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Research Article

Caffeine-Boosted Cryopreservation: Improving Post-Thaw Motility and Kinetics of Red Junglefowl Sperm

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Abstract

This study investigated the improvement of post-thaw motility and kinetics of Red Junglefowl sperm cryopreserved using an extender supplemented with caffeine as an effective cryoprotectant. Semen samples collected from the cocks were cryopreserved using extenders containing 8% dimethyl sulfoxide and varying concentrations of caffeine (0, 1, 2, and 3 mg/ml) assigned to four treatment groups: T0, T1, T2, and T3, respectively. Post-thaw sperm motility and kinetic parameters were evaluated. Results showed that 2 mg/ml caffeine (T2 group) significantly ($P<0.05$) enhanced post-thaw motility, progressive motility, and velocity compared to other treatment groups (T0, T1 and T3). The highest values for curvilinear velocity, average path velocity, and straight-line velocity were observed in sperm treated with 2 mg/ml caffeine, indicating reduced cryodamage and improved sperm quality. Furthermore, straightness, linearity, and wobble indices were significantly ($P<0.05$) improved in the T2 group compared to other treatment groups. The amplitude of lateral head displacement and beat cross frequency were also influenced by caffeine supplementation recorded in T2 group. These findings indicate caffine is an effective cryoprotective additive in avian semen cryopreservation. The supplementation of caffeine, particularly at 2 mg/ml, enhanced post-thaw sperm motility and kinetic properties, making it a promising candidate for improving the effectiveness of semen preservation protocols. Further studies are required to assess its impact on fertilization success rates in artificial insemination and long-term genetic conservation programs. This protocol could revolutionize avian genetic resource banking, particularly for threatened species like Red Junglefowl.

Keywords: Caffeine, cryopreservation, sperm kinetics, Red Junglefowl sperm, sperm motility.

Introduction

The International Union for Conservation of Nature (IUCN) identifies over 28,000 species as threatened, including approximately 14% of avian species (IUCN, 2019). Climate change, habitat loss, and unsustainable human activities increase extinction risk (BirdLife International, 2020). Conservation efforts, including ex-situ methods such as cryopreservation, play a crucial role in preserving genetic

diversity and ensuring the survival of threatened avian populations (Donoghue and Wishart, 2000).

Cryopreservation is widely used in mammalian species; however, its application in avian species faces several challenges due to the unique morphological and physiological characteristics of avian spermatozoa. Avian spermatozoa, characterized by their filiform shape, are particularly susceptible to cryodamage, leading to reduced motility, viability, and fertilization potential post-thaw (Blesbois, 2012). One of the primary factors influencing sperm cryosurvivability is the selection of appropriate cryoprotectants and extenders, which help mitigate the harmful effects of freezing and thawing processes (Madeddu *et al.*, 2009). Dimethyl sulfoxide (DMSO) is a commonly used cryoprotectant for avian sperm cryopreservation, as it demonstrates lower toxicity and better post-thaw sperm quality compared to other cryoprotectants such as glycerol (Sontakke *et al.*, 2004). However, additional protective agents, such as antioxidants and energy substrates are needed to improve post-thaw survival and motility (Rakha *et al.*, 2016). Among these additives, caffeine, a known stimulant and phosphodiesterase inhibitor, has been explored to enhance sperm function during cryopreservation (Pariz and Hallak, 2016). Caffeine functions by inhibiting phosphodiesterase enzymes, thereby increasing intracellular cyclic AMP (cAMP) levels, which play a crucial role in sustaining dynein-driven motility in sperm cells (Lindemann *et al.*, 1983). Additionally, caffeine exhibits antioxidant properties that help mitigate oxidative stress, a major contributor to cryodamage in frozen-thawed sperm (Yashin *et al.*, 2010). Studies on mammalian sperm demonstrate that caffeine supplementation improves post-thaw motility and viability, leading to increased fertilization success rates (Maia and Bicudo, 2009). However, limited research has been conducted on the effects of caffeine on cryosurvivability in avian species, particularly in native genetic resources such as the Red Junglefowl (Gallus gallus).

