesting as progressive declines in motility and viability across the 24–72 h storage (Aitken, 2017, 2020; Rizkallah *et al.*, 2022; Ji *et al.*, 2024). Although Tris-egg yolk extenders remain the mainstay for 3–5 ˚C storage, time dependent quality loss is consistently reported and mechanistically linked to oxidative stress and perturbed osmotic homeostasis in rams (Zhang *et al.*, 2023; Akhter *et al.*, 2023). Evidence from Indonesian AI programs echoes these trends, highlighting the centrality of ROS control and standardized handling to sustain insemination performance in tropical chains (Suyadi *et al.*, 2020; Nugraha *et al.*, 2019).

These observations are consistent with a broader mechanistic consensus that oxidative stress is the primary driver of quality loss during dilution and chilling. Spermatozoa, which are rich in polyunsaturated fatty acids and possess limited cytoplasmic antioxidant defenses, are particularly susceptible to reactive oxygen species (ROS)-induced damage to lipids, proteins, and DNA (Díaz-Hernández *et al.*, 2024; Faheem *et al.*, 2023; Sohail *et al.*, 2024; Sun *et al.*, 2024). Empirically, elevated ROS levels are correlated with decreased motility and viability, as well as compromised plasma membrane and acrosomal integrity during cold storage. These findings provide strong justification for supplementing Tris-based extenders with exogenous antioxidants to restore redox balance and maintain sperm fertilizing capacity (Abdelnour *et al.*, 2022; Berean et al., 2024).

Among phytochemical antioxidants, quercetin, a dietary polyphenolic flavonoid, exhibits free-radical scavenging, metal-chelating, membrane-stabilizing, and redox-signaling modulation properties that are relevant to sperm function. Recent evidence indicates that quercetin supplementation improves post-preservation sperm kinematics, membrane and acrosome integrity, as well as antioxidant status in small ruminants (goat/buck models), and enhances in vivo fertility at optimized doses (Batool *et al.*, 2024; Kumar *et al.*, 2024). The dose–response range is narrow because supra-optimal concentrations may exert pro-oxidant or enzyme-inhibitory effects; therefore, breed- and extender-specific titration is essential for field application (Batool *et al.*, 2024). Indonesian research groups have also begun investigating the use of quercetin in small-ruminant semen preservation, highlighting practical considerations for its implementation under local logistical conditions (Athalla *et al.*, 2023).

A focused investigation into the role of quercetin in Tris-egg yolk extenders during chilled storage is warranted because oxidative stress remains a principal cause of functional decline in ram semen under 3–5 ˚C conditions, leading to reduced motility, viability, membrane integrity, and ultimately fertility. Despite the widespread use of Tris-egg yolk extenders for short-term preservation, evidence specifically addressing whether quercetin's free radical scavenging, metal chelating, and membrane stabilizing properties can effectively mitigate storage-induced damage in Dorper semen remains limited, particularly under logistics-constrained tropical AI systems. Therefore, this study aimed to evaluate the effects of graded concentrations of quercetin added to a Tris-egg yolk extender on motility, viability, plasma membrane integrity, and acrosome status of Dorper ram semen during 72 h of storage at 5 ˚C. Systematic evaluation of quercetin within this extender matrix is essential to determine its capacity to stabilize key semen quality parameters, establish a practically applicable supplementation strategy, and ultimately enhance the reproducibility and field performance of liquid-stored ovine semen.

