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Other

Successful Cryoprotectant-Free Vitrification of Honey Bee (*Apis mellifera*) Drone Sperm With Royal Jelly Supplemented Extender

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ABSTRACT

In trying to protect honey bee species and maintain genetic diversity, employing effective approaches for drone sperm conservation is crucial. Based on literature, drone sperm cryopreservation extenders and methods have not been fully optimized. Our research aim was to enhance drone bee sperm preservation by incorporating royal jelly (RJ) in the extender of the cryoprotectant-free vitrification method. Drone sperm was collected from adult drone bees (36 days old) using the manual inversion method. Different concentrations of RJ (0%, 0.5%, 1%, 2.5% and 5%) were added to the extender. Diluted sperm were cryopreserved using a cryoprotectant-free vitrification technique where 10 μL aliquots of the diluted sperm were directly dropped into the liquid nitrogen and then stored. Data were analysed based on a completely randomized design with ten replications. Sperm quality parameters, including motility, viability and DNA damage, were evaluated in vitro. Queens were artificially inseminated to measure the ability of motile sperm to reach the spermathecae. The results showed that 1% and 2.5% of RJ supplementation significantly enhanced sperm motility and viability and reduced DNA fragmentation compared to control and higher RJ concentrations. Specifically, the 1% RJ group resulted in the highest sperm viability, while both the 1% and 2.5% groups maintained lower DNA fragmentation rates. Queens inseminated with sperm treated with 1% and 2.5% RJ showed a notably higher number of motile sperm in their spermathecae. In conclusion, supplementation of 1% RJ to the cryoprotectant-free vitrification media may improve drone sperm quality parameters post-warming. Our findings provide valuable insights into optimizing drone bee sperm preservation, contributing to the conservation of these vital pollinators.

1 | Introduction

Cryopreservation of bee sperm, scientifically known as *Apis mellifera*, has been explored as a substantial technique for preserving genetic diversity in honeybee populations (Smilga-Spalvina et al. 2023; Paillard et al. 2017; Brutscher et al. 2019; A. N. Gulov et al. 2023). Based on literature, cryopreserved sperm of drone honey bees can keep viability and fertilizing ability, with successful artificial insemination outcomes (Ostroverkhova et al. 2015; Paillard et al. 2017). This preservation technique offers a promising solution for the long-term survival of honeybee germplasm, aiding in the creation of disease-resistant lines and ensuring genetic diversity (Bolton et al. 2022). Sperm banking in honeybees by various methods results in the protection of genetic material, the possibility of reintroduction of lost genetics back into populations when needed, and therefore the enhancement

of genetic diversity in honeybee colonies (Wegener et al. 2014; Paillard et al. 2017).

Royal jelly (RJ) is a gelatinous and creamy secretion produced by the hypopharyngeal and mandibular glands of young honeybee (*A. mellifera* L.) workers, also known as nurse bees. RJ primarily consists of water (60%−70%), carbohydrates, proteins, free amino acids, lipids, minerals, vitamins and polyphenols (Y. Wang et al. 2023). Flavonoids, particularly naringenin, acacetin, apigenin, chrysin, genistein and 10-hydroxy-2-decenoic acid (10-HDA) (J.-R. Liu et al. 2008; Kocot et al. 2018; Collazo et al. 2021; Botezan et al. 2023), are key antioxidant components of RJ which is believed to be in charge of its health-promoting aspects, including anti-inflammatory, anti-microbial and anti-ageing effects (J.-R. Liu et al. 2008; Kocot et al. 2018; Collazo et al. 2021). RJ has been employed for sperm cryopreservation and improved post-thawing sperm quality parameters, boosted antioxidant capacity and enhanced conception rates in cattle bulls (Elsheshtawy et al. 2023), buffalo bulls (Shahzad et al. 2016), roosters (Hadavand Mirzaei et al. 2021), bee drones (Alcay et al. 2019) and boars (Iljenkaite et al. 2020) upon RJ supplementation to the extender. RJ supplementation at specific concentrations (5 mg/mL in roosters and 1% in boars) has been particularly effective in maintaining sperm motility, viability and membrane integrity during liquid and frozen storage (Shahzad et al. 2016; Hadavand Mirzaei et al. 2021). Furthermore, RJ has been successfully used for drone sperm cryopreservation. Results indicated that the addition of 1% RJ to the extender elevated the post-thaw quality of drone sperm (Abdelnour et al. 2020; Iljenkaite et al. 2020; Rahnama et al. 2020; Hadavand Mirzaei et al. 2021; Elsheshtawy et al. 2023).

