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Research Article
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Effect of extenders supplemented with varying levels of royal jelly on caprine semen quality at different chilling storage times

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Abstract: This study investigated the impact of royal jelly (RJ) supplementation on the quality of caprine semen during chilled storage. Twelve semen samples were collected from three mature bucks and divided into four treatment groups. Each group was diluted using a tris–citric acid–fructose–egg yolk (TCFY) extender supplemented with different concentrations of RJ (0%, 0.05%, 0.10%, and 0.15%). Semen quality parameters, including total motility, progressive motility, viability, morphology, sperm concentration, semen volume, and pH, were evaluated at 0, 24, and 48 hours of storage at 4 °C. The results demonstrated that RJ supplementation influenced semen quality in a concentration-dependent manner. Low to moderate concentrations of RJ (0.05% and 0.10%) showed a partial protective effect by maintaining higher sperm membrane viability at extended storage (48 h) compared to the highest concentration, but did not consistently improve motility parameters. In contrast, the highest concentration (0.15%) resulted in a significant reduction ($p < 0.05$) in sperm motility and viability, particularly after 48 hours of storage. Sperm morphology was generally unaffected by RJ supplementation, while semen volume remained stable and pH showed a slight decline with increased storage duration. Overall, RJ did not improve total or progressive motility compared to the T0, but exhibited limited concentration-dependent effects on sperm viability. These findings indicate that RJ supplementation cannot be considered a general enhancer of caprine semen quality during chilled storage, as its effects vary depending on concentration and parameter measured. Therefore, its application as a semen extender additive requires careful optimisation, particularly to avoid potential inhibitory effects at higher concentrations. Further studies are recommended to evaluate its effects on fertilisation success and reproductive performance under field conditions.

Keywords: caprine semen, chilled storage, royal jelly, semen extender, sperm motility

1. Introduction

Artificial insemination (AI) is a key technique in goat breeding, allowing for the effective use of high-quality male genetics to boost productivity and genetic diversity across the livestock industry. The effectiveness of AI largely depends on how well semen quality is maintained during storage, particularly in terms of motility, structural integrity, and fertilising ability. In

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tropical climates like Malaysia, especially in states like Sabah, improving semen preservation methods is crucial to prevent heat-related damage to sperm and to ensure ongoing improvements in goat production and food security (Batool et al., 2024). Semen extenders are essential solutions designed to maintain sperm viability during storage by shielding cells from cold shock, microbial contamination, and oxidative damage. However, caprine sperm are uniquely sensitive to preservation stresses compared to other species. Goat semen contains bulbourethral gland secretions, specifically phospholipase A, which interacts with egg-yolk-based extenders to produce toxic fatty acids that compromise sperm membranes (Pintus & Ros-Santaella, 2021).

During chilled storage at 4°C to 5°C, sperm cells undergo "cold shock," which triggers an overproduction of Reactive Oxygen Species (ROS). This biochemical shift leads to lipid peroxidation, which damages the polyunsaturated fatty acids in the sperm plasma membrane, resulting in a decline in motility and DNA stability (Mocé et al., 2020). To counter these issues, natural additives with antioxidant properties have gained interest as sustainable alternatives to synthetic supplements. Royal Jelly (RJ), a substance secreted by nurse bees, is well known for its rich content of proteins, vitamins, and strong antioxidants such as flavonoids and phenolic compounds (Peykova-Shapkova et al., 2024). These bioactive components, particularly the unique fatty acid 10-hydroxy-2-decenoic acid (10-HDA), provide RJ with antimicrobial and anti-inflammatory properties that are thought to protect sperm from oxidative damage during storage (Moradi et al., 2013).

Despite these benefits, excessive use of synthetic additives has caused concerns regarding chemical residues and long-term sperm toxicity. Moreover, maintaining the quality of goat semen during chilled storage continues to be a major challenge because the quality still declines sharply after 24 to 48 hours, limiting the success of AI programs. Previous research on RJ use in caprine species has been inconsistent, with limited scientific guidance on optimal concentrations. Specifically, the optimal amount of RJ and its impact across different chilling durations remain uncertain; while low doses may protect the cell, high concentrations may alter the osmotic balance or cause cytotoxic effects that accelerate cell death (Coskun Cetin & Karaca, 2023). This lack of precise protocol has reduced breeders' confidence in using RJ as a reliable and sustainable biological additive.