MATERIALS AND METHODS

Research material

Semen was collected from a healthy, sexually mature Dorper ram (3 years old, ±80 kg) maintained at CV. Kambing Burja, Malang, Indonesia (7˚50'20" S, 112˚37'50" E). The diet consisted of fresh Pakchong grass (*Pennisetum purpureum* cv. Thailand) offered at 10% of body weight per day. This was supplemented with a concentrate mixture (14% crude protein; approximately 3100 kcal/kg metabolizable energy) formulated from locally available ingredients: cassava cobs, coffee husks, corn, kapok seeds, cassava peels, coconut meal, corn gluten feed (CGF), soybean meal (SBM), distillers dried grains (DDGS), soy sauce residue, iodized salt, lime, sheep premix, molasses, fermented mother liquor (FML), sodium bicarbonate, and pollard. To support reproductive performance during mating, rams were given nutritional supplements: sprouts on day 7 and a traditional Indonesian herbal mixture (jamu) on day 10. The jamu formulation was prepared by the partner farm (CV Kambing Burja) from locally sourced ingredients including palm sugar, native chicken eggs, garlic, honey, and vitamin E-selenium. Forage and clean drinking water were provided *ad libitum*.

Semen collection

Five ejaculates were collected at one-week intervals using an artificial vagina. The device was prepared by filling it

with water at 42–45 °C to maintain an internal temperature of ~38 °C (Nugraha et al., 2023). The inner liner was lubricated and a collection tube was attached to the rear end (Elsayed *et al*., 2019). Collections were performed by a trained technician at Burja Goat Farm, Malang. Ejaculates were initially assessed visually for physical characteristics, including milky-white color and a minimum volume of ±1 mL. Initial quality was then evaluated under a light microscope for individual motility, viability, and morphology. Only ejaculates with mass motility ≥2+, individual motility ≥70%, and abnormalities <20% were included for further analyses (Suyadi *et al*., 2020).

Diluent preparation

The basic diluent (tris-aminomethane; Merck, Germany) was prepared at the Biotechnology Laboratory, Faculty of Animal Science, Universitas Brawijaya. Quercetin (Sigma-Aldrich, USA) and sodium chloride (Sigma-Aldrich, Germany) were used for the preparation of the HOST solution (Nugraha et al., 2023).

The extender used in this study was a Tris-aminomethane egg yolk. The Tris-buffer solution (100 ml) was prepared by dissolving 1.363 g Tris-aminomethane, 0.762 g citric acid, 1.5 g lactose, 2.7 g raffinose, and 0.5 g fructose in aquabidest. To inhibit microbial growth, 0.1 g of streptomycin and 0.1 g of penicillin were added to the solution. The final extender was formulated by mixing 80% of this Tris-buffer solution with 20% fresh egg yolk, which was collected using a sterile syringe to ensure purity. Working concentrations (0, 30, 60, 90 µM) were prepared by dilution according to the formula $M_1V_1 = M_2V_2$.

Preparation of quercetin treatment and semen dilution

For the antioxidant treatments, Quercetin (Sigma Aldrich, MW 302.24 g/mol) was first dissolved in a solvent of 10% DMSO and 90% aquadest to create a 1000 µM stock solution. To achieve the final working concentrations of 30, 60, and 90 µM in a 5 ml (5000 µL) final volume, specific aliquots of the stock solution (150, 300, and 450 µL, respectively) were added. The concentration of DMSO was standardized at 0.9% (v/v) across all groups. This was achieved by adjusting the total volume of the 10% DMSO-based solution to 450 µL in every treatment; the 0 µM control group received 450 µL of the vehicle only (10% DMSO/90% aquadest), while the 30 and 60 µM groups were supplemented with 300 and 150 µL of pure vehicle, respectively, to reach the 450 µL threshold. The mixture was homogenized using a magnetic stirrer at 1270 rpm for 15–20 minutes and subsequently Tris-egg yolk with quercetin treatment was centrifuged at 1500 rpm for 30 minutes. The supernatant was collected as the final medium and stored at 4–5°C for 24 hours before use.