For the first time, Nawroth et al. (2002) introduced a novel method of ice- and cryoprotectant (CPA)-free vitrification of human spermatozoa by direct dropping of a sperm suspension into liquid nitrogen. They reported extremely high cooling and warming rates of up to hundreds of thousands of °C/min. In this method only non-permeable additives like sugars are employed, resulting in the elimination of lethal osmotic and toxic effects of high CPA concentrations. Several studies showed higher recovery rates of motility, sperm qualities and fewer biological changes, as well as reduced DNA fragmentation in sperm cells in cryoprotectant-free vitrification method (Aizpurua et al. 2017; Isachenko et al. 2019; Rayea et al. 2019; Amer et al. 2021; M. Wang et al. 2022). Other sperm features, including acrosome integrity and cytoskeleton structure, were better preserved by this type of vitrification method, making it a promising alternative for sperm preservation in assisted reproductive technologies (Aizpurua et al. 2017).

The purpose of this investigation is to explore the effects of different concentrations of RJ in drone sperm extenders on cryopreservation outcomes (motility, viability, DNA fragmentation and number of spermatozoa in queen spermathecae) using the cryoprotectant-free vitrification method.

2 | Materials and Methods

All chemicals used in this study were purchased from Sigma unless otherwise specified.

2.1 | Drone Management

This study was conducted at the Research and Education Center for Agriculture and Natural Resources in Hamedan Province, Iran. In the beginning, 25 honey bee hives were separated for breeding bee drones with the aid of wax sheets having bigger-sized hexagonal cells. More than 1000 bee drones (36 days old) were randomly collected from hives. This was done using a side frame chamber at the hive entrance when they returned from an orientation flight. Immediately before sperm collection, the bee drones were placed in a handmade cage measuring 30 × 30 × 30 cm with a 46 mm mesh screen. The matured bee drones' sperm was collected in 0.2-mL capillaries using a 10-mL syringe. The collected sperm was then transferred to the laboratory for microscopic evaluation.

2.2 | Sperm Collection and Dilution

Sperm of sexually mature drones (36 days and older) were selected. To induce ejaculation, pressure was applied to the chest and then the abdominal area while gently compressed. Approximately 1 μL of sperm was collected from each drone using a 20 mL syringe under a stereo microscope. The syringe was attached to a 50 mL glass capillary with a thin, handmade glass tip to collect semen. Previously, all parts of the syringe were washed with 70% alcohol and distilled water. The syringe was filled with normal saline and antibiotic solution (10 g sodium chloride in 1000 mL distilled water with 0.25 (v/v) dihydrostreptomycin). To prevent the mixing of normal saline solution and sperm, a space between saline solution and sperm was established. During several days, sperm was collected and stored in a refrigerated incubator at +16°C before testing. All collected sperm were pooled to eliminate individual differences. The volume of pooled sperm was divided into five equal volumes. Each volume of sperm was diluted 1:15 (v/v) with the Hopkins' modified extender solution (Table 1) supplemented with different concentrations of RJ (0%, 0.5%, 1%, 2.5% and 5%) to a final concentration of about 150×10^6 spermatozoa per mL.

2.3 | Preparation of Vitrification Solution

Vitrification solution was prepared by dissolving 0.5 M sucrose (Merck, Darmstadt, Germany) in ultra-pure water. This solution was then filtered through a 0.22 μm filter (Millipore, Darmstadt, Germany). The final vitrification solution containing sperm was prepared as follows: 0.5 M sucrose solution was diluted 1:1 with the modified Hopkins extender solution (as shown in Table 1), resulting in a final sucrose concentration of 0.25 M.

2.4 | Experimental Design

The study design is illustrated in Figure 1. The pooled sperm samples were divided into five groups. Each group cryopreserved in granules by directly dropping samples into liquid nitrogen. The quality of the spermatozoa after cryopreservation processes (vitrification and warming) was evaluated as described below (refer to Figure 1).

TABLE 1 | Hopkin's modified extender used to dilute drone sperm.

