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Research Article

Quercetin in Tris-Aminomethane Extender Preserves Motility Ram Semen During Room Temperature Storage

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Abstract | The use of antioxidants in semen extenders plays a crucial role in preventing quality deterioration during dilution and storage. This study investigates the effects of quercetin supplementation in Tris-Aminomethane egg yolk extender on the semen quality of Dorper and Awassi rams stored at room temperature. Semen was collected twice weekly from Dorper and Awassi rams using an artificial vagina. A factorial experimental design was applied to evaluate the impact of quercetin concentrations (0, 30, 60, and 90 µM) and storage durations (0, 2, 4, 6, and 8 hours) at room temperature. The assessed parameters included individual motility, viability, abnormality, and membrane integrity. Data were analyzed using ANOVA and Duncan's multiple range test. The results indicated that quercetin significantly enhanced individual motility in Dorper ($P < 0.01$) and Awassi ($P < 0.05$) rams, with the optimal effect observed at 30 µM. However, viability, abnormality, and membrane integrity were not significantly affected by quercetin supplementation. Storage duration significantly influenced motility and membrane integrity in both breeds ($P < 0.01$). The addition of 30 µM quercetin maintained sperm motility for up to 8 hours at room temperature, with motility rates of 76.4 ± 5.11% (Dorper) and 74.6 ± 8.28% (Awassi). In conclusion, quercetin supplementation at 30 µM in Tris-Aminomethane extender effectively preserves the semen quality of Dorper and Awassi rams during room temperature storage, with potential applications for improving artificial insemination success rates.

Keywords | Quercetin, Semen extender, Sperm motility, Dorper rams, Awassi rams

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INTRODUCTION

Ram (*Ovis aries*) are among the most significant domesticated ruminants, contributing substantially to global livestock production. With over 1,200 recognized breeds, ram farming provides an essential source of meat, milk, and wool (Kizilaslan *et al*., 2024). In Indonesia, ram farming is a growing industry due to its economic viability, adaptability, and genetic potential for high reproductive performance (Chetroiu *et al*., 2024). Among the most promising breeds for meat production, Dorper and Awassi ram are widely recognized for their rapid growth, high

fertility rates, and adaptability to diverse environmental conditions. The success of breeding programs for these ram relies on reproductive biotechnologies, particularly Artificial Insemination (AI), which enables the genetic improvement of livestock populations by facilitating selective breeding.

AI is an indispensable technology in modern livestock production, allowing for superior genetic dissemination without direct male-female interaction. AI offers multiple advantages, including controlled breeding, improved genetic selection, and the prevention of sexually transmitted diseases (Bustani and Baiee, 2021). However, AI success is highly dependent on semen quality, which is compromised by storage-related oxidative stress and spermatozoa damage. Cryopreservation is commonly used for long-term semen storage but is associated with significant sperm motility loss (30–40%) due to cold shock and oxidative damage (Khan *et al*., 2021). While liquid semen storage at room temperature is a viable alternative, it remains a challenge to maintain sperm viability over time (Zhang *et al*., 2022).

Spermatozoa are highly susceptible to oxidative stress, primarily caused by excessive *Reactive Oxygen Species* (ROS) accumulation during storage. ROS lead to lipid peroxidation, membrane disruption, and reduced sperm viability, which negatively affect fertility outcomes (Sohail *et al*., 2024). Compared to other livestock species, ram spermatozoa contain higher concentrations of polyunsaturated fatty acids and exhibit lower intrinsic antioxidant defences, making them particularly vulnerable to oxidative damage (Wang *et al*., 2021). To mitigate these effects, antioxidant supplementation has been explored as a strategy to maintain semen quality during storage. Recent studies suggest that natural antioxidants incorporated into semen extenders can effectively improve sperm longevity and motility (Ewuola *et al*., 2021). Quercetin, a flavonoid with potent antioxidant properties, has shown promise in counteracting oxidative stress in spermatozoa. It effectively scavenges ROS and inhibits lipid peroxidation, preserving sperm membrane integrity and motility (Wei *et al*., 2024). While previous studies, such as Athalla *et al*. (2023), demonstrated that quercetin improves goat semen quality during cold storage (5°C), storage at room temperature presents a distinct physiological challenge. Ambient temperature storage is characterized by higher metabolic rates and accelerated ROS generation, creating intensified oxidative conditions that require specific evaluation (Wei *et al*., 2024). Several studies have investigated the role of quercetin in semen preservation. Similarly, hydroxytyrosol, another natural antioxidant, has been found to enhance sperm quality in pigs at room temperature (Li *et al*., 2022). These findings suggest that quercetin may serve as an effective bioactive compound for optimizing semen storage conditions in ram.

To enhance semen storage outcomes, Tris-Aminomethane egg yolk extenders have been widely utilized due to their buffering capacity and nutrient composition. Tris serves as an efficient pH stabilizer, while egg yolk provides cryoprotective benefits through its phospholipids and lipoproteins Bustani and Baiee (2021). Incorporating quercetin into this extender formulation could provide additional protection against oxidative stress, further prolonging sperm motility and viability during room temperature storage. However, the optimal concentration of quercetin for preserving Dorper and Awassi ram semen remains unexplored, necessitating a systematic evaluation of its effects over different storage durations.

While previous studies have investigated various antioxidants in semen preservation, gaps remain regarding their application in ram semen storage at room temperature. Taurine supplementation has demonstrated beneficial effects in preserving sperm quality in *Hu* ram Wang *et al*. (2021), and the Hypo-Osmotic Swelling Test (HOST) has been refined to assess sperm membrane integrity in felines (Prochowska *et al*., 2022). However, limited research has explored quercetin's specific impact on Dorper and Awassi ram semen. Moreover, while antioxidants such as vitamin C, vitamin E, and resveratrol have been widely studied, comparative evaluations of quercetin's effectiveness under non-cryogenic storage conditions remain insufficient (Seifi-Jamadi *et al*., 2017). Given the unique physiological properties of ram spermatozoa, further research is needed to establish optimal antioxidant concentrations and their long-term implications for reproductive success.

This study aims to evaluate the effects of quercetin supplementation on Dorper and Awassi ram semen quality during room temperature st orage. Specifically, it examines different quercetin concentrations. The novelty of this study lies in its focus on optimizing a non-cryogenic semen storage method using a naturally derived antioxidant, offering practical applications for AI programs in resource-limited settings. By addressing a critical gap in ram reproductive biotechnology, this research contributes to the advancement of AI methodologies and genetic selection strategies, ultimately enhancing livestock productivity and sustainability.

MATERIALS AND METHODS

Research location

The research was conducted at two different locations to facilitate semen collection and laboratory analysis. Semen was collected at Boerja Goat Farm, Lawang, Malang Regency, and subsequently analyzed at the Biotechnology Laboratory, Faculty of Animal Science, Brawijaya University, Malang. The rams were housed individually and were provided a diet consisting of elephant grass

(*Pennisetum purpureum*) and concentrate in a 60:40 ratio with ad libitum access to water.