The RJF is considered the wild ancestor of domestic chickens and holds significant genetic and ecological importance. Conservation efforts focusing on maintaining RJF genetic diversity are essential for preserving its unique traits and potential contributions to future poultry breeding programs (Nishibori *et al.*, 2005). Given the challenges associated with avian sperm cryopreservation, this study aims to investigate the effect of caffeine on the cryosurvivability of RJF sperm. Specifically, the study evaluates post-thaw sperm motility and kinetic parameters following caffeine treatment at varying concentrations.

By optimizing cryopreservation protocols with effective cryoprotectants and additives, including caffeine, this research seeks to improve the success rate of avian sperm cryopreservation and support the long-term conservation of RJF genetic resources. The findings of this study also contribute to advances in assisted reproductive technologies (ART) for avian species, providing new insights into sperm physiology and cryobiology.

Materials and Methods

Study location and duration

The study was conducted at the Advanced Avian Research Farm, HSTU, Dinajpur, Bangladesh, over six months (December 2021 – June 2022). The location was selected for its well-equipped facilities for avian reproductive studies, ensuring optimal environmental control and experimental accuracy.

Experimental birds and management

Twelve mature RJF cocks (average weight: 1800 g) were housed in individual cages (60× 60×70 cm^3) under an intensive management system. Housing was maintained at 30°C with 55–75% humidity, and a lighting schedule of 16 hours of light and 8 hours of darkness was ventilation and biosecurity measures, including restricted access, routine disinfection, and footbaths, were implemented to prevent disease outbreaks. Birds were provided a balanced breeder diet (100 g/day) containing maize, soybean meal, wheat bran, fish meal, vegetable oil, and essential vitamins and minerals. Water was provided ad libitum; dispensers cleaned daily. Feed was supplied in a restricted manner to prevent obesity-related fertility issues. Bedding was changed weekly to maintain cleanliness, and an automated air circulation system-controlled ammonia levels.

Routine Routine monitoring ensured early disease detection or abnormalities. Any signs of distress were documented, and appropriate interventions were administered. Efforts were made to minimize aggression by housing birds individually, aligning the lighting schedule with their natural rhythms, and ensuring environmental stability. Temperature and humidity were continuously monitored, with additional heating or cooling provided when necessary. A detailed log was maintained to record environmental parameters, health status, feed intake, and observations, ensuring optimal conditions for bird welfare and semen quality.

Training of cocks for semen collection

The birds underwent training for semen collection through abdominal massage, as described by Miah *et al.* (2024). Training was initiated to prepare the birds for semen collection and to ensure a clean ejaculate, free from feces. The training continued until sufficient semen was collected for the planned experiments. Successful ejaculates were obtained after four weeks of training. Semen collection was performed using six cocks that showed the best performance after training.

Semen collection and processing

Semen was collected twice weekly using the abdominal massage technique, a widely accepted non-invasive method for avian species (Fig 1). This method was chosen due to its efficiency in obtaining high-quality ejaculates without the need for anesthesia or excessive handling stress. Each collection was performed by experienced personnel in a quiet environment to reduce stress and maximize yield.

Immediately after collection, the semen was transferred into pre-warmed (37°C) collection tubes to prevent sudden temperature fluctuations that could affect sperm viability. The volume of each ejaculate was measured using a calibrated micropipette, while the color was evaluated to detect abnormalities such as blood contamination or excessive debris. The pH of each sample was determined using a digital pH meter, ensuring values remained within the optimal range (7.0–7.5) to maintain sperm integrity. Sperm concentration was determined using a hemocytometer, following appropriate dilution with a sodium citrate solution to facilitate accurate counting. Mass motility assessment was conducted using a phase-contrast microscope under a heated stage to prevent temperature-related motility fluctuations. These measures ensured use of high-quality samples for cryopreservation, minimizing variability in subsequent analyses.