Materials	Amount	Materials	Amount
Penicillin	0.0125 g	Glycine	0.1 mM
Streptomycin	0.011 g	Proline	4.3 mM
EDTA	0.01 mM	Catalase	0.5 mg
Sodium phosphate dibasic	1 mM	BSA	2 mg
Sodium citrate	1 mM	KCl	82 mM
Glucose	2.7 mM	NaCl	83 mM
Arginine	0.57 mM	$NaHCO_3$	5 mM
Tris base *N*-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid (TES) buffer	30 mM	Royal jelly	(0%, 0.5%, 1%, 2.5% and 5%)

Note: Products were dissolved in a final volume of 100 mL distilled water.

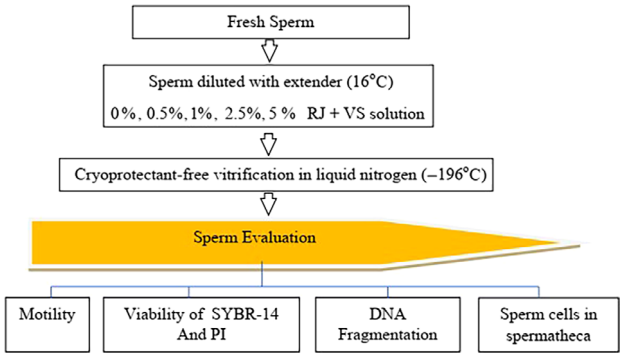

FIGURE 1 | Experimental design.

2.5 | Statistical Method

Data were analysed using analysis of variance (ANOVA) in SAS 9.4 software after confirming homogeneity and normality. The experiments were conducted in a completely randomized design with five treatments (0%, 0.5%, 1%, 2.5% and 5%) and 10 repetitions. The results are presented as mean ± standard deviation. If significant differences were observed, Duncan's test was used to compare the means ($p < 0.05$).

2.6 | Cryopreservation and Thawing of Spermatozoa at −196°C

After preparing the sample, a micropipette was used to drop 10 µL aliquots of the spermatozoa suspension directly into the cooling agent from a distance of 10 cm (M. Wang et al. 2020). A sphere was formed immediately and floated to the surface. After 6 s in liquid nitrogen, the sphere solidified and sank to the bottom of the strainer (refer to Figure 2). Once solidified, the spheres were collected, packaged into 1.8 mL cryotubes, and stored in a tank with liquid nitrogen before warming. The warming process was carried out by quickly submerging five spheres, one by one (no more than five spheres at a time), into 3 mL of modified Hopkins extender solution that had been prewarmed to 37°C. This was accompanied by gentle vortexing for 10 s. The suspension kept at 37°C in a 5% CO_2 environment for 10 min, was followed by centrifugation at 1000 × *g* for 10 min. At the end, resuspension of the sperm pellet was completed in 20 µL of modified Hopkins extender solution for quality evaluation (Hidalgo et al. 2020; M. Wang et al. 2020).

2.7 | Assessment of Spermatozoa Motility

The sperm sample was diluted at a 1:30 ratio with Hopkins solution and incubated for 30 min at 35°C. Following this, the sample was placed on a glass slide, protected with a coverslip and evaluated under a microscope with heat stage (35°C) for motility. Motility is expressed either as a percentage or on a scale from 0 to 5, indicating the proportion of sperm cells in the sample that perform circular movements and vibrations (Taylor et al. 2009; Gül et al. 2017; Alcay et al. 2019, 2020, 2021; Auth and Hopkins 2021). Sperm motility was estimated subjectively by a single observer in a blinded manner. At least 100 cells were examined per sample.

2.8 | Assessment of Viability by SYBR-14 and Propidium Iodide

Sperm viability was evaluated after cryoprotectant-free vitrification and warming. This was determined using the LIVE/DEAD dual fluorescent staining method (Collins and Donoghue 1999). Before use, SYBR-14 and propidium iodide (PI) stock solutions were diluted with DMSO (1:50) and distilled water (1:4), respectively. Then, SYBR-14 and PI stains were added to each sperm sample in the microcentrifuge tube and kept in incubator at 36°C for 10 min. Subsequently, a 5 µL aliquot of the stained sample was placed on a glass slide, covered with a cover slip and observed by a microscope equipped with a fluorescent light source and filter at 400× magnification. The spermatozoa in six aliquots of each sample were scored as green (live) or red (dead). At least 300 spermatozoa were counted for each sample to determine sperm viability, which was defined as the percentage of live spermatozoa out of the total spermatozoa counted. In total, about (Locke et al. 1990; Burley et al. 2008). A total of 15,000 sperm were counted.