Semen collection

The study utilized fresh semen from two three-year-old purebred rams of Dorper and Awassi breeds, with body weights of 80 kg until 90 kg, respectively. Semen collection was performed twice weekly (Monday and Thursday at 10:00 AM) within 2 months, using an Artificial Vagina (AV) following standard semen collection protocols (Sari *et al*., 2024). The artificial vagina was preheated to 45°C, lubricated with Vgel, and positioned appropriately. Upon collection, semen samples were transported within 15 minutes to the Biotechnology Laboratory of the Faculty of Animal Science, Universitas Brawijaya, under controlled temperature conditions.

Semen processing and extender preparation

Upon arrival at the laboratory, both macroscopic and microscopic evaluations were conducted to assess the quality of the semen and determine its suitability for further processing. The semen was subsequently diluted using a Tris-Aminomethane extender, which was supplemented with egg yolk as a cryoprotectant and quercetin as an antioxidant. This formulation has been widely employed to maintain sperm quality during storage by preventing oxidative stress and preserving membrane integrity.

The preparation of the Tris-Aminomethane extender involved several sequential steps to ensure optimal composition and homogeneity. First, a Tris-Aminomethane stock solution was prepared by dissolving Tris buffer in distilled water, followed by pH adjustment to 7.2–7.4 to maintain an optimal environment for spermatozoa. Once the buffer was adequately prepared, fresh egg yolk was incorporated into the solution at a concentration of 20%, acting as a protective agent against cold shock during storage. The mixture was then homogenized at 1270 rpm for 15 minutes to ensure uniform distribution of the protective components within the extender.

For antioxidant supplementation, quercetin (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock concentration of 1000 µM. This stock solution was subsequently diluted to achieve final treatment concentrations. The prepared extender solutions were then transferred into sterile centrifuge tubes and stored at 4°C until use to maintain their stability and bioactive properties.

Experimental design

This study employed a factorial Completely Randomized Design (CRD) to evaluate the impact of quercetin antioxidant supplementation and storage duration on semen quality. The experiment was structured around two independent factors: Factor A, representing the concentration of quercetin in the Tris-Aminomethane extender, and Factor B, denoting the duration of semen storage at room temperature (24–26°C). The Quercetin antioxidant concentrations tested included of 0 µM, 30 µM, 60 µM, and 90 µM. The semen samples were stored for 0, 2, 4, 6 and 8 hours at room temperature. Each treatment was replicated for 5 replications.

Semen quality evaluation

Macroscopic analysis

Following semen collection, macroscopic assessments were conducted to determine the initial quality and suitability of the samples for further processing. The volume was measured in millilitres (mL) using a graduated collection tube, while colour was visually inspected and categorized within a normal range from milky-white to creamy. The odour was evaluated based on its characteristic seminal scent, ensuring the absence of contamination or signs of infection. Additionally, consistency was assessed to determine viscosity and fluidity, as these parameters influence semen handling and preservation efficiency (Sari, *et al*., 2024).

Microscopic evaluation

Microscopic evaluations were performed using a phase-contrast microscope at 400× magnification to analyze the functional characteristics of spermatozoa according to (Sari *et al*., 2024).

Individual motility

The assessment of individual motility was conducted by collecting a semen sample using an inoculation loop (ose) and placing it onto a glass slide, which was subsequently covered with a cover slip. The sample was observed under a microscope at 400x magnification to evaluate the individual movement of spermatozoa. The assessment focused on identifying spermatozoa that exhibited progressive forward movement across five fields of view. Individual motility was expressed as a percentage, ranging from 0% to 100%.

Viability

Sperm viability was assessed by placing a drop of semen onto the edge of a glass slide using an inoculation loop. A drop of *eosin-nigrosin* stain was then added, and the mixture was homogenized in a clockwise direction. A smear was prepared using another glass slide held at a 45° angle. The prepared slide was either air-dried or briefly exposed to a Bunsen flame for 1–2 seconds to accelerate drying. The sample was subsequently examined under a microscope at 400x magnification. Viability assessment was performed by counting the number of spermatozoa that had absorbed the stain out of a total of 200 spermatozoa observed, using a Hand Tally Counter (HTC).

Abnormality

The assessment of sperm abnormalities followed the *eosin-nigrosin* staining method. A drop of semen was placed at the edge of a glass slide using an inoculation loop, followed by the addition of a drop of *eosin-nigrosin* stain. The mixture was homogenized in a clockwise motion before preparing a smear using another glass slide positioned at a 45° angle. The slide was then left to air-dry or briefly heated over a Bunsen flame for 1–2 seconds. The prepared sample was observed under a microscope at 400x magnification. The number of normal and abnormal spermatozoa was counted using a Hand Tally Counter (HTC).

Membrane integrity

The evaluation of sperm membrane integrity was performed by mixing 0.1 ml of semen with a hypotonic solution (HOS) at a concentration of 150 mOsml/liter. The dilution ratio used was 1:10 for fresh semen and 1:4 for semen mixed with an extender. The mixture was incubated at 37°C for 30 minutes. Following incubation, a drop of the sample was collected using an inoculation loop and placed onto a glass slide, which was then covered with a cover slip. The sample was observed under a microscope at 400x magnification to determine the number of spermatozoa exhibiting a coiled tail, which served as an indicator of membrane integrity. The percentage of spermatozoa with a coiled tail was calculated based on the total number of spermatozoa observed.

Statistical analysis

Data collected from all experimental groups were analyzed using Analysis of Variance (ANOVA) in SPSS (Version 23) to determine the effects of quercetin supplementation and storage duration on semen quality parameters. If a statistically significant effect was detected, Duncan's Multiple Range Test (DMRT, $P < 0.05$) was applied to further compare treatment means and establish significant differences between groups. This statistical approach ensured robust data interpretation and validation of the observed effects of antioxidant supplementation and storage duration on sperm quality.

RESULTS

Fresh semen quality

The examination of fresh ram semen is crucial before conducting research that evaluates the effect of antioxidant levels and storage duration at room temperature. This assessment determines the feasibility of semen as research material. The microscopic and macroscopic evaluation results of fresh semen from Dorper and Awassi rams collected at CV. Kambing Boerja, Lawang, Malang, are presented in Table 1.

Table 1: The characteristic fresh dorper and Awassi ram semen.