Semen dilution and cryopreservation

To prepare the semen for cryopreservation, the collected ejaculates were first diluted using a Ringer's solution-based extender (Table 1). This extender provided an optimal physiological environment for sperm survival during the cryopreservation process. The extender was supplemented with 8% dimethyl sulfoxide (DMSO), a widely used cryoprotectant that prevents ice crystal formation and protects sperm from cryodamage during freezing.

To evaluate the effects of caffeine on sperm cryosurvivability, caffeine was added to the extender at four different concentrations considering four treatment groups, T0: 0 mg/ml (control), T1: 1 mg/ml, T2: 2 mg/ml, and T3: 3 mg/ml. Each treatment group consisted of three replicates with semen pooled from multiple birds to minimize individual variation.

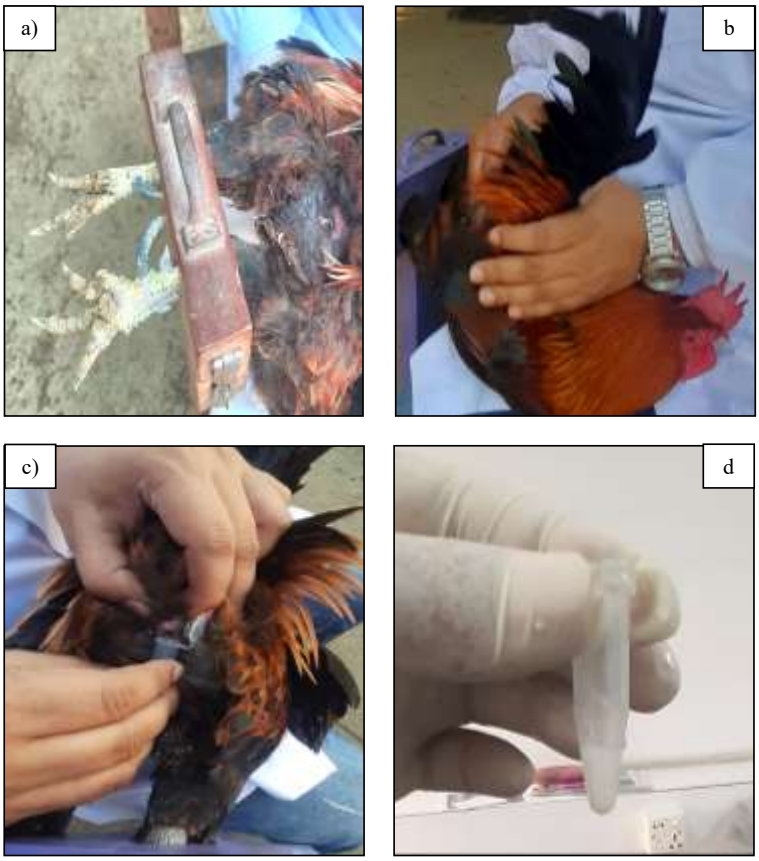

Fig 1: Sequential activities for semen collection a) locking of leg before semen collection, b) massaging of cock, c) collection of semen and d) semen content

Table 1: Composition of the semen diluents

Ingredients (units)	Amount
Sodium chloride (g)	9.50
Potassium chloride (g)	0.20
Calcium chloride (g)	0.26
Sodium bicarbonate (g)	0.20
Glucose (g)	1.00
Distilled water (litre)	1.00

Akcay *et al*., 2006

Following dilution, the semen samples were subjected to an equilibration period at 4°C for 60 minutes. This step was crucial to allow the cryoprotectant to permeate the sperm membrane gradually, reducing osmotic stress and enhancing the ability of sperm cells to withstand freezing. The pH of the extender was carefully maintained between 7.2 and 7.4 throughout the process, ensuring an optimal biochemical environment that supports sperm viability and function.