Therefore, this study aimed to evaluate the effects of varying rates of RJ supplementation in extenders on the quality and functional integrity of caprine semen. The study sought to determine the most effective concentration for improving sperm motility, viability, and membrane integrity across different chilled storage times. Specifically, the objectives were: (i) to determine the effects of different concentrations of RJ (0.05%, 0.10%, and 0.15%) on caprine sperm motility, morphology, and viability; and (ii) to assess the impact of these levels on sperm membrane integrity over 0, 24, and 48 hours of storage.

2. Materials and Methods

2.1 Study location

This research was conducted at the Faculty of Sustainable Agriculture, University Malaysia Sabah, Sandakan, Sabah.

2.2 Semen collection

Three sexually mature bucks (approximate 2 years old) under intensive management at FPL were used. Semen was collected biweekly for 8 weeks, then pooled and divided into four treatments (T0–T3). The artificial vagina (AV) was used with the temperature of approximately 45 °C before being closed with a rubber stopper. The open side of the AV was connected with a 15 mL graduated semen collecting tube. The lubricant was placed at the AV before collection took place.

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2.3 Preparation of extender

The T0 extender was prepared using the tris-citric acid-fructose-egg yolk (TCFY) formula, which consisted of 3.028 g of tris, 1.675 g of citric acid, 1.250 g of fructose, 20 mL of egg yolk, and 0.1 g of penicillin (Ng et al., 2022). First, the tris, citric acid, fructose, and penicillin were dissolved in approximately 70 mL of distilled water and stirred on a hot plate for 10 minutes. The egg yolk was then added to the same beaker, and the volume was adjusted to 100 mL with distilled water. The mixture was stirred again on a hot plate for another 10 minutes. The resulting TCFY solution was filtered first using filter paper and then using a 0.45 µm syringe filter. After filtration, the extender was stored in a chiller at 4°C until further use.

Royal jelly group (TCFYRJ): To prepare the RJ extender, 3.028 g of tris, 1.675 g of citric acid, 1.250 g of fructose, and 0.1 g of penicillin were dissolved in approximately 70 mL of distilled water. The solution was stirred on a hot plate for 10 minutes. Royal jelly was added at the appropriate concentrations (0.05 g, 0.10 g, and 0.15 g) to achieve final concentrations of 0.05% (T1), 0.1% (T2), and 0.15% (T3), respectively. The mixture was stirred thoroughly to ensure the RJ is fully dispersed or dissolved. Afterward, 20 mL of egg yolk was added to the same beaker, and the final volume was adjusted to 100 mL using distilled water. The solution was stirred again on a hot plate for another 10 minutes using gentle heat to avoid protein denaturation. The final extender was first filtered using filter paper and subsequently passed through a 0.45 µm syringe filter. While some loss of larger bioactive components in RJ (e.g., certain proteins and lipid-associated fractions) may occur during filtration, the selected pore size was used to ensure sterility while retaining most low–molecular-weight bioactive compounds. The prepared extender was stored at 4°C until use.

2.4 Semen dilution

Six percent glycerol was prepared by mixing 0.6 mL glycerol with 9.4 mL distilled water. For each treatment, 10 mL of semen extender was mixed with 5 mL of 6% glycerol, 4 mL of TCFY (T0) or TCFYRJ (T1–T3), and 1 mL of semen, resulting in a final glycerol concentration of approximately 2%. This concentration was selected based on previous study (Sabri et al., 2024), indicating that lower glycerol levels can reduce osmotic and toxic effects on sperm while still providing adequate cryoprotection.

2.5 Experimental design

All semen samples were diluted with either TCFY (T0) or TCFY supplemented with RJ (TCFYRJ: T1, T2, and T3). The diluted semen samples were equilibrated in a water bath at 25°C for 30 minutes before being transferred to a refrigerator at 4°C for storage. The samples were stored for 0, 24, and 48 hours prior to semen analysis. For evaluation, chilled semen samples were rewarmed in a water bath at approximately 37 °C for 30 seconds before sperm quality assessments were conducted.