Variable	Dorper ram semen	Awassi ram semen
Volume (ml)	0.93±0.12	1.1±0.14
Colour	Milky white/cream	Milky white/ cream
Odor	Characteristic ram odor	Characteristic ram odor
Consistency	Thick/viscous	Thick/viscous
Mass Motility	(+++)	(+++)
Individual Motility (%)	83.33±5.77	85±7.07
Concentration (10^6/ml)	2778.33±1022.13	3720±438.41
Viability (%)	65.74±5.61	98.98±0.26
Abnormalities (%)	0.6±0.31	0.3±0.42
Membrane integrity (%)	62.46±3.01	55.18±4.6.2

Sperm motility during room temperature storage

The evaluation of spermatozoa motility variation in Dorper rams with the different addition level of antioxidant quercetin at concentrations and different storage durations (0 hours, 2 hours, 4 hours, 6 hours, and 8 hours) is presented in Table 2. and Awassi rams is presented in Table 3.

The variance analysis of individual spermatozoa motility in Dorper rams (Table 2) indicates that the addition of 30 µM quercetin yielded the highest average motility (76.4±5.11%), while the 90 µM quercetin treatment

Table 2: Percentage of individual motility in dorper ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					Average
	0	2	4	6	8	
0 µM	83.00±4.47	74.00±5.48	68.00±4.47	69.00±5.48	66.00±5.48	72.00±7.77^{c}
30 µM	82.00±2.74	79.00±2.24	78.00±4.47	71.00±2.24	72.00±2.74	76.40d±5.11^{d}
60 µM	76.00±5.48	72.00±4.47	71.00±2.24	64.00±5.48	61.00±2.24	68.80±6.81^{b}
90 µM	78.00±4.47	70.00±6.12	63.00±4.47	61.00±2.24	58.00±4.47	66.00±8.42^{a}
Average	79.75±4.99^{d}	73.75±5.59^{c}	70.00±6.69^{b}	66.25±5.59^{a}	64.25±6.54^{a}	

Note: Different notations in the same row and column indicate a highly significant difference ($P < 0.01$).

Table 3: Percentage of individual motility in Awassi ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					
	0	2	4	6	8	Average
0 µM	84.00±5.48	76.00±5.48	70.00±6.12	67.00±6.71	65.00±5.00	72.40±8.79[a]
30 µM	82.00±4.47	76.00±5.48	76.00±5.48	78.00±2.74	61.00±2.24	74.60d±8.28[b]
60 µM	81.00±2.24	72.00±4.47	72.00±4.47	70.00±7.07	62.00±2.74	71.40±7.43[a]
90 µM	82.00±2.74	74.00±5.48	66.00±5.48	67.00±8.37	62.00±2.74	70.10±8.72[a]
Average	82.25±3.80[d]	74.50±5.10[c]	71.00±6.20[b]	70.50±7.59[b]	62.50±3.44[d]	

Note: Different notations in the same row and column indicate a significant difference (P < 0.05).

Table 4: Percentage of spermatozoa viability in dorper ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					
	0	2	4	6	8	Average
0 µM	97.97±1.18	95.46±1.27	96.54±1.99	96.78±3.45	95.23±1.67	96.40±2.15
30 µM	97.00±1.03	95.92±2.50	96.6±3.15	93.49±8.00	95.57±4.81	95.72±4.35
60 µM	97.79±1.12	96.66±3.11	97.62±1.15	93.17±3.30	96.01±3.68	96.25±3.00
90 µM	95.87±3.21	93.95±4.21	96.57±2.70	95.94±2.97	95.82±2.17	95.63±2.99
Average	97.16±1.92	95.50±2.91	96.83±2.23	94.85±4.76	95.23±1.67	

Table 5: Percentage of spermatozoa viability in Awassi ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					
	0	2	4	6	8	Average
0 µM	96.12±2.44	95.56±2.06	93.2±3.98	95.22±3.1	96.41±1.79	95.3±2.79
30 µM	94.83±3.46	95.66±2.21	96.06±1.88	95.94±3.39	96.86±1.66	95.86±2.50
60 µM	97.79±1.12	95.61±2.4	96.64±1.6	95.82±2.46	97.04±2.19	96.58±2.02
90 µM	95.44±2.39	95.4±2.05	96.74±1.68	94.28±4.1	97.81±1.27	95.93±2.60
Average	96.05±2.55	95.56±2.01	95.66±2.72	95.32±3.12	97.03±1.70	

resulted in the lowest average motility (66±8.42%). The analysis further confirms that quercetin supplementation significantly influences spermatozoa motility across all concentrations (P < 0.01). The sequential mean motility values at 0, 2, and 4 hours of storage were 79.75±4.99%, 73.75±5.59%, and 70.00±6.69%, respectively. The highest motility was recorded at 0 hours (79.75±4.99%), while the lowest was at 8 hours (64.25±6.54%). These results demonstrate that prolonged storage at room temperature significantly reduces spermatozoa motility in Dorper rams (P < 0.01).

The variance analysis of individual spermatozoa motility in Awassi rams (Table 3) confirms that quercetin supplementation significantly affects spermatozoa motility (P < 0.05). The addition of 30 µM quercetin resulted in the highest motility (74.6±8.28%), while the 90 µM treatment had the lowest (70.1±8.72%). The highest mean motility was observed at 0 hours (82.25±3.80%), while the lowest was at 8 hours (62.5±3.44%). This suggests that storage duration significantly impacts spermatozoa motility in Awassi rams (P < 0.01).

Spermatozoa viability during room temperature storage

The evaluation of spermatozoa viability variation in Dorper rams with the different addition level of antioxidant quercetin at concentrations and different storage durations (0 hours, 2 hours, 4 hours, 6 hours, and 8 hours) is presented in Table 4 and Awassi rams is presented in Table 5.

The variance analysis of Dorper ram semen viability during room temperature storage (Table 4) shows that the addition of antioxidant quercetin at concentrations of 0 µM, 30 µM, 60 µM, and 90 µM did not result in significant differences in semen viability. The highest average viability was observed in the 0 µM quercetin treatment (without quercetin) at 96.40±2.15%, while the lowest was recorded in the 90 µM quercetin treatment at 95.63±2.99%. Furthermore, storage duration did not significantly impact semen viability, with the highest mean viability at 0 hours (97.16±1.92%) and the lowest at 6 hours (94.85±4.76%).