The dilution process was conducted in two stages (VA1 and VA2) to maintain sperm viability during cold storage. Fresh semen was first diluted with the treated extender (VA1) at a 1:1 ratio at 37°C. The tube was placed in a water jacket and moved to a refrigerator to ensure a gradual temperature decline. Once the mixture reached 12–15°C, the second stage extender (VA2) was added slowly along the tube wall until the final calculated volume was reached to minimize mechanical stress. Throughout the 72-hour storage period, the samples were kept in centrifuge tubes wrapped in aluminum foil to provide protection from light and prevent photo-oxidative degradation of quercetin. The temperature was strictly monitored at a constant 4–5°C within the refrigerator to prevent cold shock and maintain the physiological integrity of the sperm membranes.

Research design

This study employed a Completely Randomized Design (CRD) with a 4x4 factorial arrangement. The first factor was the concentration of quercetin (0, 30, 60, and 90 µM), and the second factor was the storage duration (0, 24, 48, and 72 hours). This design was selected to simultaneously evaluate the effects of various quercetin concentrations and storage durations on semen quality, enabling the identification of an optimal dosage that stabilizes sperm parameters over a 72-hour period. This design allows for a systematic observation of how each factor independently contributes to the preservation of sperm functional integrity during cold storage, which is essential for identifying the narrow optimal window of antioxidant supplementation.

Semen quality evaluation

Semen quality was assessed to determine the fertilizing potential of spermatozoa during storage. Evaluations were conducted at 0, 24, 48, and 72 h and included measurements of individual motility, viability, morphological abnormalities, and plasma-membrane integrity following quercetin supplementation.

Evaluation of sperm motility

Sperm motility was assessed as mass motility and individual motility (Suyadi et al., 2026). For mass motility, a drop of fresh semen was placed on a clean glass slide without a coverslip and observed under a light microscope at 100× magnification (Masoudi *et al*., 2021). Assessment was performed subjectively based on the wave motion generated by collective sperm movement and classified as excellent (+++), good (++), fair (+), or poor (0), according to wave intensity, speed, and uniformity (Rehman *et al*., 2023). Individual motility was evaluated under a light microscope at 400× magnification, counting at least 200 spermatozoa per field to ensure a representative estimate (Towhidi *et al*., 2020; Masoudi *et al*., 2021).

Evaluation of sperm viability

Sperm viability was assessed using eosin-nigrosin staining. One drop of semen was mixed with one drop of eosin-nigrosin on a glass slide using an inoculating loop. The mixture was spread into a thin smear with another slide held at a 45° angle and air-dried for 15 min. Smears were examined under a light microscope at 400× magnification. Spermatozoa that excluded the dye and appeared white were classified as viable (live), whereas those that took up the stain and appeared pink or red were classified as non-viable (dead) due to membrane damage (Suyadi *et al.*, 2020). Viability was calculated by counting 200 sperm cells and recording live (unstained) versus dead (stained) cells (Masoudi *et al.*, 2021).

Evaluation of sperm abnormalities

Abnormality refers to a condition of abnormality or damage that occurs to the head, tail, and acrosome of spermatozoa that can be detected through special staining. Sperm abnormalities were evaluated using e. One drop of semen was placed at the edge of a glass slide with an inoculating loop and mixed with one drop of eosin–nigrosin solution. A smear was prepared using a second slide at a 45° angle and air-dried for 15 min. The slide was examined under a light microscope at 400× magnification to identify morphological defects such as small heads, double heads, coiled tails, or headless spermatozoa (El-Sheshtawy *et al.*, 2020).

Evaluation of sperm membrane integrity

Plasma-membrane integrity was assessed using the hypo-osmotic swelling test (HOST) following Zubair *et al.* (2013) as cited in Nugraha et al. (2023). The HOST solution was prepared by dissolving 0.55 g sodium citrate and 1.35 g fructose in 1,000 mL distilled water to obtain an osmotic pressure of 150 mOsm/L. A 0.5 mL semen sample was mixed with 1 mL HOST solution and incubated at 37 °C for 30 min. One drop of the incubated mixture was placed on a slide, covered with a coverslip, and examined under a light microscope at 400× magnification. Spermatozoa with intact membranes were identified by swollen or coiled tails, indicating resistance to hypo-osmotic conditions (Prochowska *et al.*, 2022; Suyadi et al., 2026).