2.9 | Evaluation of Sperm DNA Damage

TUNEL technique was used to assay DNA fragmentation of sperm cells using In Situ Cell Death Detection Kit with fluorescein

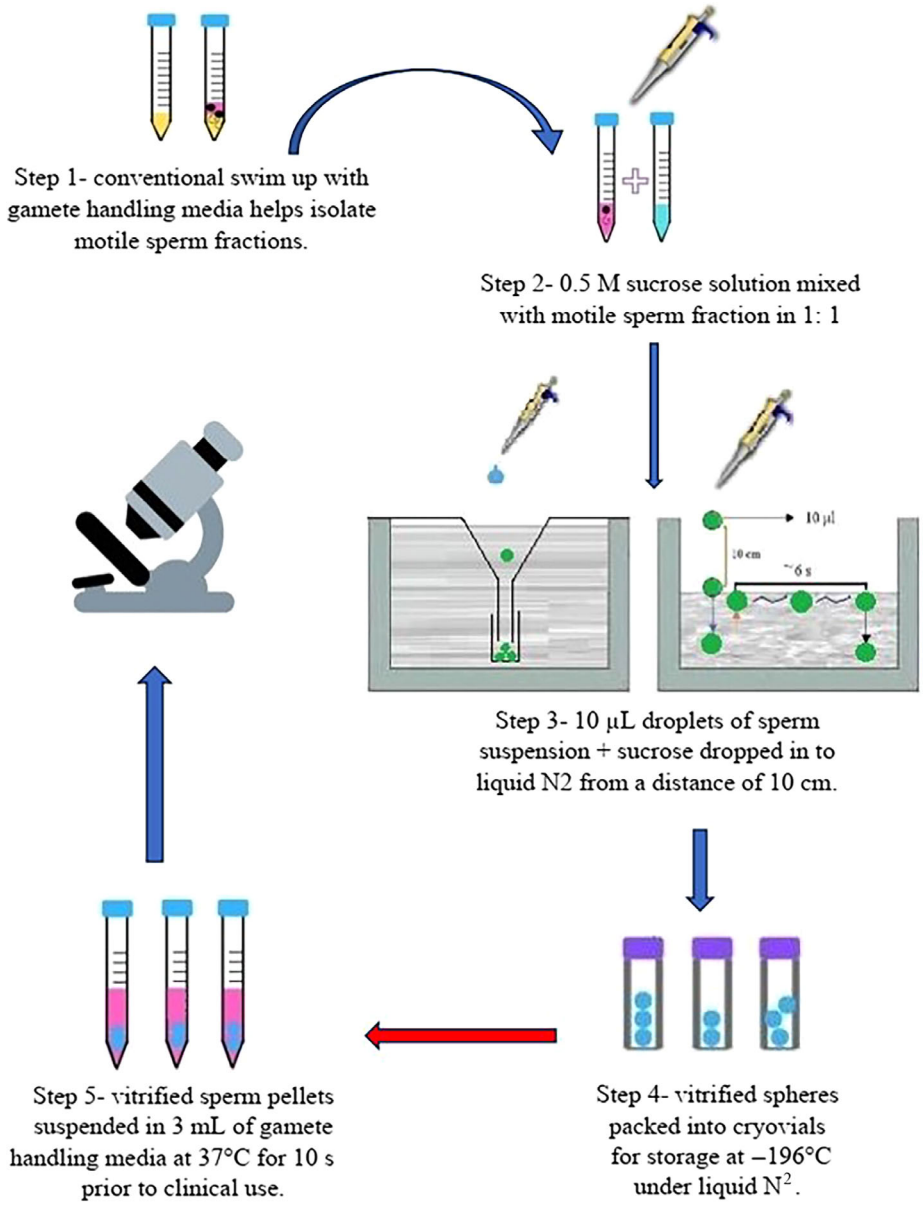

FIGURE 2 | Schematic representation of cryoprotectant-free vitrification and thawing of drone spermatozoa using the droplet method.