Table 6: Percentage of abnormalities in dorper ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					
	0	2	4	6	8	Average
0 µM	0.78±0.27	0.64±0.26	0.60±0.23	0.66±0.26	0.74±0.28	0.68±0.25
30 µM	0.72±0.27	0.57±0.23	0.73±0.23	0.63±0.26	0.85±0.21	0.70±0.24
60 µM	0.76±0.25	0.82±0.21	0.74±0.25	0.74±0.25	0.64±0.26	0.73±0.23
90 µM	0.63±0.23	0.63±0.2	0.57±0.17	0.56±0.17	0.54±0.17	0.59±0.18
Average	0.72±0.24	0.67±0.23	0.66±0.22	0.65±0.23	0.74±0.24	

Table 7: Percentage of abnormalities in Awassi ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					
	0	2	4	6	8	Average
0 µM	0.71±0.21	0.74 ±0.27	0.77±0.25	0.59±0.23	0.66±0.24	0.69±0.23
30 µM	0.67±0.2	0.84 ±0.19	0.68±0.23	0.48±0.04	0.66±0.22	0.67±0.21
60 µM	0.70±0.21	0.75±0.26	0.64±0.25	0.73±0.21	0.58±0.22	0.68±0.22
90 µM	0.60±0.23	0.63±0.27	0.66±0.25	0.67±0.23	0.47±0.03	0.61±0.22
Average	0.67±0.20	0.74±0.24	0.69±0.23	0.62±0.20	0.59±0.20	

The variance analysis of Awassi ram semen viability (Table 5) indicates that quercetin supplementation at different concentrations did not significantly affect viability. The highest viability mean was recorded in the 60 µM quercetin treatment (96.58±2.02%), whereas the lowest was in the 0 µM quercetin treatment (95.3±2.79%). Additionally, storage duration had no substantial effect on viability, with the lowest mean at 6 hours (95.32±3.12%).

Spermatozoa abnormalities after room temperature storage

Evaluation of spermatozoa abnormalities is important to determine the normality of the spermatogenesis process of a male and to know the accuracy of handling semen during the experiment. Antioxidants and duration of room temperature storage likely contributed in producing indicators of spermatozoa abnormalities, as shown in Tables 6 and 7.

The variance analysis of spermatozoa abnormalities in Dorper ram semen (Table 6) revealed that the addition of antioxidant quercetin at different concentrations (0 µM. 30 µM. 60 µM. and 90 µM) did not significantly affect sperm abnormalities. The highest average abnormality rate was recorded in the 60 µM quercetin treatment (0.73±0.23%). while the lowest was observed in the 90 µM quercetin treatment (0.59±0.18%). Storage duration also did not have a significant effect on sperm abnormalities. with the lowest average recorded at 6 hours (0.65±0.23%) and the highest at 8 hours (0.74±0.24%).

Similarly, the analysis of spermatozoa abnormalities in Awassi ram semen (Table 7) showed no significant effect of quercetin supplementation. The highest average abnormality rate was recorded in the Quercetin of 0 µM treatment (0.69±0.23%). while the lowest was in the 90 µM quercetin treatment (0.61±0.22%). The highest abnormality rate was observed at 2 hours (0.74±0.24%). while the lowest was at 8 hours (0.59±0.20%).

Spermatozoa membrane integrity after room temperature storage

The variance analysis of membrane integrity in Dorper ram semen (Table 8) revealed that the addition of antioxidant quercetin at concentrations of 0 µM, 30 µM, 60 µM, and 90 µM did not significantly affect membrane integrity. The highest average membrane integrity was observed in the 30µM Quercetin treatment (57.01±21.17%), while the lowest was recorded in the 0 µM Quercetin treatment (46.97±25.47%). Storage duration significantly affected membrane integrity ($P<0.01$), with the highest mean at 0 hours (67.98±19.46%) and the lowest at 8 hours (36.58±24.05%).

Similarly, the analysis of membrane integrity in Awassi ram semen (Table 9) showed no significant effect of quercetin supplementation. The highest mean membrane integrity was observed in the 60 µM Quercetin treatment (75.62±10.77%), while the lowest was in the 0 µM Quercetin treatment (73.52±10.37%). Storage duration significantly influenced membrane integrity ($P<0.01$), with the highest average recorded at 0 hours (83.28±5.21%) and the lowest at 6 hours (66.75±11.11%).

Table 8: Percentage of membrane integrity in dorper ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					Average
	0	2	4	6	8	
0 µM	63.31±3.92	39.91±4.11	62.76±1.01	46.48±2.93	22.39±7.6	46.97±5.47
30 µM	68.37±5.28	46.8±9.24	68.44±2.42	61.20±0.13	40.23±2.68	57.01±1.17
60 µM	75.36±5.41	37.96±6.2	64.51±4.32	37.45±3.58	45.97±9.29	52.25±7.69
90 µM	64.87±5.47	46.87±4.76	54.92±2.38	35.32±7.65	37.74±6.16	47.95±7.41
Average	67.98±9.46[b]	42.89±6.91[a]	62.66±0.37[b]	45.11±2.79[a]	22.39±7.6	

Note: Different notations in the same row indicate a highly significant difference (P < 0.01).

Table 9: Percentage of membrane integrity in Awassi ram semen at different quercetin concentrations and room temperature storage durations.

Level of quercetin	Storage time at room temperature (hours)					Average
	0	2	4	6	8	
0 µM	81.55±6.28	78.04±4.72	64.43±15.39	73.64±4.6	69.92±9.99	73.52±10.37
30 µM	81.48±1.93	78.51±4.62	67.98±14.8	67.83±10.85	74.07±7.14	73.98±10.01
60 µM	86.87±5.13	77.31±3.95	75.63±7.66	63.62±14.81	74.66±5.75	75.62±10.77
90 µM	83.21±5.95	75.87±1.67	70.12±14.24	73.9±11.68	66.96±8.99	74.01±10.40
Average	83.28±[c]5.21	77.43±3.76[b]	69.54±12.97[a]	66.75±11.11[a]	71.4±8.13[a]	

Note: Different notations in the same row and column indicate a highly significant difference (P < 0.01).

Multivariate evaluation of semen quality characteristics in dorper and Awassi rams

The Principal Component Analysis (PCA) showed a significantly different pattern of variance between the two ram nations (Table 10), where the Awassi ram had a more concentrated variance structure with the accumulation of PC1 (49.89%) and PC2 (33.10%) which explained 82.99% of the total variability. In the Awassi nation, the first component (PC1) showed a negative correlation between viability (0.700) and abnormality (-0.699), reflecting cell viability as the main differentiator of cement quality, while PC2 reflected functional properties through high positive loading values on motility (0.717) and plasma membrane integrity (IMP) (0.695). In contrast, Dorper ram exhibited a wider variance distribution with the accumulation of PC1 (34.77%) and PC2 (27.88%) accounting for only 62.65% variability, where PC1 was more influenced by motility (0.639) and IMP (0.499) with a negative contribution of viability (-0.533). Unique characteristics appear in PC2 Dorper ram which are specifically dominated by abnormalities (0.759), suggesting that morphological defect factors are a dimension of independent variation that is more prominent in this breed than in Awassi ram.