Statistical analysis

Statistical analyses were performed using R software (version 4.4.3). Hypotheses were tested using a Repeated Measures ANOVA (Split-plot in Time) design to account for longitudinal observations on the same experimental units. The assumptions and normality of data were checked through the Shapiro–Wilk test and Levene's test. Statistical significance was set at $P < 0.05$, and significant differences were analyzed using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Characteristics of fresh semen

As shown in Table 1, fresh Dorper ram semen exhibited individual motility of 82.5%, viability of 95.37%, abnormality of 0.46%, and plasma-membrane integrity of 63.47%, with an ejaculate volume of 0.73 mL and pH 5.25.

Table 1: Characteristics of fresh dorper ram semen.

Quality of Fresh Semen Dorper Ram	
Parameter	**Mean ± SD**
Macroscopic	
Volume (ml)	0.73 ± 0.21
Color	Yellowish white
Consistency	Medium
pH	5.25 ± 0.5
Microscopic	
Mass Motility	+++
Individual Motility (%)	82.5 ± 6.45
Concentration (million/ml)	3547.5 ± 948.17
Viability (%)	95.37 ± 5.03
Abnormality (%)	0.46 ± 0.04
Membrane Integrity (%)	63.47 ± 15.4

The values in Table 1 surpass these benchmarks, thereby justifying progression to treatment and time-course evaluations (Rizkallah *et al.*, 2022). Operationally, ejaculates with motility >70% and abnormalities <15% are considered suitable for subsequent processing as experimental material, since these thresholds reflect adequate functional competence of spermatozoa prior to dilution and chilled storage (Garner and Hafez, 2000; Suyadi *et al.*, 2020).

Progressive individual motility at different levels of quercetin

The effect of quercetin supplementation on individual progressive motility of Dorper ram semen during cold storage is presented in Table 2. The analysis was conducted to evaluate the effects of quercetin concentration, storage duration, and their interaction on the decline in progressive motility during storage at 4-5°C.

Treatment of quercetin levels did not exert a significant effect ($P>0.05$) on the progressive motility of spermatozoa. These findings contrast with previous reports in ram semen, where quercetin supplementation significantly enhanced sperm motility (Falchi et al., 2018; Suyadi et al., 2026), possibly due to differences in dosage, extender composition, or storage conditions. However, these findings are still similar to those of Silva *et al.* (2012) who stated that the addition of the antioxidant quercetin before or after cryopreservation

Table 2: Progressive individual motility of dorper ram semen during cold storage at different quercetin concentrations and storage durations.

Concentration of Quercetin (µM)	Time (hours)				P-value		
	0	24	48	72	G	T	G*T
0	76.00± 4.18^{a}	70.00±13.69^{ab}	69.00±11.4^{b}	67.00±9.75^{b}	0.800	0.006	0.717
30	79.00±5.48^{a}	71.00±13.42^{ab}	69.00±14.32^{b}	67.00±15.65^{b}			
60	75.00±3.54^{a}	70.00±10.00^{ab}	64.00±18.17^{b}	63.00±16.81^{b}			
90	75.00±11.3^{a}	72.00±9.08^{ab}	63.00±12.04^{b}	56.10±11.40^{b}			

Data are expressed as mean ± SD. G = group of quercetin concentrations; T = time; G×T = interaction between group of quercetin concentrations and storage time. Different superscripts (a, b) within the same row indicate significant differences (P < 0.05) among storage times within the same treatment group.

Table 3: Percentage of sperm viability of dorper rams during cold storage at different quercetin concentrations and storage durations.