The experiment was arranged in a 4×3 factorial experiment under a Completely Randomized Design (CRD), consisting of four extender treatments (T0: 0.0% RJ; T1: 0.05% RJ; T2: 0.1% RJ, and T3: 0.15% RJ) and three storage durations (0, 24 h, and 48 h). Each treatment combination was considered an experimental unit and was replicated three times, resulting in a total of 36 experimental units.

2.6 Physical evaluation

Semen volume was measured using a 1 mL syringe, where the gel mass was removed for more accurate results (El-Hanoun et al., 2014). Semen pH was measured using universal pH paper, where the colour of the paper indicated the pH of the semen (Khadr et al., 2015).

2.7 Sperm evaluation

2.7.1 Assessment of sperm count

Sperm concentration was calculated using a hemacytometer (WHO, 2010). The fresh semen was mixed with semen dilution fluid. Semen dilution fluid consists of 500 mL of distilled water, 5 mL of 35–40% formaldehyde, and 25g of sodium bicarbonate. About 380 µL of semen dilution fluid and 20 µL of diluted semen was mixed using a pipette. To enable capillary action to draw the cell suspension between the chambers, the mixture was gradually loaded underneath the coverslip. Ten times objective on a compound microscope was set on centre of the hemacytometer grid lines. Sperm cells were counted manually in one set of 16 squares, and counting was continued until all four sets of 16 corner squares had been evaluated. The measurement analysis was according to the following formula: Average of sperm count in all 4 sets × semen dilution × 10^6 = Sperm cell × 10^6 mL^{-1}.

2.7.2 Assessment of sperm viability

Semen viability was analysed using the eosin-nigrosine stain. An eosin-nigrosine stain was performed to differentiate the live and dead sperm. Live sperm do not take up the eosin stain and appear white in colour, whereas dead sperm absorb the stain and appear reddish due to the loss of membrane integrity (El-Hanoun et al., 2014). About 50 mL of distilled water and 0.5g of eosin powder was mixed in a beaker to make 1% of the eosin stain. To ensure that all the eosin powder will thoroughly be dissolved in the distilled water, the mixture was mixed uniformly using a glass rod. A drop of diluted semen was placed on a glass slide. Next, two drops (100µl) of 10% nigrosine were added along with a drop (50µl) of 1% eosin to the same slide. Another clean slide was taken and smeared. The smear was left to dry naturally. The slide was examined under a compound microscope (Biological Compound Microscope, View Solution Inc) with a 40× objective lens, the slide. Three measurements were made, each with a count of 200 sperm (Ng et al., 2022).

2.7.3 Assessment of sperm motility

About 3 µL of diluted semen was put on the warm glass slide. A coverslip was put on the warm slide. The glass slide was observed under a compound microscope (Biological Compound Microscope, View Solution Inc) with a 40X objective. Three measurements were made, each with a count of 200 sperm (Ng et al., 2022). The sperm was graded based on its movement. There were four grades of sperm motility as shown in Table 1.

Table 1. Grades of sperm motility (WHO, 2010).

Grade	Description
A	Rapid progressive motility, which sperm can swim fast in a straight line.
B	Slow or sluggish or non-linear progressive motility (the sperm move forward but in a curved or crooked motion).
C	Non-progressive motility or vibrate, (the sperm move their tail but not moving forward).
D	Immotile (fail to move at all).

2.7.4 Assessment of sperm morphology

Sperm abnormalities were observed by using the eosin-nigrosine stain. Eosin-nigrosine staining was performed to differentiate the sperm morphology. About 50 mL of distilled water and 0.5g of eosin powder was mixed in a beaker to make 1% of the eosin stain. To ensure that all the eosin powder will thoroughly be dissolved in distilled water, the mixture was mixed uniformly using a glass rod. A drop of diluted semen was placed on a glass slide. Next, add two drops of 10% nigrosine along with a drop of 1% eosin to the same slide. They then were mixed and

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waited for 10 seconds. Another clean slide was taken and smeared. The smear was left to air-dry naturally. The slide was examined under a compound microscope using a 40× objective lens. Three measurements were made, each involving a count of 200 sperm (Ng et al., 2022).

2.8 Statistical analysis

Data were analysed by using two-way analysis of variance (ANOVA) in SPSS software to evaluate the main effects of extender treatment and storage duration. The interaction effect between extender treatment and storage duration was not included in the statistical model. Differences among means were compared using Tukey's test at $p < 0.05$.