DISCUSSION

The examination of fresh semen volume from Dorper and Awassi rams (Table 1) aimed to determine the amount of semen obtained during collection. The results showed variability during collection, but the semen volume was within the normal range. Kumar *et al.* (2024) stated that the normal volume of ram semen ranges between 0.8 ml/ejaculation and 1.2 ml/ejaculation. This study found that the volume of Awassi ram semen was higher than that of Dorper rams, which can be attributed to breed differences and collection frequency. According to Solihati *et al.* (2018), variations in semen volume during collection are influenced by several factors, including breed, age, body size, feed quality, body condition, and collection frequency.

Table 10: Principal component loadings for semen quality traits in dorper and Awassi ram.

Breed	Parameters	PC1	PC2	PC3	PC4
Dorper	Motilitas	0.639	-0.110	-0.397	-0.649
	Viabilitas	-0.533	-0.312	-0.787	0.009
	Abnormalitas	0.242	0.759	-0.460	0.392
	IMP	0.499	-0.561	-0.108	0.652
	Eigenvalue	1.391	1.115	0.803	0.692
	Variance (%)	34.77	27.88	20.07	17.29
Awassi	Motility	-0.057	0.717	0.695	0.012
	Viability	0.700	-0.030	0.101	-0.707
	Abnormalities	-0.699	0.046	-0.093	-0.707
	IMP	0.136	0.695	-0.706	0.005
	Eigenvalue	1.996	1.324	0.649	0.031
	Variance (%)	49.89	33.10	16.23	0.79

The color of fresh semen from Dorper and Awassi rams was milky white/cream, which falls within the normal

range. Kumar *et al.* (2024) reported that normal ram semen color varies between milky white, whitish, or cloudy. The semen color is associated with sperm concentration, where higher sperm concentrations result in denser or cloudier semen. Color serves as an indicator of semen quality, and any color abnormalities indicate contamination. A reddish or greenish-yellow hue suggests microbial contamination or reproductive tract injuries (Athalla *et al.*, 2023).

The odor of fresh semen from both Dorper and Awassi rams was characteristic of ram semen, indicating normal conditions without contamination. The odor of fresh semen is assessed by bringing the collection tube close to the nose; good-quality semen has a distinctive fishy odor similar to that of the collected animal. Abnormal the odor of fresh semen suggests contamination (Nahriyanti *et al.*, 2017). The consistency of fresh semen from Dorper and Awassi rams was categorized as thick or viscous. Semen consistency is related to sperm concentration, where a higher sperm concentration results in a more viscous semen texture. This aligns with the findings of Nahriyanti *et al.* (2017), who stated that consistency is assessed by shaking the collection tube and observing the semen flow semen with slow flow is thick, while rapidly flowing semen is dilute. Semen consistency is influenced by collection frequency.

The mass motility examination of fresh semen from Dorper and Awassi rams was rated as 3+ (excellent), characterized by large, numerous, dark, thick, and actively moving sperm waves under the microscope. This value meets the standard for semen processing for artificial insemination, which requires a minimum mass motility of 2+ and individual motility of at least 70% (Athalla *et al.*, 2023). The individual motility of fresh semen from Dorper rams averaged 83.33±5.77%, while Awassi rams averaged 85±7.07%, indicating normal sperm motility. Most fertile males exhibit semen motility between 50% and 80%. Differences in individual motility between Dorper and Awassi rams may be attributed to environmental temperature factors.

The mean sperm concentration of fresh Dorper ram semen was 2778.33±1022.13 $x10^6$/ml, while Awassi rams had 3720±438.41 $x10^6$/ml. According to Kumar *et al.* (2024), normal ram semen concentrations range between 2000 and 6000 million/ml, indicating that the observed values fall within normal limits. The higher concentration in Awassi rams compared to Dorper rams is likely due to differences in body weight. In rams, body weight is positively correlated with scrotal circumference and testicular weight, which directly determines the volume of sperm-producing. This finding aligns with Salvado *et al.* (2024), who stated that semen concentration variability during collection is influenced by factors such as animal age and body weight.

The viability of fresh semen from Dorper rams averaged 65.74±56.11%, while that of Awassi rams was 98.98±0.26%. These values meet the minimum viability standards for semen used in artificial insemination. According to Soltanpour *et al.* (2013), the viability percentage for diluted or frozen semen should be at least 60% to 75%. Based on these averages, Awassi ram semen exhibited superior ability to maintain plasma membrane integrity compared to Dorper ram semen. Prastika *et al.* (2018) emphasized that sperm viability is determined by plasma membrane integrity, which functions to protect sperm organelles and facilitate sperm metabolism. Plasma membrane damage affects sperm metabolism, leading to decreased viability and increased sperm mortality.

The mean abnormality rates of fresh semen from Dorper and Awassi rams were 0.6±0.31% and 0.3±0.42%, respectively, which are within normal limits. Normal ram semen abnormality rates range between 5% and 20%. The most commonly observed abnormalities were tail fractures. Abnormalities serve as an indicator of sperm quality, as structural damage can impair fertility. Sperm abnormalities are characterized by head and tail deformities, such as bent, coiled, or broken tails. An abnormality rate exceeding 20% leads to reduced fertility (Perry, 2021).

The mean membrane integrity of fresh Dorper ram semen was 62.46±30.15%, while Awassi rams had 55.18±46.21%. Membrane integrity values ranging from 50% to 70%. Membrane integrity is assessed by observing the presence of swollen and coiled tails in spermatozoa, indicating good sperm condition due to intact plasma membranes.

Spermatozoa motility is a reference in determining the quality of semen before and after processing because motility is closely related to fertility. According to Van de Hoek *et al.* (2022) stated that motility is a determinant of the success of spermatozoa to reach the ovum and the simplest way to assess sperm for artificial insemination. The motility of individual semen continues to decrease during storage at room temperature, this is suspected to be due to changes in environmental conditions, especially the environmental temperature and diluent materials used and the level of adaptation of spermatozoa to these two things. The process of adaptation of spermatozoa to diluent materials can result in impaired membrane permeability, decreased metabolic activity, cell damage, and decreased motility of spermatozoa. The addition of the antioxidant quercetin 30 µM was proven to be able to maintain the motility quality of individual spermatozoa semen of Dorper and Wassi ram.

A comparison analysis between Tables 2 and 3 reveals that Awassi ram semen exhibited higher initial motility at 0 hours (82.2±3.80%) compared to Dorper ram semen (79.75±4.99%). However, both breeds demonstrated a significant decline in motility over time. Notably, the addition of 30 µM quercetin provided the highest motility retention in both breeds, suggesting its efficacy in preserving spermatozoa motility during storage. Conversely, the 90 µM quercetin treatment resulted in the lowest motility in both breeds, indicating potential cytotoxic effects at higher concentrations. The decline in motility across storage durations aligns with findings from Sari *et al*. (2024), which suggest that prolonged exposure to environmental factors and extender composition affects sperm viability. These results emphasize the importance of optimizing antioxidant concentrations to enhance semen preservation for artificial insemination applications.