(Roche Diagnostics GmbH, Germany) after cryopreservation process through the manufacturer's protocol (Wegener et al. 2014). To quantify the amount of DNA breakage through the cryopreservation process, nuclei containing DNA nicks were identified using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, as per the methodology outlined in the manufacturer's manual. Briefly, diluted sperm samples using 1:500 with PBS buffer were gently mixed at room temperature, smeared on poly-lysine-coated cover glasses (18 × 18 mm) and air-dried. For each treatment, five cover glasses were prepared—three for sample evaluation, one for a positive control (pre-treated with DNAse) and another for a negative control (no incubation with TUNEL solution, but only with fluorescein dUTP). After 1 h of fixation in a freshly prepared fixation solution (17°C; 4% paraformaldehyde in PBS with pH 7.4), the coverslips were gently rinsed with PBS. They were then incubated for exactly 2 min with a freshly prepared permeabilizing solution (0.1% Triton X-100; 0.1% sodium citrate). During the incubation, the samples were placed in pre-chilled Petri dishes on ice to maintain a temperature of about 2°C. The coverslips were then washed twice with PBS and air-dried. After adding 50 µL of TUNEL solution per coverslip, the samples were incubated in a humidified atmosphere for 60 min at 37°C in the dark. The TUNEL solution (2 µL of TdT enzyme, 1 µL of dUTP and 47 µL of buffer, totalling 50 µL for one test). After incubation with the TUNEL solution, the samples were rinsed three times with PBS and then analysed under a fluorescence microscope (excitation wavelength 450–490 nm; emission wavelength 515–565 nm). Only sperm with DNA strand breaks exhibited a distinct green fluorescence.

2.10 | Sperm Cells in the Spermatheca

Ten adult queens (6–8 days old), belonging to identical genetic strains, underwent insemination with vitrified/warmed sperm

TABLE 2 | Effect of royal jelly supplementation on drone sperm parameters after the vitrification/warming process by Droplet method.

	Sperm parameters		
RJ (%)	**Motility**	**Viability**	**DNA fragmentation**
RJ 0 (Control)	56.10 ± 2.85^{c}	60.40 ± 2.72^{d}	20.00 ± 2.49^{a}
RJ 0.5	67.10 ± 2.56^{b}	71.60 ± 3.27^{b}	15.80 ± 2.04^{b}
RJ 1	70.90 ± 2.60^{a}	75.40 ± 4.03^{a}	12.20 ± 1.32^{c}
RJ 2.5	67.30 ± 3.02^{b}	69.20 ± 1.40^{b}	14.80 ± 2.20^{b}
RJ 5	56.70 ± 3.09^{c}	63.30 ± 1.89^{c}	18.70 ± 1.49^{a}

Note: All the experiments were repeated 10 times. Data are presented as mean ± standard deviation. Data are presented as percentages and different superscript letters (a, b, c and d) indicate significant differences among experimental groups.

from each treatment. Specifically, a total of 150 queens were subjected to insemination. The day before insemination, the queens were exposed to carbon dioxide (CO_2) gas for a duration of 1–4 min. On the day of insemination, each queen received 8–10 μL of sperm in conjunction with CO_2 gas (Woyke 1983; Collins et al. 2006; Cobey et al. 2013). Subsequently, the queens were individually relocated to small cages and placed within queenless colonies. Following a period of 48 h post-insemination, the queens were positioned in a freezer, set at −20°C for a duration of 15 min for euthanasia, after which they were dissected to collect their spermathecae for sperm quantification (Wegener et al. 2012). The spermathecae were ruptured and washed in 50 μL of Hopkin's modified extender. The suspension was further diluted as required, with 1 microliter being diluted in 1 mL extender, resulting in a dilution factor of 1000 (1:1000). Sperm densities were ascertained utilizing a Neubauer counting chamber, with the mean value derived from four central squares being counted (Paillard 2016). To calculate the sperm numbers per queen, a specific formula was utilized:

$$Cells/\mu L = \text{Averagecellcountspersquare} \times \text{Dilutionfactor} \times 10$$

3 | Results

Drone sperm motility, viability and DNA Fragmentation data after the vitrification/warming process with different concentrations of RJ are presented in Table 2. The highest motility was observed in the RJ 1 group (70.90 ± 2.60) compared to the other groups after post-storage, which was significantly higher than the RJ 0 (56.10 ± 2.85) and RJ 5 (56.70 ± 3.09) groups ($p < 0.05$). The RJ 0.5 (67.10 ± 2.56) and RJ 2.5 (67.30 ± 3.02) groups also showed significantly higher motility than the control group, but no significant difference was observed between these two groups. The highest sperm viability percentage was observed in the RJ 1 group (75.40 ± 4.03), which was significantly greater than the other treatments, including RJ 0 (60.40 ± 2.72) and RJ 5 (63.30 ± 1.89) ($p < 0.05$). The RJ 0.5 (71.60 ± 3.27) and RJ 2.5 (69.20 ± 1.40) groups also showed improvements compared to the control group, but the difference between these two groups was not significant.