Concentration of quercetin (µM)	Time (hours)				P-value		
	0	24	48	72	G	T	G*T
0	95.27±3.71	95.15±2.60	92.25±7.18	91.72±4.50	0.324	0.141	0.838
30	96.42±1.60	95.63±2.19	94.72±3.27	94.41 ± 2.64			
60	96.38±2.84	95.58±1.70	95.41±2.89	92.89 ± 6.85			
90	95.90 ±2.18	95.92±3.57	93.46±4.65	92.92±7.05			

Data are expressed as mean ± SD. G = group of quercetin concentrations; T= time; G×T= interaction between group of quercetin concentrations and storage time. Viability was assessed using eosin–nigrosin exclusion staining.

did not have a significant effect on the progressive motility of spermatozoa (Jiménez-Aguilar *et al*., 2021). This may be caused by several factors such as the species of livestock, the diluting agents used, and the type and concentration of antioxidants (Nugraha et al., 2023).

In contrast to the storage time treatment, which actually had a very significant effect on the progressive motility of spermatozoa (P<0.01). The decrease in motility began to be seen after 24 hours and became significant after 48 hours of storage. Average progressive motility decreases gradually during cold storage. Descriptively, the addition of 30 µM quercetin showed a relatively higher motility value up to 72 h (67.00 ± 15.65) compared to 60 µM (63.00 ± 16.81) and 90 µM (56.10 ± 11.40%). However, the difference was not statistically significant (P>0.05). The addition of 90 µM actually resulted in the highest decrease in motility (56.10±11.40), which is likely due to dose-dependent redox imbalances at higher quercetin levels (Aitken, 2020). This pattern favors a narrow optimal window, where antioxidant supplementation in moderate doses is able to maintain cell function, while supra-optimal doses may reduce benefits due to pro-oxidant effects or enzymatic disruptions as reported in small ruminant semen (Aitken, 2020; Batool *et al*., 2024).

The percentage of motility in this study remained above ≥40% to 72 hours of storage, so it still met the commonly applied acceptance threshold for liquid sheep semen used in artificial insemination (Rizkallah *et al*., 2022). These findings are in line with the experience of artificial insemination centers in Indonesia, where the precision of semen handling and conservative antioxidant titration contributed to the success of field applications (Suyadi *et al*., 2020; Nugraha *et al*., 2019). Overall, the observed motility decline trajectory was consistent with the central role of oxidative stress in semen stored in cold conditions. Mitochondrial metabolic activity that remains at low levels during storage results in ROS that can damage membrane and flagella function; the addition of the right antioxidants can slow, though not completely prevent, time-dependent damage (Aitken, 2020; Ji *et al*., 2024). Thus, although not statistically significant, the 30 µM concentration of quercetin descriptively shows the most favorable balance in maintaining motility during 72 hours of storage.

Sperm viability at different levels of quercetin

The percentage of Dorper ram spermatozoa viability during cold storage at various quercetin concentrations is presented in Table 3. The analysis was performed to evaluate the effects of quercetin concentration, storage duration, and their interaction on sperm plasma membrane stability during storage at 4-5°C. Quercetin significantly affected viability over 72 h of chilled storage (P < 0.05).

Storage time quercetin concentration treatment and interaction of the two (P>0.05) had no significant effect on the percentage of spermatozoa viability. The most descriptively pronounced decreases were seen in the 0 µM and 90 µM groups at the 72nd hour (91.72 ± 4.50% and 92.92 ± 7.05%, respectively), while the 30 µM and 60 µM groups showed a more sloping pattern of decline until the end of storage.

Table 4: Percentage of sperm abnormalities in dorper ram semen during cold storage at different quercetin concentrations and storage durations.

Concentration of quercetin (µM)	Time (hours)				P-value		
	0	24	48	72	G	T	G*T
0	0.91±0.57	0.93±0.31	1.11±0.56	1.73±1.22	0.287	0.069	0.055
30	0.66±0.25	0.84±0.36	1.00±0.33	1.23±0.41			
60	0.67±0.25	1.00 ± 0.51	1.21 ± 0.64	1.51±1.25			
90	0.86±0.25	0.96±0.36	1.35±0.82	2.35±1.27			

Data are expressed as mean ± SD. G = group of quercetin concentrations; T= time; G×T = interaction between group of quercetin concentrations and storage time. Abnormalities were evaluated using morphological staining under light microscopy.