3. Results and Discussion

3.1 Sperm quality

Semen volume and pH are presented in Table 2. The mean semen volume was 0.4 mL ejaculation^{-1}, indicating moderate variability among samples. The semen pH remained constant at 7.0, suggesting a stable and neutral environment across ejaculates. Sperm concentration during chilled storage is shown in Table 3. At 0 hours, the mean concentration was 521.7 × 10^6 mL^{-1}. After 24 hours of storage, the concentration decreased to 325.7 × 10^6 mL^{-1}, representing approximately a 37% reduction. A further decline was observed at 48 hours, reaching 164.7 × 10^6 mL^{-1}. Overall, sperm concentration progressively decreased with increasing storage time. The relatively high standard deviations at each time point indicate considerable variability among samples.

Table 2. The semen volume and semen pH of caprine.

Parameter	Mean ± standard deviation
Volume (mL ejaculation^{-1})	0.4 ± 0.3
pH	7.0 ± 0.0

Table 3. The sperm concentration (×10^6 mL^{-1}).

Chilling times	Mean ± standard deviation
0 hour	521.7 ± 200.1
24 hours	325.7 ± 101.1
48 hours	164.7 ± 91.5

Table 4. Percentage (mean ± SD) of the total sperm motility parameters in a different concentration of royal jelly extender regardless of chilled storage hour.

Chilling times	Royal jelly concentration				p-value
	T0	T1	T2	T3	
0 hour	51.0^{a} ± 8.1	40.1^{ab} ± 11.8	26.0^{bc} ± 5.3	21.9^{c} ± 0.3	0.006
24 hours	23.5 ± 1.6	20.1 ± 3.2	29.7 ± 1.2	13.5 ± 14.2	0.134
48 hours	17.5^{a} ± 0.8	14.7^{b} ± 0.6	6.8^{c} ± 0.3	4.8^{d} ± 0.2	<0.001

SD, Standard deviation; T0, Tris-citrate-fructose-yolk, T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%. Means with different superscripts in a same row differ significantly ($p < 0.05$).

3.2 Total sperm motility (%)

Contrary to the expected beneficial effects, RJ supplementation did not consistently improve total sperm motility compared to the control (Table 4). At 0 and 48 hours of storage, the control (TCFY) exhibited significantly higher motility than all RJ-treated groups ($p < 0.05$), indicating a detrimental or non-beneficial effect of RJ at the tested concentrations. Although T2 showed a

numerically higher value at 24 hours, the difference was not statistically significant ($P > 0.05$), suggesting no clear advantage of RJ supplementation during storage.

Motility is utilised as an initial and reliable indicator of sperm damage during storage, particularly under conditions of cold or extended storage. The reduced motility observed in RJ-treated groups may be associated with suboptimal concentrations or possible interactions between RJ components and the extender, which could negatively affect sperm membrane stability or metabolic activity.

Sperm are highly vulnerable to oxidative stress due to their limited cytoplasmic antioxidant defences and membranes rich in polyunsaturated fatty acids (Agarwal et al., 2019; Pintus & Ros-Santaella, 2021). Excessive reactive oxygen species generated during semen handling and storage attack membrane lipids and axonemal proteins, leading to impaired flagellar motion. Royal jelly contains flavonoids, phenolic acids, peptides, vitamins, and unique fatty acids such as 10-hydroxy-2-decenoic acid, all of which possess strong free radical–scavenging activity (El-Guendouz et al., 2020; Peykova-Shapkova et al., 2024).

Despite these known antioxidant properties, the present findings suggest that the inclusion levels of RJ used in this study were not optimal to confer protective effects on sperm motility. It is possible that higher concentrations may exert pro-oxidant effects or alter osmotic balance, thereby impairing sperm function. Previous studies have reported improved motility with RJ supplementation in ram, goat, and boar semen (Moradi et al., 2013; Iljenkaite et al., 2020; Coskun Cetin & Karaca, 2023); however, the discrepancy with the current results may be attributed to differences in species, extender composition, dosage, or storage conditions. Therefore, the present findings do not support a positive effect of RJ on sperm motility under the conditions tested and highlight the need for further optimisation of its inclusion level.