Viability is a determinant of semen quality indicators by looking at the percentage of live spermatozoa. The viability of Dorper and Awassi ram semen was observed using eosinnegrosin staining where live spermatozoa were characterized by not absorbing eosin-negrosin color. Spermatozoa that burn eosin-negrosin color indicate that the plasma membrane of the spermatozoa is damaged. Spermatozoa plasma membrane functions in maintaining intracellular and extracellular electrolyte balance, plasma membrane damage will disrupt metabolic processes and cause death. The average viability during quercetin treatment is above 90% so that Dorper and Awassi ram semen are eligible for artificial insemination where the viability is at least 70%. The results showed that the diluent Tris-aminomethane egg yolk and the antioxidant quercetin were able to maintain and provide protection against the viability of Dorper ram spermatozoa during room temperature storage. The viability of Dorper ram spermatozoa is very good because the diluent Tris-Aminomethane has substances needed by spermatozoa consisting of fructose, lactose, raffinosa, amino acids, and vitamins. The yolk acts as an extracellular cryoprotectan where the content of lipoprotein and lecithin prevents cold shock and the content of amino acids, carbohydrates, vitamins, and minerals as the necessities of spermatozoa (Saifudin *et al*., 2018).

In comparison of Tables 4 and 5 reveals that both Dorper and Awassi ram semen maintained high viability throughout storage, with mean values consistently above 90%. However, Awassi ram semen demonstrated slightly higher overall viability, particularly at the 8-hour mark, where the average viability was 97.03±1.70% compared to 95.23±1.67% for Dorper rams. The supplementation of 60 µM quercetin exhibited the highest viability in both breeds, indicating its potential as an effective antioxidant for preserving sperm integrity. These findings align with previous studies, which reported that the inclusion of antioxidants such as quercetin in semen extenders enhances sperm survival during storage (Nugraha *et al*., 2023). Additionally, Tris-aminomethane and egg yolk extenders provided essential nutrients such as fructose, lactose, raffinose, amino acids, and vitamins necessary for sperm survival, while the lipoproteins and lecithin content of egg yolk acted as extracellular cryoprotectants, reducing cold shock damage (Saifudin *et al*., 2018). These results underscore the importance of optimizing semen extender composition to enhance artificial insemination success rates.

Abnormalities are related to the structure of spermatozoa and affect the fertility of spermatozoa. High abnormalities will reduce the pregnancy rate. According to Sterbenc *et al*. (2019) the forms of abnormalities are divided into 2, namely primary and secondary abnormalities. Primary abnormalities are abnormalities caused by failures in the process of spermatogenesis. Abnormality is due to hereditary factors and poor environment. Secondary abnormalities are caused by the storage and cryopreservation process of spermatozoa as well as treatment during semen processing. Factors that affect the high and low abnormality are temperature, storage time, and genetic factors. The average abnormality produced by each treatment during the study was 1%, which indicates that the abnormality of dorper and Awassi ram semen is very low. According to Afiati (2015) stated that the abnormality of ram spermatozoa ranged from 5-20%, abnormalities that exceeded 20% indicated symptoms of infertility in housed males.

A comparison of Tables 6 and 7 data indicates that both Dorper and Awassi ram semen exhibited low abnormality rates. Remaining below 1% throughout the storage period. The overall abnormality levels in Dorper rams were slightly higher than in Awassi rams. Particularly in the 60 µM quercetin treatment. Where the highest value was observed. However, in both breeds. The 90 µM quercetin treatment consistently showed the lowest abnormality rates. Suggesting that higher quercetin concentrations might have a protective effect against spermatozoa structural damage. These findings are consistent with previous studies indicating that controlled temperature changes slow the rate of spermatozoa abnormality development. Allowing for better adaptation to new environmental conditions (Sari *et al*., 2024). Additionally. Research by Afiati (2015) suggests that sperm abnormalities are influenced by genetic factors, storage temperature, and preservation duration. The consistently low abnormality rates across treatments demonstrate that quercetin supplementation. Particularly at higher concentrations, could play a role in maintaining sperm integrity and viability during room temperature storage. Further supporting its application in artificial

insemination protocols.

Membrane integrity refers to the ability of spermatozoa to maintain the integrity of the plasma membrane as one of the important factors in determining spermatozoa fertility. The integrity of the spermatozoa membrane can be tested using the HOS Test (Hypoosmotic Swelling Test) test. The plasma membrane functions to maintain the electrolyte balance in spermatozoa. Damage to the membrane will cause a decrease in fertility rates and even death for spermatozoa. Spermatozoa with a damaged membrane cannot adjust their osmose pressure so that the tail does not bubble while spermatozoa with an intact membrane of the tail will experience bending or swelling (Hameed *et al*., 2024). According to Solihati *et al*. (2018) semen can be said to be normal if it has plasma membrane integrity ranging from 55% to 60%. Based on this opinion, Dorper ram semen that is classified as normal is 30 µM Quercetin. Egg yolk Tris aminomethane diluent can maintain the integrity of the plasma membrane during storage. The buffer material in the Tris Aminomethane diluent in the form of raffinosa provides a source of nutrients for spermatozoa and raffinosa will associate with carbohydrates in the spermatozoa cell sheath so that the plasma membrane can be protected during processing (Iskandari *et al*., 2020). Plasma membrane damage in 0µM, 60 µM, and 90µM Quercetin treatments due to spermatozoa's adaptation to low diluents will reduce the permeability level of spermatozoa membrane.

Differences in the pattern of membrane integrity decline between Dorper and Awassi rams during storage up to 8 hours, as well as the large standard deviations observed in Dorper data, primarily reflect biological variation among sires and breed-specific physiological characteristics of the sperm membrane. In Dorper rams, the higher heterogeneity of initial ejaculate quality and a membrane composition richer in unsaturated fatty acids make their spermatozoa more susceptible to lipid peroxidation and oxidative stress during storage, resulting in a sharp decline in membrane integrity and wider variability in HOST responses (Holt, 2000). In contrast, Awassi spermatozoa showed better membrane stability up to 8 hours, likely due to a more stable lipid composition and a lower cellular metabolic rate, which contribute to reduced membrane damage and more consistent data (Watson, 2000). These findings support previous reports indicating that genetic and physiological differences between breeds play a major role in determining plasma membrane resilience during liquid semen storage.

Comparison analysis of Tables 8 and 9 indicates that Awassi ram semen consistently exhibited higher membrane integrity across all treatments and storage durations than Dorper ram semen. The highest membrane integrity was observed at 0 hours for both breeds, with Awassi semen maintaining a superior mean (83.28±5.21%) compared to Dorper semen (67.98±19.46%). However, a significant decline in membrane integrity was noted over time, with Dorper semen showing a more pronounced reduction, particularly at 8 hours (36.58±24.05%) compared to Awassi semen (71.4±8.13%). This suggests that Awassi ram spermatozoa might have greater resilience against environmental stressors during room temperature storage. Previous studies have reported that antioxidant supplementation, including quercetin, helps mitigate oxidative stress and maintain membrane integrity during semen preservation (Mahgoub *et al*., 2022). These findings emphasize the potential benefits of optimizing antioxidant concentrations to enhance semen storage conditions and improve artificial insemination outcomes.