Table 5: Percentage of sperm plasma membrane integrity in dorper ram semen during cold storage at different quercetin concentrations and storage durations.

Concentration of quercetin (µM)	Time (hours)				P-value		
	0	24	48	72	G	T	G*T
0	73.24±4.12^{ab}	58.23±6.40^{a}	53.98±16.48^{b}	52.69±28.04^{b}	0.339	0.002	0.409
30	78.88±3.55^{ab}	66.43±13.11^{a}	66.43±13.11^{b}	52.88±19.62^{b}			
60	80.21±14.38^{ab}	74.36±16.32^{a}	72.31±10.25^{b}	49.42±28.09^{b}			
90	77.16±7.75^{ab}	70.62±24.44^{a}	62.92±19.72^{b}	48.72±14.5^{b}			

Data are expressed as mean ± SD. G = group of quercetin concentrations; T = time; G×T = interaction between group of quercetin concentrations and storage time. Different superscript letters (a, b) within the same row indicate significant differences (P < 0.01) among storage times.

This pattern shows optimum dose response in the range of 30–60 µM, with no additional benefit at 90 µM. Although not statistically significant, concentrations of 30–60 µM maintain relatively more stable viability than controls and the highest doses. These findings are in line with reports that antioxidant titration in moderate doses is able to improve parameters related to membrane integrity, while supra-optimal doses have the potential to reduce gains through redox overshoot or enzymatic interference (Batool *et al*., 2024; Aitken, 2020).

Across all treatments, viability remained high (> 91%) but showed modest, time-dependent declines by 72 h, most pronounced in 0 µM and 90 µM groups (Table 3). By contrast, 30–60 µM maintained the flattest decline, consistent with quercetin's ROS-scavenging and membrane-stabilizing actions under refrigerated metabolism (Rizkallah *et al*., 2022; Wei *et al*., 2024). In operational terms, maintaining high viability complements the ≥ 40% motility acceptability threshold used for liquid-stored ram semen supporting downstream usability of the 30–60 µM treatments over 72 h (Rizkallah *et al*., 2022). Observations from Indonesian AI programs similarly emphasize that antioxidant-aware handling is pivotal to sustain semen quality in tropical supply chains (Suyadi *et al*., 2020; Nugraha *et al*., 2019).

Viability (eosin–nigrosin exclusion) reflects plasma-membrane intactness. During chilled storage, low-rate mitochondrial metabolism persists, generating ROS that compromise membranes and judicious antioxidant supplementation mitigates these losses (Aitken, 2020; Rizkallah *et al*., 2022). Although differences were not statistically significant, a numerical trend suggested relatively better to preserved progressive motility at 30-60 µM, without incurring dose-related liabilities, aligning with recent small ruminant evidence that targeted flavonoid dosing preserves functional sperm endpoints (Batool *et al*., 2024; Wei *et al*., 2024).

Sperm abnormalities at different levels of quercetin

The percentage of sperm abnormalities during cold storage at various quercetin concentrations is presented in Table 4. The analysis was conducted to evaluate the effects of quercetin concentration, storage duration, and their interaction on sperm morphological changes at 4-5 °C.