3.3 Progressive motility (%)

Progressive motility was not consistently improved by RJ supplementation, and in most cases showed comparable or lower values than the control, particularly at extended storage periods (Table 5). Although a significant difference was observed at 0 hour ($p < 0.05$), this effect was not sustained during storage, and overall, RJ supplementation did not demonstrate a stable beneficial effect on progressive motility.

Progressive motility indicates successful forward propulsion, a crucial factor for sperm mobility within the female reproductive system and subsequent fertilisation (Villani et al., 2022). Therefore, it is considered a key indicator of semen quality. The sperm midpiece's mitochondrial ATP production is essential for progressive movement. Oxidative stress damages mitochondrial membranes, impedes electron transport, and diminishes ATP availability, thereby causing sluggish or non-progressive sperm movement (Carrageta et al., 2023).

Although RJ is known for its antioxidant properties, the inconsistent and generally reduced progressive motility observed in RJ-treated groups suggests that it did not effectively support mitochondrial function under the present conditions. Instead, the decline in motility at higher concentrations and longer storage time may indicate possible osmotic imbalance or metabolic stress induced by RJ supplementation.

The marked reduction in progressive motility, particularly at higher RJ concentration (T3), further suggests that excessive supplementation may negatively affect sperm function rather than provide protection. This indicates that RJ may have disrupted the extender’s physiological balance, affecting sperm energy metabolism and motility.

While previous studies have reported beneficial effects of antioxidant supplementation on sperm motility, such effects are strongly dose-dependent and highly species- and extender-specific. Over-supplementation may disturb redox homeostasis and impair mitochondrial activity. Similar dose-dependent responses have been reported for RJ, green tea extract, quercetin, and crocin in semen extenders (Amini et al., 2019; Susilowati et al., 2022; Batool et

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al., 2024). However, in the present study, RJ supplementation did not provide consistent protective effects and instead showed reduced performance at higher levels and longer storage duration.

Table 5. Percentage (mean ± SD) of the progressive motility in a different concentration of royal jelly extender.

Chilling times	Royal jelly concentration				p-value
	T0	T1	T2	T3	
0 hour	$6.3^{b} \pm 1.4$	$13.2^{a} \pm 1.8$	$6.1^{b} \pm 2.6$	$4.3^{b} \pm 0.1$	0.005
24 hours	$5.9^{a} \pm 0.2$	$3.9^{a} \pm 0.4$	$4.1^{a} \pm 0.1$	$1.3^{b} \pm 2.3$	0.010
48 hours	$3.9^{a} \pm 0.1$	$3.0^{b} \pm 0.1$	$1.1^{c} \pm 0.1$	$0.0^{d} \pm 0.0$	<0.001

SD, Standard deviation; T0, Tris-citrate-fructose-yolk, T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%. Means with different superscripts in a same row differ significantly ($p < 0.05$).

3.4 Sperm viability

Royal jelly supplementation influenced sperm viability in a concentration-dependent manner (Table 6). At 48 hours of chilled storage, semen supplemented with low to moderate concentrations of RJ (T1 and T2) showed significantly higher percentages of live sperm and lower percentages of dead sperm compared with the control and the highest RJ concentration (T3) ($p < 0.05$). In contrast, the highest concentration of RJ (0.15%) resulted in the lowest percentage of live sperm and the highest percentage of dead sperm, indicating a detrimental effect at excessive concentrations. Sperm viability, which reflects plasma membrane integrity, is negatively affected during storage due to oxidative stress, osmotic imbalance, and cold shock (Aitken et al., 2022). The plasma membrane plays an essential role in maintaining ion balance, enzyme activity, and fertilising capacity. The improved viability observed in T1 and T2 may be attributed to the antioxidant properties of RJ, which can stabilise membrane lipids and neutralise ROS, thereby reducing membrane damage and sperm death during storage.

Table 6. Percentage (mean ± SD) of the sperm viability in a different concentration of royal jelly extender.