After the equilibration phase, the diluted semen was aliquoted into 0.5 ml cryovials, ensuring uniform sample distribution. The cryovials were then subjected to a controlled cooling protocol, which involved pre-cooling in liquid nitrogen vapor for 30 minutes. This step facilitated a gradual temperature decline, preventing intracellular ice crystal formation, which is a major cause of cryodamage.

Once the pre-cooling step was completed, the cryovials were rapidly plunged into liquid nitrogen (-196°C) for long-term storage. The frozen semen samples were stored under these conditions until further analysis, ensuring their stability and preservation for post-thaw assessments.

Thawing and post-thaw analysis

After two days of storage, semen samples were thawed at 38.5°C for 40 seconds in a water bath. For the analysis, Post-thaw semen aliquots were diluted at a 1:20 ratio using the basic modified Ringer's solution. This dilution ratio was optimized for CASA (Fig. 2; Miah *et al*., 2020). A precise volume of 0.5 µl of diluted semen was carefully placed on a clean, flat microscopic slide to ensure even distribution. The sperm movement parameters were analyzed using the CASA system fitted with an avian-specific condenser (ph-1) and a 10× objective. The parameters were evaluated including total motility, progressive motility, and sperm kinetics such as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN=VSL/VCL), straightness (STR=VSL/VAP), wobble (WOB=VAP/VCL), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF). For this analysis, the SCA motility software (Microptic Automatic Diagnostic System, Barcelona, Spain) was employed to ensure statistical reliability of the sample, at least 500 sperms per sample were counted as this large sample size enhances the reliability and robustness of the results.

Statistical Analysis

The data for total motility, progressive motility and sperm kinetics (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) were analyzed using a Completely Randomized Design (CRD). The analyses were conducted using the Generalized Linear Model (GLM) procedure in SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA).

To determine the significance of differences among the means of toms or hen groups, Duncan's Multiple Range Test (DMRT) was employed within the same software package. Results were presented as the Mean ± Standard Error of the Mean (SEM), and a significance level of $P<0.05$ was considered to indicate statistically significant differences.

Ethical considerations

The study was conducted following ethical guidelines for animal research, ensuring minimal discomfort to the birds. Approval was obtained from the Institutional Animal Ethics Committee of HSTU, and all procedures adhered to international guidelines for animal welfare in reproductive studies. Birds were handled by trained personnel, and semen collection was performed using humane techniques to minimize stress. Post-experiment, the birds were monitored for any adverse effects and were returned to the breeding flock under standard care conditions.

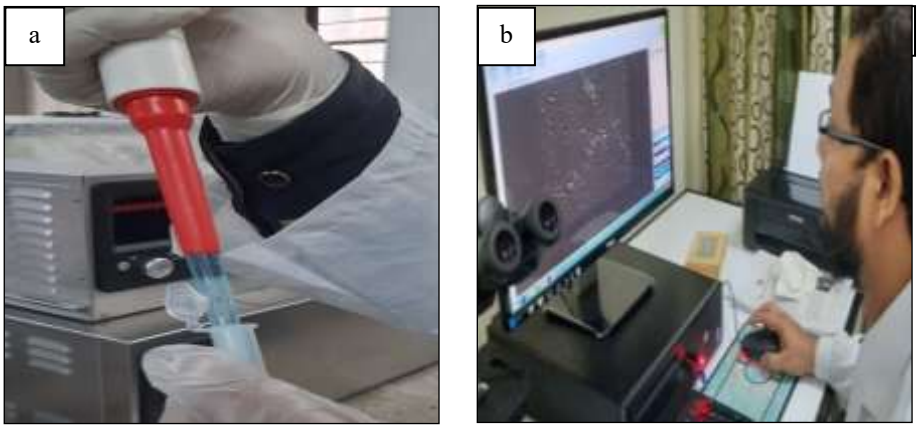

Fig. 2: Analysis of semen by CASA: a) pipetting of semen b) evaluation of semen by CASA