Comparison analysis of Table 10 indicated that PCA loading plot analysis showed a marked difference in the structure of variance in semen quality between Dorper and Awassi rams. Awassi rams showed a much higher concentration of variance in the first two main components (PC1 and PC2) of 82.99% compared to Dorper rams, which was only 62.65% (Figure 1). In the Awassi breed, the main dimension of variation was driven by a very strong antagonistic relationship between viability and abnormality, indicating that the structural integrity of the cell is a key determinant of quality in this breed. This finding aligns with previous studies indicating that different sheep breeds exhibit distinct sperm morphometric and kinematic profiles due to genetic variations in testicular physiology and spermatogenesis efficiency (Goshme *et al*., 2020; Yana *et al*., 2023). In contrast, in Dorper rams, PC1 was more affected by functional parameters such as Motility and IMP, while abnormalities emerged as independent variables in PC2. This suggests that the sperm physiological profiles of the two breeds have different correlations with environmental factors, where Dorper semen quality assessment relies heavily on kinetic functionality, consistent with reports on the variability of motility subpopulations in specific ram breeds (Santiani *et al*., 2016).

Visualization of the PCA biplot based on quercetin supplementation levels showed clustering that tended to overlap but had different vector direction tendencies in the two ram breeds (Figure 2). In Dorper rams, treatment clusters with specific quercetin doses (P2 and P3) tended to shift in the direction of plasma membrane motility and integrity (IMP) vectors. This signals the crucial role of this antioxidant in mitigating lipid peroxidation damage to cell membranes and maintaining mitochondrial function. Quercetin has been proven to protect the plasma membrane rich in polyunsaturated fatty acids

from oxidative stress, thereby preserving sperm motility parameters (Seifi-Jamadi *et al*., 2017; Rather *et al*., 2016). Meanwhile, in the Awassi biplot, the effect of quercetin was more pronounced in maintaining viability stability and suppressing the rate of abnormalities during the incubation process. This proves that quercetin acts as an effective protective agent in neutralizing reactive oxygen species (ROS), thereby preventing oxidative damage to the sperm head and tail structures which often leads to morphological defects (Batool *et al*., 2024).

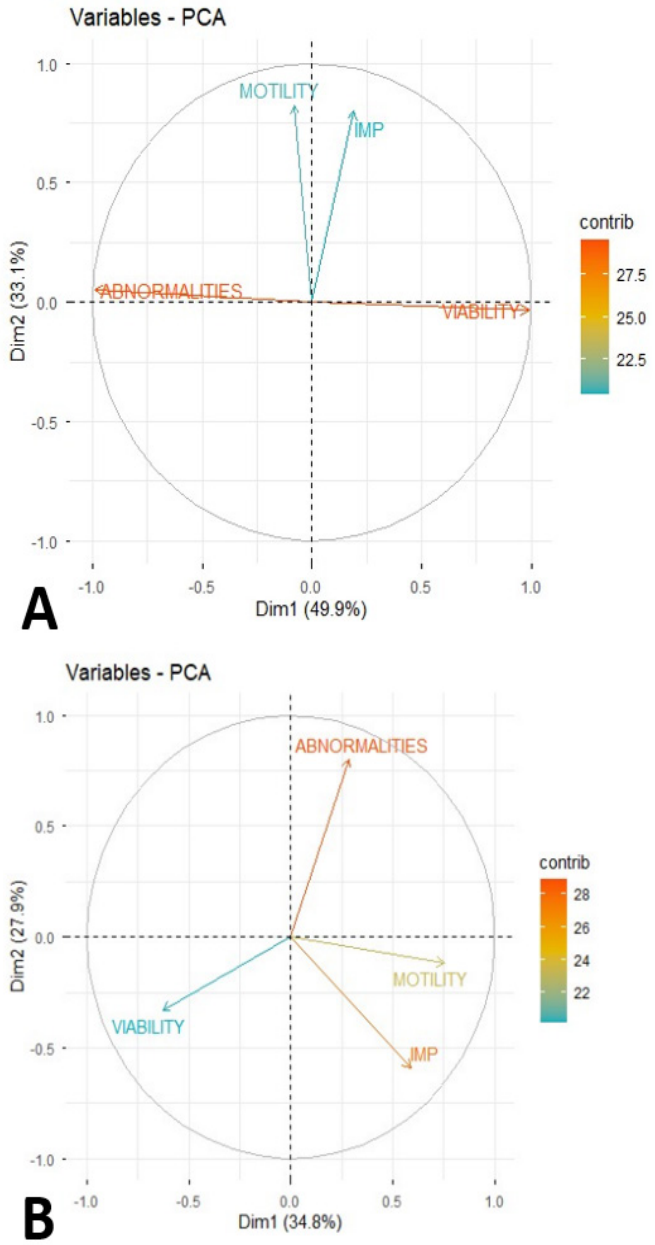

Figure 1: Principal Component Analysis (PCA) Loading Plot of Semen Characteristics in Dorper Ram (**A**) and Awassi Ram (**B**). Variables included are motility, viability, abnormality, and Plasma Membrane Integrity (IMP).

PCA analysis based on semen quality during liquid storage for 8 hours showed a significant convergence pattern, where the sample at 0 hours had the widest elliptical variance and was decisively separated from the rest of the storage times (Figure 3). As the storage duration increases, the position of the sample cluster gradually shifts away from the motility and viability vectors, reflecting a progressive decline in the metabolic activity and thermodynamic stability of the sperm membrane. This time-dependent deterioration is a common phenomenon caused by the accumulation of lactic acid and ROS in the extender, which compromises sperm viability over time (Bustani and Baiee, 2021; Allai *et al*., 2018). In Awassi rams, this shift is mainly influenced by a sharp decline in viability, whereas in Dorper rams, the progressive loss of motility is a key indicator of cell aging. This phenomenon confirms that storage time is a critical factor that alters the correlation structure between semen parameters, where the interaction between exogenous antioxidants and incubation time determines the rate of decline in sperm functionality (Rizkallah *et al*., 2022).