Sperm viability	Chilling times	Royal jelly concentration				p-value
		T0	T1	T2	T3	
Live	0 hour	71.0 ± 11.0	72.7 ± 9.5	67.4 ± 5.5	77.0 ± 6.5	0.590
	24 hours	65.4 ± 2.0	69.0 ± 33.4	83.7 ± 7.6	73.0 ± 6.0	0.622
	48 hours	$73.0^{c} \pm 5.5$	$92.0^{a} \pm 0.0$	$90.0^{b} \pm 1.7$	$45.6^{d} \pm 1.5$	<0.001
Dead	0 hour	29.0 ± 11.0	27.3 ± 9.5	32.6 ± 5.5	23.0 ± 6.5	0.590
	24 hours	34.6 ± 2.0	31.0 ± 33.4	16.3 ± 7.6	27.0 ± 6.0	0.622
	48 hours	$27.0^{b} \pm 5.5$	$8.0^{c} \pm 5.7$	$10.0^{c} \pm 4.0$	$54.4^{a} \pm 1.5$	<0.001

SD, Standard deviation; T0, Tris-citrate-fructose-yolk, T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%. Means with different superscripts in a same row differ significantly ($p < 0.05$).

However, the reduced viability observed in T3 suggests that excessive RJ supplementation may induce osmotic or metabolic stress, leading to impaired membrane stability and increased sperm mortality. Similar concentration-dependent effects of antioxidant supplementation have been reported in previous semen preservation studies (Coskun Cetin & Karaca, 2023). Therefore, the present findings indicate that low to moderate concentrations of RJ may provide partial protection to sperm membrane integrity during chilled storage, whereas higher concentrations may exert detrimental effects.

3.5 Morphology

Royal jelly supplementation did not significantly affect the percentage of bent tail defects during chilled storage (Table 7), although numerically lower values were observed in some RJ treated groups at 0 hour compared with the control. Tail abnormalities commonly arise from osmotic imbalance, membrane destabilisation, and oxidative stress during semen preservation (Pelzman & Sandlow, 2024). These abnormalities may impair sperm motility and fertilising ability. Although RJ contains antioxidant compounds that may help stabilize membrane structure and protect cytoskeletal proteins from oxidative damage, the present findings suggest that the concentrations used were insufficient to produce statistically significant improvements in bent tail morphology. Similar variability and non-significant responses in sperm morphological traits have been reported in studies evaluating antioxidant supplementation in semen extenders (Longobardi et al., 2020). Therefore, under the present experimental conditions, RJ supplementation did not demonstrate a significant protective effect against bent tail abnormalities during chilled storage.

Table 7. Percentage (mean ± SD) of the sperm morphology (Bent Tail) parameters in a different concentration of royal jelly extender.

Chilling times	Royal jelly concentration				p-value
	T0	T1	T2	T3	
0 hour	14.3 ± 2.5	10.6 ± 4.9	11.3 ± 6.1	11.6 ± 0.5	0.721
24 hours	11.3 ± 2.8	13.3 ± 3.7	15.3 ± 2.5	13.0 ± 1.0	0.412
48 hours	13.3 ± 1.5	16.0 ± 4.0	17.0 ± 1.0	15.6 ± 0.5	0.294

SD, Standard deviation; T0, Tris-citrate-fructose-yolk; T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%.

Table 8. Percentage (mean ± SD) of the sperm morphology (midpiece defects) parameters in a different concentration of royal jelly extender.

Chilling times	Royal jelly concentration				p-value
	T0	T1	T2	T3	
0 hour	18.0 ± 2.6	14.3 ± 6.1	12.6 ± 1.5	12.6 ± 1.5	0.276
24 hours	15.0 ± 2.6	17.6 ± 4.5	21.6 ± 0.5	17.3 ± 1.5	0.091
48 hours	17.3 ± 3.5	20.3 ± 1.5	20.0 ± 1.0	18.3 ± 5.5	0.679

SD, Standard deviation; T0, Tris-citrate-fructose-yolk; T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%.

Midpiece abnormalities were not significantly affected by RJ supplementation during chilled storage (Table 8), although numerically lower values were observed in some RJ-treated groups at 0 hour compared with the control. The sperm midpiece contains mitochondria responsible for ATP production required for sperm motility; therefore, damage to this area may impair sperm function and fertilising ability (Carrageta et al., 2023). Oxidative stress is known to contribute to midpiece defects during semen storage through mitochondrial membrane damage and disruption of cellular metabolism. Although RJ possesses antioxidant properties that may help protect mitochondrial membranes against oxidative injury, the present findings suggest that the concentrations used in this study did not produce statistically significant improvements in midpiece morphology. Similar non-significant responses in sperm morphological characteristics have also been reported in studies evaluating antioxidant supplementation in semen extenders (Moradi et al., 2013; Amini et al., 2019; Coskun Cetin &

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Karaca, 2023). Therefore, under the present storage conditions, RJ supplementation did not demonstrate a significant protective effect against midpiece defects.