Results and Discussion

Total motility and progressive motility

The total sperm motility and progressive motility of four treatments are shown in the Fig. 3. Total motility, defined as the percentage of sperm exhibiting any form of movement, is a key parameter for post-thaw function. The findings of this study indicate that total motility was significantly higher (P<0.05) in the 2 mg/ml caffeine-treated group (T2) compared to the control (T0) and other treatment groups (T1 and T3). This suggests caffeine plays a key role in enhancing sperm motility, essential for fertilization. Similar findings have been reported in studies involving bovine and equine sperm, where caffeine supplementation led to increased total motility, likely due to its stimulatory effects on energy metabolism (Sanchez *et al*., 2022).

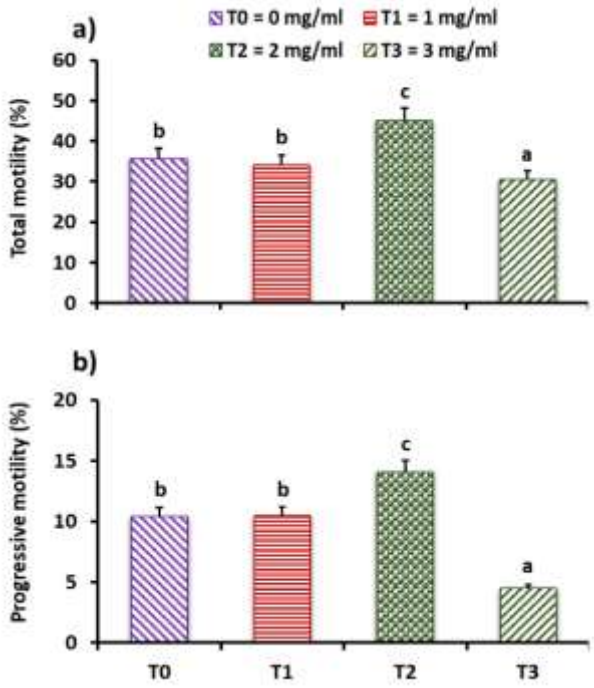

Fig. 3: Effect of pre-freezing caffeine supplementation (0, 1, 2, and 3 mg/ml) on a) Total motility, and b) Progressive motility of frozen-thawed RJF sperm. Each bar with an error bar represents the mean±SEM. Different letters on error bars indicate significant differences (P<0.05) among treatments.

Progressive motility, which refers to the proportion of sperm moving in a straight trajectory, is a crucial determinant of fertilization success. The present study revealed a significant improvement (P<0.05) in progressive motility in the 2 mg/ml caffeine-treated group (T2), achieving a rate of 14.07%. This enhancement in directional movement is particularly beneficial for artificial insemination and *in vitro* fertilization (IVF), where high progressive motility is correlated with increased fertilization rates (Choi *et al*., 2023). Caffeine's role in improving progressive motility can be attributed to its impact on sperm flagellar function. Flagellar movement is powered by dynein ATPase, which requires a steady supply of ATP to sustain motility. By enhancing mitochondrial efficiency, caffeine ensures a consistent ATP supply, thereby facilitating sustained sperm propulsion (Moraes *et al*., 2022). Moreover, caffeine-induced increases in intracellular calcium levels have been shown to activate key signaling pathways that regulate sperm movement, further promoting progressive motility (Jiang *et al*., 2023).

This suggests caffeine plays a key role inhibiting phosphodiesterase (PDE), leading to elevated levels of cyclic adenosine monophosphate (cAMP). The increased cAMP stimulates protein kinase A (PKA), which enhances calcium influx and mitochondrial function, ultimately improving sperm motility (Kwon *et al*., 2022). Additionally, oxidative stress is a significant factor affecting sperm function during cryopreservation. Caffeine's antioxidant properties help mitigate oxidative damage by reducing reactive oxygen species (ROS) accumulation, thereby preserving sperm motility (Rahman *et al*., 2023).