The lowest DNA fragmentation was observed in the RJ 1 group (12.20 ± 1.32), which was significantly lower than the other groups ($p < 0.05$). The RJ 0 (20.00 ± 2.49) and RJ 5 (18.70 ± 1.49) groups exhibited the most significant damage to DNA integrity, while the RJ 0.5 (15.80 ± 2.04) and RJ 2.5 (14.80 ± 2.20) groups showed significantly better results than the control group. Subsequent post-thawing outcomes indicated that RJ 1 had the potential to enhance the preservation of DNA integrity throughout the freezing procedure.

TABLE 3 | Effect of royal jelly supplementation on the number of motile sperm cells in queen's spermatheca after insemination of vitrified/warmed sperm.

RJ (%)	**Number of motile sperm cells in queen's spermatheca (millions/μL)**
RJ 0 (Control)	2.92 ± 0.115^{b}
RJ 0.5	3.01 ± 0.100^{b}
RJ 1	3.21 ± 0.138^{a}
RJ 2.5	3.06 ± 0.093^{a}
RJ 5	2.86 ± 0.112^{b}

Note: All the experiments were repeated 10 times. Data are presented as mean ± standard deviation. Different superscript letters (a and b) indicate significant differences among experimental groups.

Overall, the RJ 1 group demonstrated the best results across all sperm parameters, while the RJ 5 group had negative effects on sperm parameters.

The data presented in Table 3 illustrates the quantity of motile sperm cells moved to the queen's spermatheca after artificial insemination of vitrified and warmed sperm. The high concentration of RJ in the RJ 5 group had negative impact on the quantity of motile sperm cells in the spermathecae across all storage periods. On the other hand, the RJ 1 and RJ 2.5 groups demonstrated a notably higher number of motile sperm cells in the spermatheca post-cryo-storage ($p < 0.05$).

4 | Discussion

Oxidative stress that occurs during cryopreservation, negatively affects the sperm quality parameters, morphology and ultrastructure (A. Gulov and Kolchaeva 2023). Sperm damage during cryopreservation is largely caused by an imbalance between

reactive oxygen species (ROS) and antioxidant levels (X. Liu et al. 2021). The antioxidant properties of RJ and its potential advantages in cryopreservation procedures have been the subject of much research. Studies have indicated that adding RJ to semen extenders can enhance the redox status and therefore, properties of the semen (Alcay et al. 2019).

One of the critical quality parameters of cryopreserved sperm is their post-thaw motility, resulting in successful artificial insemination and, therefore, optimal queen fertility. Our results showed that supplementation of the extender with 1% RJ significantly increased motility percentage in comparison to the other groups after storage. This finding is consistent with that of Alcay et al. (2019), who showed that supplementation of extender with one percent RJ significantly improved drone sperm motility by about 59% after thawing. Previous studies show that due to oxidative stress, the level of ROS increases, therefore leading to impaired sperm function, particularly sperm motility (Nowicka-Bauer and Nixon 2020; Ribeiro et al. 2022; Hussain et al. 2023). In addition, ROS activates the lipid peroxidation cascade, negatively affects the properties of the sperm membrane and its composition, mitochondrial function and finally energy production (Serrano et al. 2020). Therefore, the addition of a proper amount of suitable antioxidants to the extender may prevent the adverse effects of oxidative stress. Bashir et al. (2023) showed that the supplementation with antioxidants such as ascorbic acid can improve sperm motility post-warming, resulting in higher fertility rates.

Sperm plasma membrane integrity plays a pivotal role in sperm functioning, particularly when it comes to the assessment of sperm quality after cryopreservation and applied cryopreservation methods, and conditions including appropriate cryoprotectants, antioxidant agents, base extenders, and so forth may impose huge effects on sperm viability. The results of this study showed that the addition of 1% RJ could preserve sperm plasma membrane integrity to about 75% and supplementation of 1%–2.5% resulted in a higher percentage of intact membrane compared to the other groups. These results are consistent with those of Alcay et al. (2019) who reported a viability of about 68% with the addition of 1% RJ. A little higher percentage of plasma membrane integrity in the current research might be due to improved protection against cold shock by the cryoprotectant-free vitrification method. The only cryoprotective agent used in this method is sucrose, which can interact directly with sperm membrane lipids and proteins, altering their phase transition behaviour and hydration state, therefore helping to stabilize the membrane during the freezing and thawing process (Farshad and Akhondzadeh 2008; Thananurak et al. 2019; Anjos et al. 2021). Needless to say, when sperm cells are exposed to cold shock, the probability of phase transition of membrane lipids significantly increases, leading to a significant reduction in viability (Drobnis et al. 1993; Ricker et al. 2006).