In these studies, no significant effect (P>0.05) was found on quercetin treatment, storage time, and interaction between the two. Descriptively, spermatozoa abnormalities gradually increased during cold storage in the entire treatment group. At 0 hours, the abnormality value was relatively low in all groups (0.66–0.91%). After 72 hours of storage, there was an increase in all treatments, with the highest values at 90 µM (2.35±1.27), followed by 0 µM (1.73±1.22), 60 µM (1.51±1.25), and 30 µM (1.23±0.41). Although not statistically significant, this pattern suggests that concentrations of 30–60 µM tend to maintain a lower level of abnormality compared to controls and 90 µM until

the end of storage. An increase at 90 µM after 72 hours indicates a possible unfavorable high-dose effect over a longer storage period. This is in line with the concept that antioxidants at supra-optimal concentrations have the potential to cause redox imbalances or mild pro-oxidant effects under certain conditions (Aitken, 2020).

All treatment averages well below operational thresholds (<20%), supporting suitability for advanced processing and in line with the view that prudent antioxidant titration maintains structural integrity during liquid storage (Aitken, 2020; Rizkallah *et al*., 2022). Abnormalities increased with storage time in all groups, with the sharpest increase observed at 90 µM after 72 hours. This trajectory is consistent with ROS-mediated damage that accumulates during mitochondrial metabolism at a low rate under cooling, leading to membrane injury and flagella defects, the addition of antioxidants can reduce, but not eliminate, such time-dependent injuries (Aitken, 2020; Takalani *et al*., 2023). The comparative stability of 30–60 µM indicates an effective emphasis on lipid peroxidation and preservation of membrane function, findings that are operationally relevant to the AI program and reflect the experience of the Indonesian center emphasizing quality control before processing.

Membrane integrity at different levels of quercetin

Sperm plasma membrane integrity, measured using the hypo-osmotic swelling test (HOST), during cold storage at various quercetin concentrations is presented in Table 5. The analysis was performed to assess the effects of quercetin concentration, storage duration, and their interaction on plasma membrane stability during storage at 4-5°C.

Quercetin supplementation had no significant effect on the percentage of HOST-positive spermatozoa during 72 h of chilled storage ($P > 0.05$). A highly significant effect of storage duration on plasma membrane integrity was evident ($P < 0.01$), whereas no significant treatment × time interaction was detected ($P > 0.05$). These results demonstrate that storage duration is the predominant factor influencing membrane integrity under cold storage conditions. The 30 µM group achieved the highest average integrity up to 72 hours (52.88±19.62) and consistently outperformed the 0µM control (52.69 ± 28.04), with best time reduction at 24 hours (74.36 ± 16.32) and 48 hours (72.31 ± 10.25).Taken together, these data support a dose-response optimum centered around 60 µM, consistent with the concept that moderate antioxidant titration helps preserve membrane functionality, whereas insufficient supplementation leaves redox damage unchecked and supra-optimal dosing confers no additional benefit (Rizkallah *et al*., 2022; Aitken, 2020; Wei *et al*., 2024).

Plasma membrane integrity of coiled and straight sperm tails can showed in Figure 1.

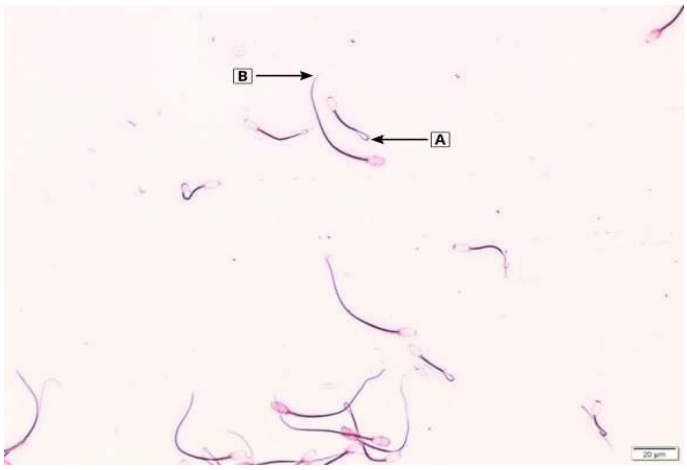

Figure 1: Microscopic evaluation of sperm plasma membrane integrity using the hypo-osmotic swelling (HOS) test at 400x magnification. (A) Spermatozoa with intact membranes exhibit characteristic tail curling as a result of functional osmoregulatory capacity and endosmosis in a hypotonic medium. (B) Spermatozoa with damaged membranes display a straight tail morphology due to compromised membrane semi-permeability, which allows the unrestricted diffusion of the hypo-osmotic solution and prevents the induction of the swelling response.