Cryopreserved sperm often show membrane damage, leading to compromised motility. Caffeine may counteract these effects by stabilizing sperm membrane integrity and maintaining lipid composition, essential for post-thaw functionality (Martinez *et al*., 2023). Previous studies have demonstrated that caffeine enhances membrane fluidity, reducing cryoinjury and improving overall sperm performance (Ortega-Ferrusola *et al*., 2021).

Kinetic parameters

Effect of pre-freezing caffeine supplementation (0, 1, 2, and 3 mg/ml) on curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) of frozen-thawed RJF sperm are shown in Fig. 4. The curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) were significantly improved (P<0.05) in the 2 mg/ml caffeine group (T2). The highest VCL (35.5 µm/s) and VAP (22.08 µm/s) were recorded in this treatment group, suggesting that caffeine positively influences sperm kinetics. Similar trends have been observed in other mammalian species, where caffeine supplementation enhanced sperm velocity through increased ATP production and improved intracellular calcium signaling (Samanta *et al*., 2019; Bezerra *et al*., 2022).

The straightness (STR), linearity (LIN), and wobble (WOB) are demonstrated in Fig. 5. The kinetic indices were significantly elevated in the caffeine-treated groups. The highest LIN (34.76%) and WOB (56.48%) values observed suggest improved sperm trajectory and reduced flagellar oscillations, factors that contribute to successful sperm-egg interactions (Del Gallego *et al*., 2020; Kherraf *et al*., 2023).

The amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) were also influenced by caffeine supplementation with BCF reaching a peak of 2.98 Hz in the 2 mg/ml caffeine group (Fig. 6). Increased BCF is indicative of enhanced flagellar beat frequency, which plays a crucial role in sperm penetration through the female reproductive tract (Aitken and Drevet, 2021). These findings are consistent with reports on cryopreserved bovine and ovine sperm, where caffeine improved post-thaw motility and kinetic properties (Oliveira *et al*., 2021).