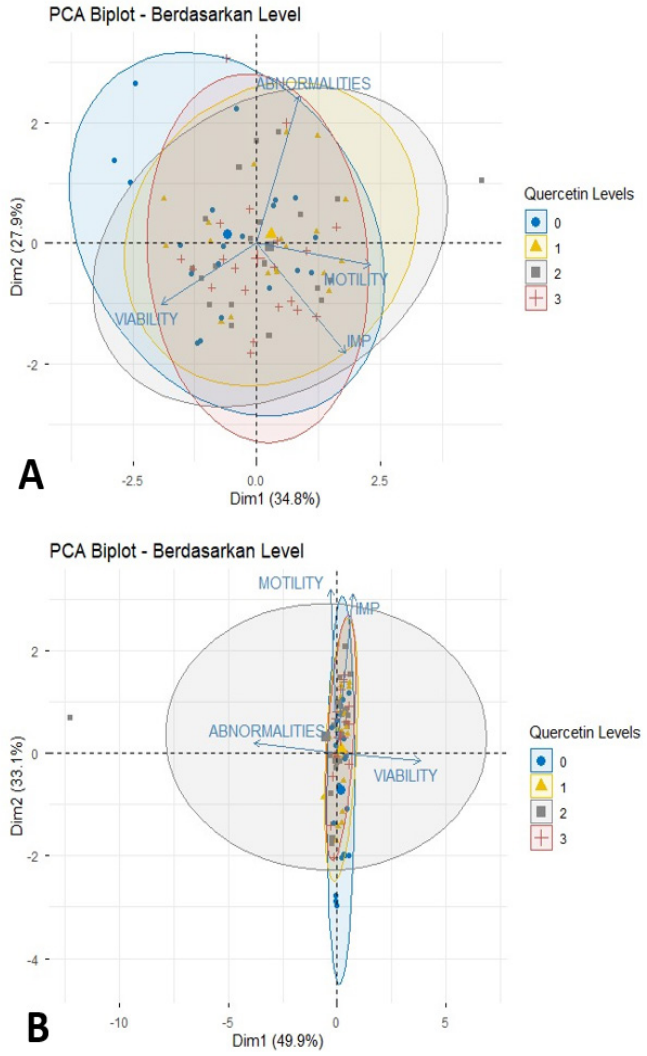

Figure 2: Principal Component Analysis (PCA) Biplot of Dorper Ram (**A**) and Awassi Ram (**B**) Semen Quality Characteristics Across Different Quercetin Levels (P0, P1, P2,P3).

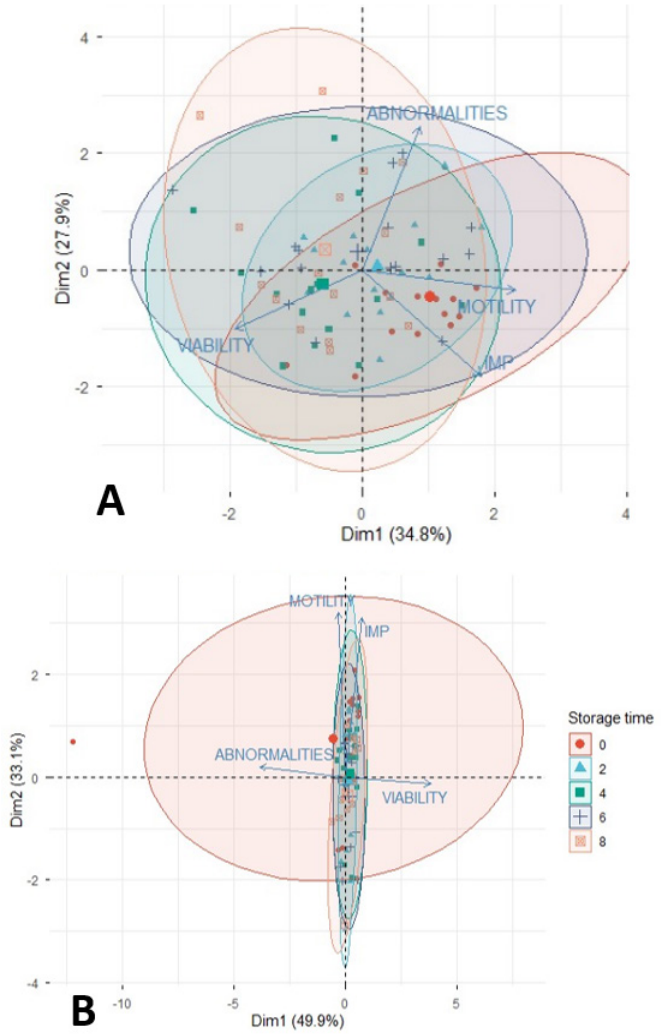

Figure 3: Principal Component Analysis (PCA) biplot of Dorper ram (A) and Awassi ram (B) semen quality characteristics across different storage durations (0, 2, 4, 6, and 8 hours).

CONCLUSION

Penelitian ini menyimpulkan bahwa suplementasi Quercetin dalam pengencer Tris-aminomethane kuning telur secara efektif mempertahankan kualitas semen domba Dorper dan Awassi selama penyimpanan suhu ruang, dengan konsentrasi 30 µM terbukti optimal untuk mempertahankan motilitas dan 60 µM untuk viabilitas. Meskipun kedua bangsa domba mengalami penurunan kualitas sperma secara progresif selama periode penyimpanan 8 jam, domba Awassi menunjukkan ketahanan yang lebih unggul, yang ditandai dengan viabilitas dan integritas membran plasma yang jauh lebih tinggi dibandingkan domba Dorper. Sebaliknya, konsentrasi yang lebih tinggi (90 µM) cenderung menurunkan kualitas selama preservasi.

Analisis PCA menyimpulkan bahwa domba Awassi memiliki struktur variansi kualitas semen yang lebih seragam dan terkonsentrasi dibandingkan domba Dorper, dengan parameter viabilitas dan abnormalitas sebagai pembeda utama. Secara keseluruhan, suplementasi astaxantin terbukti efektif dalam menjaga stabilitas fungsional sperma kedua bangsa domba terhadap degradasi kualitas akibat durasi penyimpanan hingga 8 jam.

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NOVELTY STATEMENT

This study the first to report the effect of Quercetin that focus on optimizing a non-cryogenic semen storage method using a naturally derived antioxidant, offering practical applications for AI programs in resourcelimited settings

AUTHOR'S CONTRIBUTION

SS & ANV: Conceptualization, data curation, data analysis, writing original draft, laboratory analysis, data interpretation, manuscript drafting, and revision.
AAA, CDN, RFP, AA: Conceptualization, manuscript review, data interpretation.
WAS : Manuscript review, data analyzed.
TAK: Writing final review.
SS: Corresponding author, conceptualization, manuscript review

Generative AI and AI-assisted technology statement

The authors acknowledge that generative AI tools (e.g., Google Gemini, language editing software) were used only to improve grammar and language clarity. No AI tools were used for data analysis, interpretation or scientific conclution. The authors take full responsibility for the content of this article.

Conflict of interest

The authors have declared no conflict of interest related to the publication of this article.

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