At all treatments, membrane integrity declined over time at temperatures of 3–5 °C, reflecting the well-described accumulation of reactive oxygen species (ROS) produced by mitochondrial metabolism at a low rate during storage. ROS-mediated lipid peroxidation compromises the fluidity and permeability of the bilayer, thereby reducing the responsiveness of the HOST even though the morphology remains stable (Aitken, 2020; Rizkallah *et al*., 2022). A relatively flatter trajectory at 60 µM suggests that quercetin's action in capturing ROS and stabilizing the membrane is most effective at this concentration, an observation that is in line with recent evidence from small ruminant animals that flavonoid antioxidants can maintain plasma membranes and acrosome endpoints when the dose is optimal (Wei *et al*., 2024). Methodologically, the use of HOST to distinguish functionally intact sperm (coiled tail) from damaged (straight tail) provides biologically meaningful readings of membrane performance during storage (Prochowska *et al*., 2022).

The observed improvement in semen quality in quercetin-treated groups can be attributed to its potent antioxidant activity. Spermatozoa membranes are rich in polyunsaturated fatty acids (PUFAs), making them highly susceptible to lipid peroxidation caused by Reactive Oxygen Species (ROS) (Akhter *et al*., 2023). Quercetin

acts by scavenging these excess ROS, thereby preventing oxidative damage to the lipid bilayer (Falchi *et al*., 2018). This protection preserves membrane structural integrity and fluidity, which are critical for maintaining sperm viability and motility during storage

CONCLUSION

The conclusion of this experimental study was that quercetin supplementation at all treatments did not significantly improve progressive motility, viability, abnormalities, and plasma membrane integrity during cold storage for 72 hours. However, numerical trends show that the semen quality in cold storage is more stable at moderate concentrations of 30-60 μM compared to control and high doses (90 μM). While storage duration is the main factor that affects the decline in plasma membrane integrity. These findings indicate that quercetin supplementation is biologically safe and may require further investigation using larger sample sizes and oxidative stress biomarkers for short-term liquid storage in AI programs for the future studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Rector of Universitas Brawijaya for supporting this study through the Professor Research Grant (2023–2024) administered by the Directorate of Research and Committee Service. We also thank CV. Kambing Burja, Lawang Malang, for providing Dorper rams for semen collection and UB STARS Program for supporting mobilitation in National Research and Innovation Agency Republic of Indonesia.

AUTHOR'S CONTRIBUTION

ANV: Conceptualization, data curation, data analysis, writing original draft, laboratory analysis, data interpretation, manuscript drafting, and revision. CDN, MSN, WAS, RFP, AA: Conceptualization, manuscript review, data interpretation. DS, AAs, GLS: Manuscript review, data analyzed. SW, IN: Writing final review. AR: supervision, writing final review, data curation. SS: Corresponding author, conceptualization, supervision, manuscript review.

NOVELTY STATEMENT

This study establishes the first systematic evaluation of quercetin supplementation specifically for Dorper ram semen using a Tris-egg yolk extender during 72-hour cold storage. It identifies a narrow optimal dose (30–60 μM) that provides a numerical advantage in stabilizing sperm progressive motility and membrane integrity, offering a practical antioxidant strategy tailored for short-term liquid preservation in tropical artificial insemination programs.

Ethical approval

The protocol of this study was approved by the Ethics Committee of the Animal Care and Use Committee University of Brawijaya, Indonesia (Ethical Clearance No. 192-KEP-UB-2024). All procedures were conducted in accordance with institutional guidelines for the care and use of animals in research.

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.

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