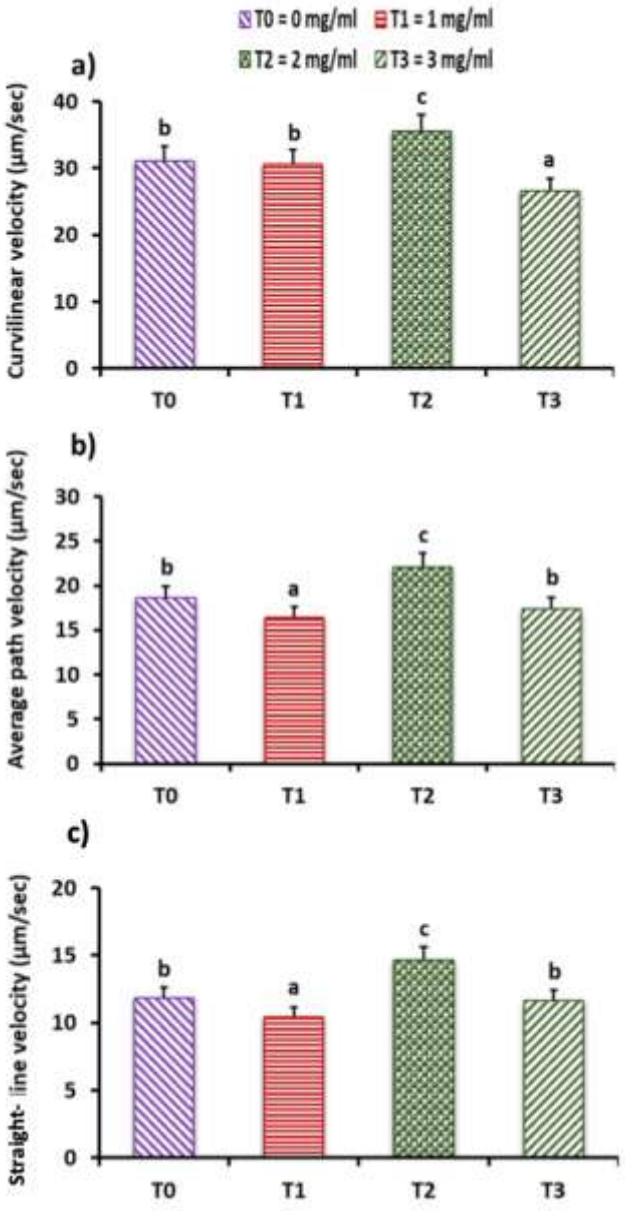

Fig. 4: Effect of pre-freezing caffeine supplementation (0, 1, 2, and 3 mg/ml) on a) Curvilinear velocity (VCL), b) Average path velocity (VAP), and c) Straight-line velocity (VSL) of frozen-thawed RJF sperm. Each bar with an error bar represents the mean±SEM. Different letters on error bars indicate significant differences (P<0.05) among treatments.

Cryosurvivability and mechanisms of caffeine action

Sperm cryosurvivability is influenced by multiple factors, including the choice of cryoprotectant, freezing-thawing protocols, and exogenous supplementation. The current study supports previous findings that caffeine supplementation enhances sperm motility and kinetic parameters post-thaw. One of the key mechanisms behind this effect is caffeine's function as a PDE inhibitor, which prevents the degradation of cAMP, thereby sustaining sperm motility (Gervasi and Visconti, 2017). Additionally, caffeine's ability to reduce oxidative stress helps prevent cryoinjury by preserving mitochondrial function and reducing lipid peroxidation (Wang *et al*., 2019).

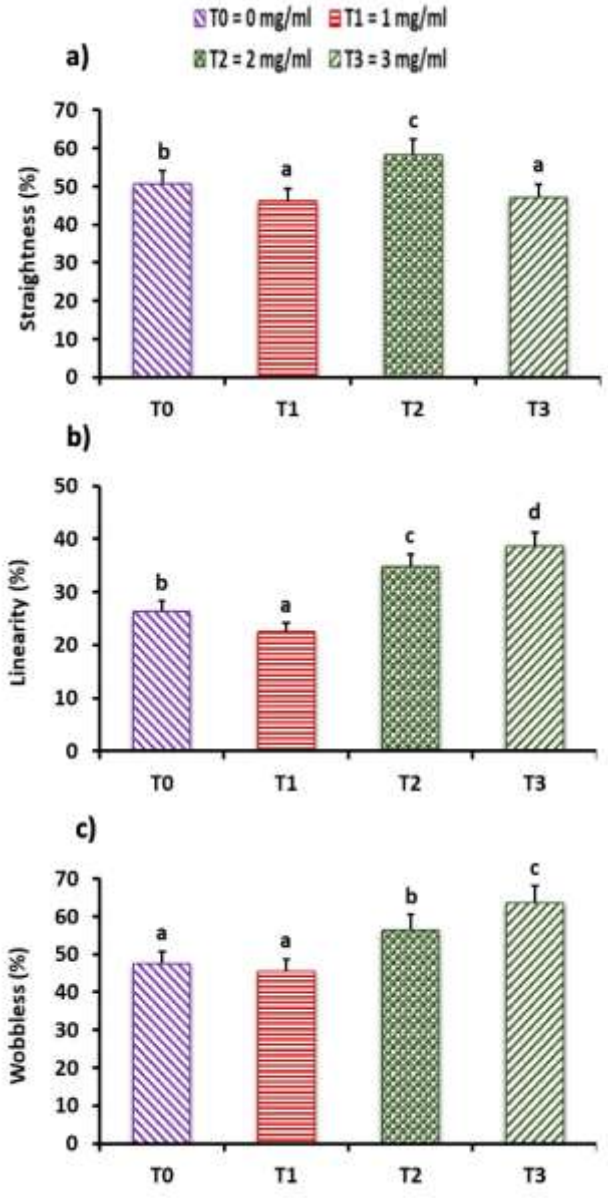

Fig. 5: Effect of pre-freezing caffeine supplementation (0, 1, 2, and 3 mg/ml) on a) Straightness (STR), b) Linearity (LIN), and c) Wobbles (WOB) of frozen-thawed RJF sperm: Each bar with an error bar represents the mean±SEM. Different letters on error bars indicate significant differences (P<0.05) among treatments.