Our finding indicated that RJ 1 had better protective effects on DNA integrity. In contrast, the higher concentrations of RJ 5 significantly increased DNA fragmentation during the frozen-thawed process of drone sperm. Following the present results, previous studies have demonstrated that incorporation of RJ in the rooster diet (Rahnama et al. 2020; Hadavand Mirzaei et al. 2021) or as a supplement to the cryopreservation extender improved sperm DNA integrity by reducing cryodamage's in other species (Abdelnour et al. 2020; Saberivand et al. 2022; Elsheshtawy et al. 2023) due to its antioxidant properties. Potent antioxidant properties of the RJ derivates from its phenolic compounds such as linuron, andrographolide, 1,2-benzene dicarboxylic acid, diisooctyl ester, estragol and *trans*-anethole. It seems that these compounds could protect sperm from detrimental effects of oxidative stress. These polyphenolic constituents can impede oxidative processes through various mechanisms, such as directly scavenging ROS, inhibiting enzymes and binding metal ions (Lodovici et al. 2001). Furthermore, these compounds can decrease lipid peroxidation. This helps in maintaining the activity of mitochondria and the integrity of sperm DNA (Guo et al. 2008; Karadeniz et al. 2011). On the other hand, the high levels of RJ in the current study decreased the DNA integrity of the drone honey bee spermatozoa during cryopreservation. This finding suggests that the excessive amount of antioxidants at high doses could disrupt the redox balance leading to cellular dysfunction (Bouayed and Bohn 2010) which ultimately results in DNA fragmentation and chromosomal abnormality (Marchetti et al. 2002).

Generally, the spermathecae of queen contain 4–7 million sperm cells after mating to several drones. After sperm cryopreservation, roughly calculating the number of cryopreserved/warmed motile sperms reached and maintain in the spermathecae, is a determining factor of forthcoming reproductive capacity of the queen bee (Mackensen 1955; Delaney et al. 2010; Tarpy et al. 2012; Brutscher et al. 2019). This factor is crucial in determining the lifespan of the queen, as once the sperm supply is exhausted, queen bees are typically replaced by a younger queen when they start laying unfertilized eggs, that give rise to drones (Cobey 2007; Amiri et al. 2017). Our results indicated that the addition of RJ to the extender resulted in significantly higher numbers of sperms in spermathecae of preservation. Compared to the other studies on drone sperm cryopreservation methods, the average number of spermatozoa in the spermatheca of queen bees using our cryoprotectant-free droplet method exceeded the result of the other researchers using the conventional method with egg yolk (Gül et al. 2017), honey (A. N. Gulov et al. 2023) and egg yolk with 25% DMSO method (Harbo 1983). In our approach, only non-permeating cryoprotective agents were employed to enhance the flexibility of the plasma membrane in the process of cryopreservation. The elimination of permeating cryoprotective agents, known to adversely affect the plasma membrane, acrosome, DNA and motility of spermatozoa, may account for the augmented sperm count within the spermathecae (Essani 2024; Bisconti et al. 2022; Murray and Gibson 2022).

5 | Conclusion

Our study shows that addition of RJ at concentrations of 1% and 2.5% into the extender, significantly enhances the quality of vitrified/warmed honeybee drone sperm. These improvements emphasize the potential of RJ as a valuable supplement in sperm cryopreservation protocols offers a promising path for maintaining genetic diversity within declining honeybee populations. Future research should focus on refining these cryopreservation techniques and exploring overall queen fertility to better support sustainable apiculture and biodiversity.

Author Contributions

Aliakbar Mohammadi: conceptualization, writing–original draft, methodology, formal analysis, investigation, resources. **Adel Sabrivand:** conceptualization, writing–original draft, methodology, formal analysis, supervision. **Hadi Hajarian:** conceptualization, writing–original draft, writing–review and editing, formal analysis, supervision.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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