Head defects were less frequent in semen supplemented with RJ, although the statistical significance of this finding differed across the treatment groups (Table 9). The morphology of sperm heads is largely defined throughout spermatogenesis within the testes, and is significantly shaped by genetic determinants, testicular health, and hormonal control factors rather than by conditions encountered after ejaculation (Pelzman & Sandlow, 2024). Therefore, the efficacy of extender supplementation in rectifying pre-existing head abnormalities is typically limited. Sperm head abnormalities, encompassing irregular morphology, detached heads, and acrosomal damage, frequently correlate with compromised chromatin packaging, DNA fragmentation, and impaired acrosomal development during spermatogenesis (WHO, 2010). Given that these defects appear before ejaculation, the lack of robust statistical significance observed in certain treatment comparisons is biologically plausible. Likewise, non-significant or modest impacts on head morphology have been identified in experiments assessing antioxidant supplementation within goat and ram semen extenders (Moradi et al., 2013; Susilowati et al., 2022).

Table 9. Percentage (mean ± SD) of the sperm morphology (head defect) parameters in a different concentration of royal jelly extender.

Chilling times	Royal jelly concentration				p-value
	T0	T1	T2	T3	
0 hour	66.7 ± 10.7	67.3 ± 11.0	68.6 ± 6.6	67.6 ± 3.0	0.993
24 hours	67.0^{a} ± 6.0	60.6^{b} ± 4.1	54.0^{b} ± 5.0	63.6^{ab} ± 3.0	0.048
48 hours	62.6 ± 4.9	56.6 ± 4.5	52.3 ± 3.5	57.3 ± 1.5	0.064

SD, Standard deviation; T0, Tris-citrate-fructose-yolk, T1, Tris-citrate-fructose-yolk-royal jelly 0.05%; T2, Tris-citrate-fructose-yolk-royal jelly 0.10%; T3, Tris-citrate-fructose-yolk-royal jelly 0.15%. Means with different superscripts in a same row differ significantly ($p < 0.05$).

4. Conclusion

In conclusion, the present study demonstrated that RJ supplementation does not consistently enhance semen quality during chilled storage, but exerts a differential effect depending on concentration and parameter evaluated. The data indicate that low to moderate concentrations of RJ (0.05–0.10%) were able to significantly preserve sperm membrane viability at 48 hours of storage compared with higher concentration (0.15%), suggesting a partial protective effect on cell membrane integrity. However, in contrast to this protective effect on viability, RJ supplementation did not improve sperm motility parameters. Both total and progressive motility were generally lower in RJ-treated groups compared to the T0, indicating an inhibitory effect of RJ on sperm kinetic activity during storage. Conversely, elevated concentrations, specifically 0.15%, proved more detrimental to sperm quality, particularly with respect to motility and overall preservation of sperm function. The experimental results further indicate that, while RJ may help maintain certain structural aspects such as membrane integrity under chilled conditions, its effect on sperm movement is negative or non-beneficial under the tested conditions. Overall, the findings suggest that RJ cannot be considered a universal enhancer of caprine semen quality during liquid storage. Its effects are concentration-dependent, with limited membrane-protective activity at lower doses but inhibitory effects on sperm motility compared with TCFY alone.

Data Availability

The data generated during the study are included in the manuscript.

Author Contributions

Wilsa Gly Mojuwil: Conceptualization, Methodology, Writing- Original draft preparation. **Nur Hafizah Syafiah Abdullah:** Data curation. **Mohamad Mijanur Rahman:** Investigation, Supervision. **Rovina Kobun:** Visualization, Validation. **Nur Syafeezah Janong** and **Renny Fatmyah Utamy:** Writing- Reviewing and Editing.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Statement

All animal procedures were conducted in accordance with the guidelines approved by the Animal Ethics Committee of Universiti Malaysia Sabah (JEHUMS), Malaysia (Approval No. AEC 0016/2025).

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