The significant improvements observed in sperm motility and kinetic parameters suggest that 2 mg/ml caffeine is an optimal concentration for enhancing RJF sperm cryosurvivability when combined with 8% dimethyl sulfoxide (DMSO). This combination likely minimizes cryodamage by maintaining mitochondrial function, stabilizing membrane integrity, and reducing oxidative stress (De Andrade *et al*., 2022).

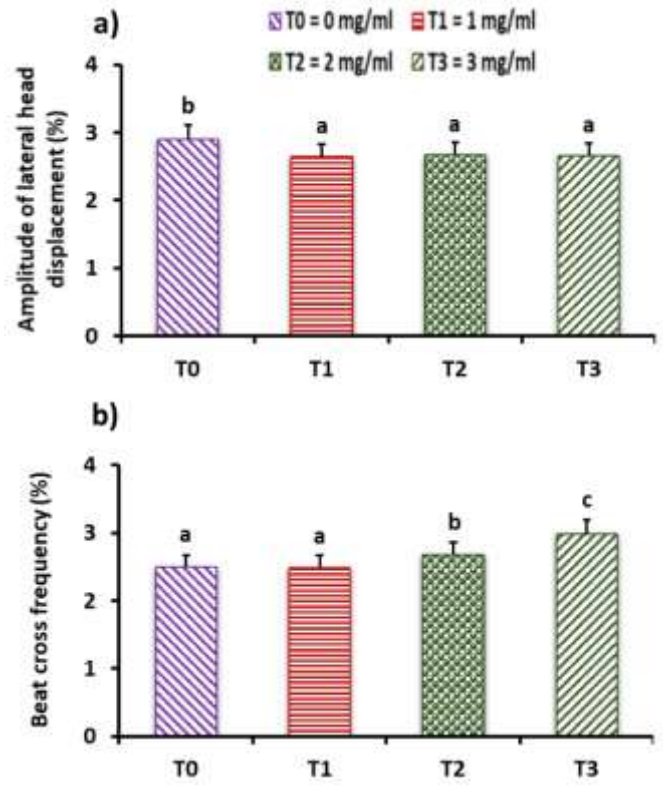

Fig. 6. Effect of pre-freezing caffeine supplementation (0, 1, 2, and 3 mg/ml) on a) Amplitude of lateral head displacement (ALH) and b) Beat cross frequency (BCF) of frozen-thawed RJF sperm. Each bar with an error bar represents the mean±SEM. Different letters on error bars indicate significant differences ($P<0.05$) among treatments.

Conclusion

This study shows benefits of 2 mg/ml caffeine in improving sperm motility, kinetic parameters, and overall cryosurvivability in RJF sperm. The observed improvements to the role role of caffeine in modulating energy metabolism, intracellular signaling, and membrane integrity. By enhancing ATP production, reducing oxidative stress, and preventing cryodamage, caffeine serves as a promising additive for optimizing semen cryopreservation protocols. The findings provide insights into reproductive biotechnology, particularly for species conservation and artificial breeding programs. Given these improvements observed in sperm quality post-thaw, caffeine could be a useful supplement in cryopreservation strategies for both RJF and other avian species. However, further studies are required to explore its long-term effects, optimal dosages across species, and potential interactions with different cryoprotectants. Its effects on fertilization and embryo development require evaluation in determining its practical applications in animal reproduction.

Conflict of Interest

Authors declare no conflict of interest with the present research.

Author's Contribution

All authors contributed equally at all stages of research and manuscript preparation. Final version of the manuscript is approved by all authors.

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